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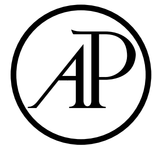

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NY-ESO-1: Review of an Immunogenic Tumor Antigen

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In the 9 years since its discovery, cancer-testis antigen NY-ESO-1 has made one of the fastest transitions from molecular, cellular, and immunological description to vaccine and immunotherapy candidate, already tested in various formulations in more than 30 clinical trials worldwide. Its main characteristic resides in its capacity to elicit spontaneous antibody and T-cell responses in a proportion of cancer patients. An overview of immunological findings and immunotherapeutic approaches with NY-ESO-1, as well the role of regulation in NY-ESO-1 immunogenicity, is presented here.
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I. IDENTITY CARD: BACKGROUND INFORMATION FOR NY-ESO-1

A. Name and Family History

The etymology of *NY-ESO-1* tells the story of its origins: NY stands for the city in which it was discovered by Chen *et al.* (1997), at the Ludwig Institute for Cancer Research and Weill Medical College of Cornell University in New York, in 1997; ESO is for esophageal cancer where it was originally described by screening a tumor-derived cDNA expression library with autologous serum of an esophageal cancer patient; and this was the first member of a new gene family.

The gene coding for *NY-ESO-1*, also known as *CTAG1*, exhibits characteristics that define a common family trait: genes with expression limited to germ cells and no normal somatic tissue, but frequently expressed in cancer, thus coding for products named cancer-testis (CT) antigens (Scanlan *et al.*, 2004). The original gene fitting the characteristic CT expression pattern was found in T. Boon's laboratory in Brussels and named *MAGE-1* (van der Bruggen *et al.*, 1991). It was described in an effort to identify antigens from tumor cells recognized specifically by CD8 T cells in a melanoma cancer patient. Homology searches and further discoveries identified additional MAGE antigens as immediate family members (Gaugler *et al.*, 1994) and other genes with similar characteristics named *GAGE*, *BAGE* .... One of them named *LAGE-1* was found briefly after *NY-ESO-1*, and showed 84% homology with *NY-ESO-1* (Lethé *et al.*, 1998). Both NY-ESO-1 and LAGE-1 have the capacity to encode shorter products from an alternative reading frame, one of which is referred to as CAMEL, and LAGE-1 has additional splice variants, named LAGE-1a and LAGE-1b (Lethé *et al.*, 1998).

Finally, in a desire to unify terminologies and attempt to cluster CT antigens by homology, a CT nomenclature was introduced and it defined NY-ESO-1 and LAGE-1 as belonging to the CT6 antigen family (http://www.cancerimmunity.org/CTdatabase). Besides CTAG1 and CT6, NY-ESO-1 has a couple of other aliases: LAGE-2, in keeping with the MAGE nomenclature, and *CAG-3* for a gene cloned by S. Rosenberg's group and coding for a product targeted by tumor-infiltrating lymphocytes (TILs) (Wang *et al.*, 1998).

B. Address: Gene Localization, Expression Pattern in Normal Tissues

The *NY-ESO-1* gene maps to the Xq28 region of the X chromosome and codes for several products (Chen *et al.*, 1997). The main product,

NY-ESO-1, is a 180 aminoacid long protein of 18 kDa, with a glycine-rich N-terminal region and an extremely hydrophobic C-terminal region, so insoluble that it can be confused for a transmembrane domain. There is however no evidence of membrane association at the cellular level (see below). Both NY-ESO-1 and LAGE-1 coding regions also yield several products from alternative reading frames, including a smaller 109 AA product named CAMEL (Aarnoudse *et al.*, 1999; Lethé *et al.*, 1998). Expression pattern analysis by RT-PCR for NY-ESO-1, LAGE-1, and CAMEL has confirmed a presence restricted to testis and no other normal tissue. A characteristic of many CT antigens, including NY-ESO-1, is that they are coded by the X chromosome, and strikingly, CT antigens were found to represent 10% of all genes in this chromosome, which was recently fully sequenced (Ross *et al.*, 2005). In cancer, genome-wide demethylation, and more specifically demethylation of *CT* gene promoter regions, was shown to be responsible for induction of CT antigen expression (De Smet *et al.*, 1996; Weber *et al.*, 1994).

The development of monoclonal antibodies to NY-ESO-1 has further confirmed its restricted expression pattern in normal tissues. Three monoclonal antibodies, ES121, E978, and B9.8, are available for studying the presence of NY-ESO-1 protein (Jungbluth *et al.*, 2001c; Schultz-Thater *et al.*, 2000; Vaughan *et al.*, 2004). These serological reagents are believed to specifically react with NY-ESO-1, and not LAGE-1, since recognized epitopes have been mapped to areas with the greatest sequence discrepancies. By immunohistochemistry, NY-ESO-1 is only found in early spermatogonia and is gradually lost with sperm cell differentiation (Fig. 1A). Its expression in these cells is predominantly cytoplasmic. Non gametogenic cells of the testis, including Sertoli cells, do not express NY-ESO-1 and neither do other somatic cells. The presence of NY-ESO-1 was found on a protein level in testicular fetal germ cells as early as week 18 (Satie *et al.*, 2002) and as well as in germ cells of the fetal ovary (Jungbluth *et al.*, 2001b). Some discrepancies exist for NY-ESO-1 expression in other normal adult tissues, since trace levels can be found in brain and uterus but they are usually considered insignificant. Nevertheless, placenta is a noncancer tissue showing expression of CT antigens on both mRNA and protein level (Fig. 1B) (Jungbluth *et al.*, 2001d). Another interesting but controversial finding is the expression of NY-ESO-1 protein in normal thymus (Nelson *et al.*, 2001), which is also supported by the detection of mRNA for NY-ESO-1 in medullary epithelial cells of the thymus (Gotter *et al.*, 2004).

C. Occupation: Functional Aspects

The function of NY-ESO-1 is still unknown. There is no obvious predicted functional domain or binding domain to give clues on partners in function. In normal cells, only a few CTs have been ascribed a role, either

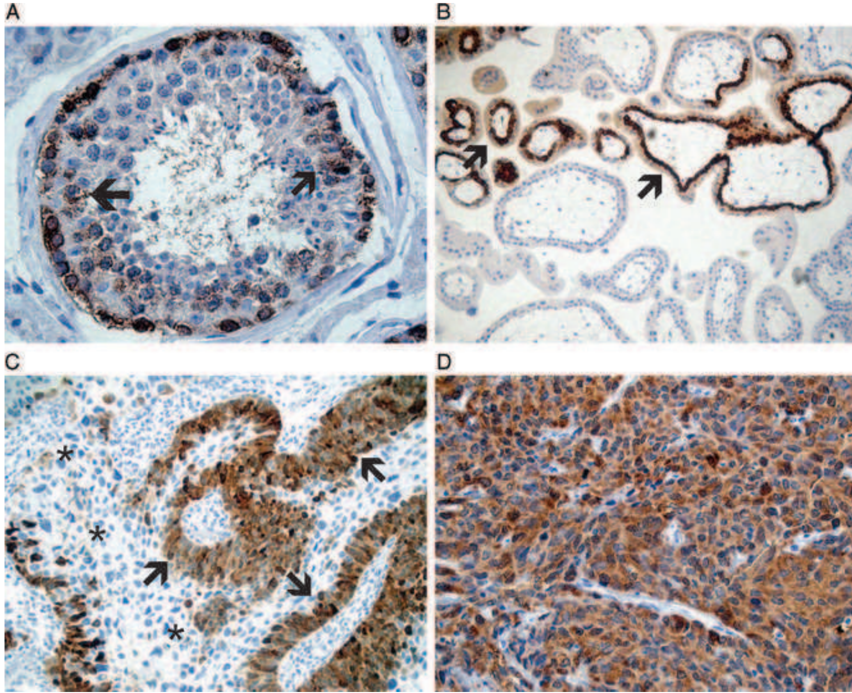

Fig. 1 Immunohistochemical staining for NY-ESO-1 with mAb E978. (A) NY-ESO-1 protein expression in testis; intense staining of spermatogonia, less intense staining of spermatocytes (arrows). (B) NY-ESO-1 immunostaining of trophoblast epithelium in placenta (arrows). (C) Heterogeneous NY-ESO-1 expression in urothelial carcinoma with scattered immunopositive areas (arrows) and negative tumor cells (asterisk). (D) Synovial sarcoma with homogeneous NY-ESO-1 immunostaining.

in meiosis (Türeci *et al.*, 1998)—which indirectly gives potential explanations on chromosomal abnormalities in cancer—or in acrosomal assembly (Ono *et al.*, 2001)—which on the contrary may indicate simple aberrant and purposeless expression in cancer. PRAME has been suggested to be involved in the response to retinoic acid, and may represent a potential resistance advantage in tumor cells (Epping *et al.*, 2005). MAGE-1 and GAGE have been found to be antiapoptotic, conferring resistance respectively to TNF-α and Fas (Cilensek *et al.*, 2002; Park *et al.*, 2002). One of the main reasons that so little is known for NY-ESO-1 stems from the absence of evolutionary homologs thus preventing immediate animal models such as knockout mice. The only distant cousin found in mice, ESO-3, is not a CT antigen but rather a possible pseudogene with universal expression in normal tissues (Alpen *et al.*, 2002). Attempts to silence NY-ESO-1 and other CT antigens using RNA interference in mammalian cells, to identify potential binding

partners, as well as to trace expression through knocking-in CT antigens with markers should hopefully soon lead to some clues. If a role can be ascribed, it will also be important to see whether it participates in a coordinate program expressed in tumor cells, as part of a cascade of events with other CT antigens (Simpson *et al.*, 2005).

II. PACKAGE INSERT: THERAPEUTIC POTENTIAL OF NY-ESO-1

A. Indications and Usage: Expression of NY-ESO-1 in Tumor Cells

The promise of NY-ESO-1 as a candidate for specific immune recognition of cancer comes from its restricted expression in normal tissues but frequent occurrence in cancer. Although originally described from the cDNA sequences of an esophageal tumor, NY-ESO-1 has shown a much more widespread incidence in a number of other tumor types. The expression of NY-ESO-1 has been analyzed in surgically removed primary or metastatic cancer tissues on two levels: message by RT-PCR and protein by immunohistochemistry or Western blot. Table I summarizes the findings of NY-ESO-1 in a variety of common cancer types. Presence of NY-ESO-1 is seen in approximately one-third to one-fourth of all melanoma, lung, esophageal, liver, gastric, prostate, ovarian, or bladder cancers (Fig. 1C). It is rare, however, in other cancer types such as colorectal, pancreatic and renal tumors or lymphoma (Jungbluth *et al.*, 2001c; Juretic *et al.*, 2003). A noteworthy cancer type with regard to NY-ESO-1 expression is synovial sarcoma (Jungbluth *et al.*, 2001a). Although a rare and aggressive disease, 80% of synovial sarcomas were found to express NY-ESO-1 (Fig. 1D) with highly homogenous distribution in tumor tissues. It is not clear what contributes to such high incidence when compared to most other tumor types but offers a hope for immunotherapeutic approaches.

The incidence of NY-ESO-1 is also interesting to compare between groups in different countries. For example, non-small cell lung cancer (NSCLC) appears to have a 20–25% expression rate of NY-ESO-1 in most US studies, while it is only rarely found in Japan (Table I). Squamous cell carcinoma of the lung is more common in Japan while adenocarcima is dominant in the United States and Europe, stressing that etiological differences may be involved and give clues to the basis of NY-ESO-1 expression in tumors. Similarly, particular carcinogens like HBV in hepatocellular carcinoma should be studied closely to understand if and how they may affect CT antigen expression.

Table I Expression of NY-ESO-1 in Various Cancers

	NY-ESO-1 mRNA positive cases	Percentage mRNA positive cases (%)	NY-ESO-1 IHC positive cases	Percentage IHC positive cases (%)	References
Bladder TCC high grade	15/43	35	22/72	31	Sharma *et al.*, 2003
Bladder TCC	20/62	32	2/14	14	Kurashige *et al.*, 2001
Bladder TCC high grade			6/33	18	Bolli *et al.*, 2005
Breast	37/88	42			Sugita *et al.*, 2004
Breast	13/129	10			Mashino *et al.*, 2001
Colorectal	2/98	2			Mashino *et al.*, 2001
Colorectal	12/121	10			Li *et al.*, 2005
Esophagus	41/123	33			Fujita *et al.*, 2004
Esophagus	11/46	24			Mashino *et al.*, 2001
Esophagus			18/56	32	Akcakanat *et al.*, 2004
Gastric	12/101	12			Wang *et al.*, 2004c
Gastric	8/102	8			Mashino *et al.*, 2001
HCC	17/62	27 (19–40)	25/132	19	Zhang *et al.*, 2005
HCC	9/24	27			Xing *et al.*, 2004
HCC	12/49	24			Korangy *et al.*, 2004
HCC	12/30	40			Chen *et al.*, 2003

HCC	31/73	42			Peng *et al.*, 2005
Head and neck			17/70	24	Prasad *et al.*, 2004
Melanoma	48/120	40	54/120	45	Vaughan *et al.*, 2004
Melanoma			12/38	32	Bolli *et al.*, 2005
Multiple myeloma	60/161	37 (31–60)			van Rhee *et al.*, 2005
Myeloma and plasmocytoma			8/29	27	Dhodapkar *et al.*, 2003
NSCLC	20/64	32			Konishi *et al.*, 2004
NSCLC	11/51	21			Wang *et al.*, 2004b
NSCLC	13/52	25	13/52	25	Jungbluth *et al.*, 2001c
NSCLC	1/46	2			Tajima *et al.*, 2003
NSCLC			15/130	12	Bolli *et al.*, 2005
Ovarian	32/107	30	62/142	43	Odunsi *et al.*, 2003
Pancreas	0/61	0			Kubuschok *et al.*, 2004
Prostate	20/53	38			Nakada *et al.*, 2003
Prostate HR			7/48	15	Fossa *et al.*, 2004
Sarcoma (all)	13/36	36			Ayyoub *et al.*, 2004
Synovial sarcoma			20/25	80	Jungbluth *et al.*, 2001a

TCC, transitional cell carcinoma; HR, hormone refractory; NSCLC, non-small cell lung cancer; HCC, hepatocarcinoma.
Ranges of numbers indicate variation in NY-ESO-1 expression according to tumor stage or grade analyzed.

Immunohistochemistry studies underline a few additional points regarding NY-ESO-1 expression. The detection of protein from cells with NY-ESO-1 mRNA expression is consistent with frequency by RT-PCR (Table I), however, the extent of cells expressing the antigen within a tumor is extremely variable. NY-ESO-1 can be expressed in a very homogeneous fashion in most tumor cells, as is often the case of synovial sarcomas (Fig. 1D), but it is more often found in patches with a high level of intratumoral heterogeneity (Fig. 1C). This observation is critical for estimating NY-ESO-1 expression frequencies in cancer and highlights the usefulness of sampling multiple areas of a tumor when evaluating expression. Some tumors also may have a focal expression pattern with only occasional cells stained for NY-ESO-1. It is not known if NY-ESO-1 heterogeneity is a sign of functional differences of expression or possibly the result of selection in tumors.

The coexpression pattern of NY-ESO-1 with other CT antigens has been established (Bolli *et al.*, 2005; Vaughan *et al.*, 2004). The most homologous gene *LAGE-1* is frequently codetected by RT-PCR in NY-ESO-1 expressing tumors, although roughly a third of cases show independent expression. Similarly, a coordinated expression of *NY-ESO-1* with *MAGE* genes reflects that such genes could have similar triggering events, such as demethylation of their promoter (De Smet *et al.*, 1996; Weber *et al.*, 1994), but many discrepancies also show that additional events may be required for expression and imply a potential gain of function in tumor cells.

B. Immunogenicity Studies

1. NY-ESO-1 ANTIBODIES

NY-ESO-1 was discovered based on its capacity to induce an antibody response *in vivo* in cancer patients. This humoral response is restricted to cancer patients and not seen in healthy individuals (Stockert *et al.*, 1998). The presence of serum antibodies to NY-ESO-1 is largely dependent on NY-ESO-1 expression within the tumor. Still, there is a small but significant number of cancer patients with humoral responses to NY-ESO-1 occurring in the absence of detectable NY-ESO-1 mRNA in the tumor which, if not due to misinterpretation from flawed sampling of tumors with heterogeneous antigen expression, could suggest some tumors may clonally evolve and lose the antigen in an editing process reflected by antibody persistence. Overall, NY-ESO-1 expressing cancers, such as melanoma or ovarian carcinomas, appear to spontaneously induce NY-ESO-1 antibodies in 5–15% of all patients with or without detectable antigen expression in tumor cells (Stockert *et al.*, 1998; van Rhee *et al.*, 2005). Accordingly, approximately 10% of operable, NSCLC patients have an antibody response to NY-ESO-1

(Gnjatic *et al.*, unpublished data). Considering the logical assumption that NY-ESO-1 is initially required to generate these antibodies, and taking into account the percentage of NY-ESO-1 expression in NSCLC to be around 20% of all surgically available cases, one can estimate that up to 50% of NSCLC patients develop an immune response to the expressed antigen. In patients with advanced melanoma, a similar observation was made in which up to 50% of patients with NY-ESO-1 expressing tumors are seropositive for NY-ESO-1 (Jäger *et al.*, 2000c). In ovarian cancer, close to 20% of all patients have spontaneous NY-ESO-1 antibodies at time of surgery. Surprisingly, other cancers with very frequent NY-ESO-1 expression like synovial sarcoma do not have a higher frequency of antibody responses, still representing less than 10% of all patients despite 80% of tumor cases expressing NY-ESO-1 (Gnjatic *et al.*, unpublished data). But on the whole, compared to other family members of CT antigens, such as MAGE antigens, NY-ESO-1 is still one of the most frequent to induce high-titered antibodies in such a broad variety of cancer types.

The humoral response to NY-ESO-1 is diverse in its immunoglobulin (Ig) subclasses, with IgG1, IgG2, IgG3, or IgG4 responses often detected in patient sera, sometimes simultaneously, and IgG1 being more frequent among isotypes studied (personal observations in a small subset of NY-ESO-1 seropositive patients). The epitopes recognized by naturally occurring antibodies are mostly mapping to the soluble N-term end of NY-ESO-1 (Fig. 2), since truncated proteins and longer peptides in this region are as efficiently recognized as the full-length product (Stockert *et al.*, 1998; Zeng *et al.*, 2005). ELISA titers to NY-ESO-1 can be very high, still detectable in some cases at a 1/1,000,000 dilution, although a majority of responses seem to be detectable up to a range from 1/6,400 to 1/100,000. Antibody responses to NY-ESO-1 are usually cross-reacting with LAGE-1 due to the high homology between the two proteins. Although IgA and IgE still need to be studied, the presence of serum IgG responses against NY-ESO-1 is an immediate indicator that there is a likely T-cell involvement explaining the class switch from IgM.

A potential role of natural antibodies to NY-ESO-1 may be that they help the initiation of cellular immune responses by forming immune complexes with their antigen, which were shown to efficiently taken up and cross-presented by dendritic cells *in vitro* (Matsuo *et al.*, 2004; Nagata *et al.*, 2002).

Fig. 2 Schematic representation of NY-ESO-1. Blue area is rich in epitopes recognized by antibodies. Red areas are rich in CD4 T-cell recognized HLA class II epitopes. These areas overlap in purple. Hatched areas represent HLA class I epitopes recognized by CD8 T cells.

2. SPONTANEOUS CD8 AND CD4 T-CELL RESPONSES TO NY-ESO-1

The first evidence of cellular recognition of NY-ESO-1 came from the group of A. Knuth and E. Jäger in Frankfurt. From a patient with metastatic melanoma, who was seropositive for NY-ESO-1, they obtained a CD8 T-cell line by autologous mixed lymphocyte-tumor culture that reacted with tumor cell lines expressing NY-ESO-1 and the HLA-A2 molecule (Jäger *et al.*, 1998). The tumor-reactive T-cell line was shown to recognize peptides 157–165, 157–167, and 155–163 of NY-ESO-1 restricted by HLA-A2. The same year, S. Rosenberg's group found that peptide 53–62 was also recognized by the patient's CD8 T cells in the context of HLA-A31 (Wang *et al.*, 1998). Since, technical improvements to measure T-cell responses using recombinant viruses expressing full-length NY-ESO-1 or long peptides derived from NY-ESO-1 have helped identify a series of peptides restricted by HLA-B and HLA-C molecules (Benlalam *et al.*, 2001; Gnjatic *et al.*, 2000; Jäger *et al.*, 2002b; Sharma *et al.*, 2003), many of which appear to be clustered around the central part of NY-ESO-1 (Fig. 2). In addition to multiple epitopes encoded by NY-ESO-1, the alternative reading frame of NY-ESO-1 was shown to code for an epitope with HLA-A2 restriction as well, known as CAMEL (Aarnoudse *et al.*, 1999). Also, some epitopes were shown to be conserved between *NY-ESO-1* and *LAGE-1* and recognized by T cells, further enriching the list of immunogenic sites from this gene family.

The first evidence of CD4 T-cell responses to NY-ESO-1 was once again demonstrated by Jäger *et al.* (2000b) with the definition of several epitopes restricted by HLA-DR4. With improvements in methodologies for CD4 T-cell analysis, many additional epitopes were defined by several groups, recognized by CD4 T cells in the context of various *HLA-DR* alleles (Gnjatic *et al.*, 2003a; Mandic *et al.*, 2005; Neumann *et al.*, 2004; Zarour *et al.*, 2000, 2002; Zeng *et al.*, 2000). Importantly, Zeng *et al.* (2001) have described CD4 T-cell responses against peptide 157–170 restricted by *HLA-DP4*, an allele found in the majority of Caucasians. Ovarian cancer patients with NY-ESO-1 antibody were found to have both Th1- and Th2-type preexisting responses to this peptide (Qian *et al.*, 2004). There are now at least 12 distinct epitopes described in the context of at least 5 HLA class II alleles (http://www.cancerimmunity.org/peptidedatabase/tumorspecific.htm). As for CD8 T-cell responses, CD4 T cells were also found against LAGE-1 and alternative reading frames of NY-ESO-1 (Mandic *et al.*, 2003; Slager *et al.*, 2003, 2004). When mapping all the peptides described to date one can nearly cover all of the central and C-term sequence of NY-ESO-1, stressing again the densely immunogenic nature of this short protein (Fig. 2).

3. INTEGRATED IMMUNOGENICITY OF NY-ESO-1

In order to estimate the frequency of CD4 and CD8 T-cell responses in relation to antibody responses against NY-ESO-1, cohorts of patients with various cancers were analyzed for IFN-γ secretion by T cells according to their serological status and expression of NY-ESO-1 in their tumors. The major finding was that antibody, CD8, and CD4 T-cell responses were observed simultaneously in an integrated manner in which one seems to depend on the other to be present (Gnjatic *et al.*, 2003a; Jäger *et al.*, 2000c). In these studies, humoral and cellular responses were generally not found in the absence of NY-ESO-1 expression in tumor cells. Despite the prevalence of HLA-DP4, there was no association between the presence of NY-ESO-1 antibody in patients and requirement for HLA-DP4 status (Huarte *et al.*, 2004). As a rule, naturally occurring NY-ESO-1 specific B and T cells appear to be induced together, although NY-ESO-1-specific CD8 T cells have been occasionally observed in individual patients sero-negative for NY-ESO-1 (Valmori *et al.*, 2000), and conversely antibody presence to NY-ESO-1 was sometimes seen in patients with no detectable T-cell responses. Still, there are no other tumor antigens as thoroughly described for their capacity to consistently induce both cellular and humoral responses at such frequency, p53 and Her2/neu being close competitors.

The generally coordinated response to NY-ESO-1 by the adaptive immune system should simplify the analysis of larger-scale correlations with clinical course of disease, since antibody can be more easily measured as a marker of both humoral and cellular reactivity.

C. Pregnancy and Gender Specificity

Despite the lack of significant NY-ESO-1 expression in any normal tissues in women, while men constitutively express it in the testis, there is no difference between sexes with regards to expression of NY-ESO-1 in tumors and resulting immunogenicity. This may indicate that expression in testicular germ cells is not causing gender-specific tolerization, and that NY-ESO-1 antigen is likely ignored by the immune system, as postulated from the lack of HLA molecules expressed by these germ cells.

Additionally, NY-ESO-1 along with other CT antigens can be found in the trophoblasts of placenta (Fig. 1B), but there is no evidence that the immune system of women who had children may have been naturally exposed to these antigens. No spontaneous antibody response was found in women, with or without children, among healthy donors. Additional studies still have to address the specific question of immune responses to NY-ESO-1 and other CT antigens during pregnancy.

Yet, recent evidence challenges the view that NY-ESO-1 is ignored by the immune system (cf. Section II.G). Rather, it appears that precursor CD4 T cells specific for NY-ESO-1 may be found in most healthy individuals, male or female, but are actively suppressed in the periphery by $CD4^{+}CD25^{+}$ regulatory T cells (Danke *et al.*, 2004; Nishikawa *et al.*, 2005a). The origin and nature of these cells is being explored, including during vaccination (Nishikawa *et al.*, 2006), but they indicate that NY-ESO-1 as a self-antigen with very limited tissue expression does not cause the central deletion of autoreactive T cells. The peripheral control of NY-ESO-1-specific T cells by Tregs may be linked with potential thymic expression of NY-ESO-1.

D. Clinical Observations

Cancer/testis antigen expression in cancer is often associated with higher-grade tumors. In bladder, breast, or lung cancer, for example, the percentage of NY-ESO-1 expression is highest in advanced tumors (Kurashige *et al.*, 2001; Sharma *et al.*, 2003; Sugita *et al.*, 2004). Since NY-ESO-1 and other CT antigens seem to be more often simultaneously expressed than not, establishing patterns in human cancer may eventually help classify tumor types, potential course of action and sensitivity to therapeutic approaches, and eventually represent a predictive tool.

Already, a number of studies have found that CT antigen expression in cancer may be associated with poorer survival in stage-matched patients (Bolli *et al.*, 2005; Güre *et al.*, 2005; Sugita *et al.*, 2002). Thus, not only do CT antigens like NY-ESO-1 potentially indicate a subset of more aggressive tumors, but they also may have a role in making the tumor less susceptible to known treatments.

If the pattern of negative association between CT antigens and survival is confirmed, should it affect the way we think of NY-ESO-1 as a naturally occurring potential rejection antigen? Should we not observe that patients with tumors expressing NY-ESO-1, especially those that show an immune response to it, have some clinical advantage? Is it worth duplicating an *in vivo* immune response that fails to rid the tumor?

To answer these questions, one must look at the antigen from the standpoint of the immune system rather than as a simple marker. It is known that serum antibodies to NY-ESO-1 are dependent on antigen expression and only occur in a subset of patients, that is, 10–50% of those with NY-ESO-1 expressing tumors. These antibodies can reach very high titers that can even be detected in urine (Jäger *et al.*, 2002a), but no function has yet been demonstrated *in vivo* for these antibodies knowing that their targets are not accessible in the cytoplasm of tumor cells. Antibodies to NY-ESO-1 could be involved in helping the initiation of immune responses by the

formation of immune complexes with antigen released from necrotic or apoptotic tumor cells (Albert *et al.*, 1998; Nagata *et al.*, 2002). Since it was shown that antibodies occur concomitantly with CD8 and CD4 T-cell responses for NY-ESO-1, their presence is an easy marker to monitor the presence of NY-ESO-1 immunity in patients and potential resulting clinical benefit.

Jäger *et al.* (1999) have shown that changes in antibody titers to NY-ESO-1 follow the clinical course of the tumor. Notably, titers to NY-ESO-1 frequently fall following removal of tumor by surgery, further confirming the relationship between antibody presence and tumor antigen expression. A humoral response to NY-ESO-1 may be a marker of progression and reflect overall tumor load. But exceptions are also seen, with patients without apparent evidence of disease but who keep high titers of NY-ESO-1 antibodies over years.

Remarkable yet incidental observations from several patients seropositive for NY-ESO-1 have shown an indolent course, not necessarily resulting in regression but sometimes associated with large necrosis areas within tumor masses. One of the first patients to be described for CD8 T-cell responses to NY-ESO-1 (NW29) is still alive with high antibody titers to NY-ESO-1 after 10 years with unresolved metastatic melanoma. If nothing else, immunotherapeutic approaches achieving such a remarkable course could make cancer a manageable disease.

An obvious critical step is now to analyze sufficient cohorts of patients matched for tumor stage or grade and compare those with immunity to NY-ESO-1 and those without for survival and time to progression following surgery. Two approaches ongoing at the New York Branch of the Ludwig Institute for Cancer Research. One is a large-scale serological analysis of antibody reactivity to NY-ESO-1 and other antigens in a cohort of nearly 1000 NSCLC patients in collaboration with N. Altorki at New York Hospital. The other, in collaboration with K. Odunsi at Roswell Park Cancer Institute, is to correlate TIL counts in epithelial ovarian cancer with disease stage, grade, and NY-ESO-1 expression among others (Sato *et al.*, 2005).

E. Vaccines Studies

The NY-ESO-1 antigen generates excitement because of its promise for immunotherapy: it has a fairly widespread occurrence in a majority of cancers, it is exquisitely specific in its expression limited to cancer tissues, and most importantly, its immunogenicity in natural settings allows to put in place tools to measure the effect of immunotherapy.

The primary challenge of experimental vaccines is to determine the best conditions for mounting immune responses: dose, frequency of vaccination, nature of the immunogen (synthetic peptide, recombinant protein, DNA ....), and requirement for adjuvants (including cytokines, mineral oils or sugars, bacterial or viral vectors ....). With the knowledge of NY-ESO-1 immunity and the techniques derived from its analysis, immunotherapy trials aiming at generating primarily cytotoxic CD8 T cells as well as helper CD4 T cells and antibodies to NY-ESO-1 have soared.

A unique, gradual approach was taken with NY-ESO-1 as a model antigen: multiple, small parallel coordinated clinical trials around NY-ESO-1 with one change of variable at a time, sponsored in majority by the Ludwig Institute for Cancer Research and Cancer Research Institute, focusing on immunological monitoring with use of comparable methodologies. This was a unique setting in a field that was dominated until the late 90s mostly by "clinical benefit" observations that were very difficult to correlate with intended immune responses from immunizations. Knowing which epitopes may be most immunogenic from hosts with naturally occurring responses has allowed the establishment of state-of-the-art sensitive approaches such as single-cell IFN-γ secretion detection by ELISPOT or phenotyping of CD8 T cells with NY-ESO-1 specificity using HLA-peptide tetramers (Atanackovic *et al.*, 2003; Jäger *et al.*, 2000c). These could then be applied to have a precise idea of the timing and formulation needed to elicit responses after peptide or full-length antigen vaccination and measure potential efficacy in terms of frequency and tumor reactivity.

The extent of trials performed or underway is now over 30 around the world, representing more than 300 patients, a selection of which is shown in Table II. The good news is that a lot has already been learned: it was demonstrated that vaccination can efficiently prime an antibody, CD8, and CD4 T-cell response to NY-ESO-1. As a rule, extended vaccination with NY-ESO-1 protein led to broader and more pronounced immune responses. On the other hand, short-term intensive vaccination with peptides was very potent at inducing the strongest CD8 T-cell responses to single epitopes.

The more difficult part in the overall evaluation of these trials is establishing a baseline level of efficacy. It is clear from studies, such as vaccination with NY-ESO-1 peptide 157–167, that misguided responses to cryptic epitope may occur, and even more concerning, that a majority of T cells to NY-ESO-1 epitopes lacked tumor reactivity (cf. Section II.F).

Very few of the trials were designed with clinical efficacy as an endpoint, simply because it was premature to ask the question without knowing how vaccination modalities may affect immune responses. As a consequence, many trials were conducted in patients with no evidence of disease following surgery in which only time to relapse may be clinically investigated.

Table II Highlight of Some Clinical Trials with NY-ESO-1 Sponsored by the Ludwig Institute for Cancer Research

Immunogen	Indication	Adjuvant	Site	Immunological response
Protein	Bladder cancer	BCG + GMSCF	MSKCC/LICR NY	Antibody and $CD4^+$ T cells
Protein	Melanoma (and others)	ISCOMatrix™	LICR Melbourne	Antibody, $CD8^+$, and $CD4^+$ T cells
Protein	Various	CHP	Mie, Japan	N/A
Peptides 157–165 and 157–167	Various	GM-CSF	Frankfurt	$CD8^+$ T cells
Peptides 157–165 and 157–167	NSCLC	GM-CSF	Cornell/LICR NY	$CD8^+$ T cells + cryptic epitope
Peptide 157–165	NSCLC and various	IFA + CpG	Cornell/LICR/Frankfurt	$CD8^+$ T cells
Peptide 157–165	Ovarian	IFA	MSKCC/LICR NY	$CD8^+$ T cells
Peptide 157–170	Ovarian	IFA	Roswell Park	$CD4^+$ T cells
Peptide 157–165 high dose	Melanoma	IFA + CpG	Lausanne	$CD8^+$ T cells
Recombinant fowlpox and vaccinia	Various	None	Frankfurt	Antibody, $CD8^+$, and $CD4^+$ T cells
Recombinant fowlpox and vaccinia	Various	None	Oxford	N/A
Recombinant fowlpox and vaccinia	Various	None	Roswell Park	N/A
DNA	NSCLC	Gold beads	Cornell/LICR NY	$CD4^+$ T cells
Planned/opening soon				
Protein	Various	IFA + CpG	Columbia U./LICR NY	N/A
Recombinant *Salmonella*	Various	None	LICR NY	N/A

BCG, Bacillus Calmette-Guerin; MSKCC, Memorial Sloan-Kettering Cancer Center; LICR, Ludwig Institute for Cancer Research in New York; IFA, Incomplete Freund's Adjuvant; CpG, Immunostimulatory DNA sequences.

1. NY-ESO-1 PEPTIDE TRIAL VACCINES

The most studied vaccine preparation for NY-ESO-1 has been so far the use of synthetic HLA-A2 peptides with various adjuvants for immunization of cancer populations with tumors expressing NY-ESO-1, including melanoma, NSCLC, ovarian cancer, synovial sarcoma .... Synthetic peptides were the first to be studied because of their relative ease of preparation for clinical investigations compared to full-length antigen. After the first clinical trial using peptides originally described by Jäger *et al.* (2000a), it was determined that NY-ESO-1 overlapping peptides 157–165 and 157–167 were frequently immunogenic when administered with GM-CSF as systemic adjuvant while peptide 155–163 was not. These overlapping peptides seemed to be able to frequently induce measurable cellular immune responses both by tetramer and by ELISPOT assays. However, it appeared clear in subsequent trials that peptide 157–167 was able to react independently from peptide 157–165, and that this was due to a cryptic epitope with dominant immunogenicity hidden within the sequence of peptide 157–167, that is, peptide 159–167. The fine analysis of vaccine-induced CD8 T cells directed to peptide 159–167 indicated that they were not able to recognize naturally processed NY-ESO-1, likely because the standard proteasome machinery did not allow cleavage after the C-term residue required for generating the epitope from full-length NY-ESO-1 (Gnjatic *et al.*, 2002). Consequently, peptide 157–167 was also removed from further clinical studies.

This left NY-ESO-1 157–165 as the only peptide representing the epitope naturally presented by HLA-A2+ NY-ESO-1+ tumor cells. It has been used in combination with various adjuvants, such as Flt3-ligand, or incomplete Freund's adjuvant, with or without addition of immunostimulatory bacterial DNA CpG sequences, and cytokines GM-CSF or IL-2 (Chen *et al.*, 2005b; Gnjatic *et al.*, 2002; Khong *et al.*, 2004; Shackleton *et al.*, 2004; Valmori *et al.*, 2003). Frequency of vaccination was also assessed with intradermal administrations ranging from monthly, weekly, to daily intensive regimen. Most studies were able to induce consistent peptide-specific CD8 T-cell response in a majority of patients. Responses can be detected as early as 2 weeks from initial injections by ELISPOT and tetramers. Stronger adjuvants and higher doses of peptide appear to improve the frequency of elicited T cells. Most responses can be detected from the peripheral blood of vaccinated patients after a single recall stimulation *in vitro* and in some exceptional cases directly *ex vivo*. However, the repertoire of T cells induced by peptide vaccines may differ from that naturally elicited (Le Gal *et al.*, 2005).

Because of simplicity and lower cost of production as well as consistent immunogenicity, more peptide approaches are being evaluated or considered, such as the use of HLA class II–restricted peptides, for vaccination or the use of 30-mer overlapping peptides that were shown to be

cross-presented and naturally processed into both class I and class II epitopes by most antigen-presenting cells (Gnjatic *et al.*, 2003b).

2. NY-ESO-1 FULL-LENGTH ANTIGEN VACCINE TRIALS

Although short peptides as vaccines offer clear advantages of rapid immunogenicity and ease of production, they also are limiting in their range of potential use by their HLA restriction requirements. In studies described in the previous section, patients had to be HLA-A2+ to be eligible, which limits the applicability of these vaccines. Even if an assortment of short peptides were to be used together and selected to bind to several diverse HLA class I molecules, the absence of CD4 help may be a critical shortcoming to achieve long-term memory responses and high quality and frequency of effectors (Hung *et al.*, 1998; Kaech and Ahmed, 2003). Moreover, peptide vaccination can give rise to responses against cryptic epitopes not representative of a naturally processed sequence of NY-ESO-1 when initial selection of peptides is not optimal (Dutoit *et al.*, 2002b).

For these reasons, approaches using full-length NY-ESO-1 antigen have been considered early on. Full-length NY-ESO-1 protein needs to be processed by professional antigen presenting cells in order to prime CD4 and CD8 T cells, which favors relevant epitope selection and thus more likely tumor reactive cells. Recombinant NY-ESO-1 protein produced in *Escherichia coli* (*E. coli*) (Murphy *et al.*, 2005) was the first to be evaluated in clinical settings and turned out to be among the most promising trials published so far with NY-ESO-1 because of broad immunological and apparently favorable clinical results. It was conducted by the Melbourne Branch of the Ludwig Institute for Cancer Research, where NY-ESO-1 protein together with lipid/saponin-based encapsulating particles named ISCOMatrix™ used as adjuvant were administered to melanoma patients (Davis *et al.*, 2004). The results showed broad integrated immune responses, consisting of NY-ESO-1-specific antibodies and polyclonal CD4 and CD8 T cells that were all dependent on the use of both antigen and adjuvant, as witnessed by placebo and control groups (Chen *et al.*, 2004; Davis *et al.*, 2004).

As this trial is moving into Phase II, comparison will be possible with other NY-ESO-1 protein vaccine trials: at Memorial Sloan-Kettering Cancer Center, NY-ESO-1 protein is evaluated with BCG as systemic adjuvant in bladder cancer patients at high risk of relapse after cystectomy, and in Mie University, Japan, NY-ESO-1 protein is used with cholesterol-bearing hydrophobized pullulan (CHP) as a lipid/sugar-based encapsulating structure. Additional plans include assessing liposomes, incomplete Freund's adjuvant, and immunostimulatory molecules targeting toll-like receptors as adjuvants for NY-ESO-1 protein.

Many other new developments are also on the close horizon. Full-length NY-ESO-1 antigen is evaluated in the form of a DNA vaccine in which DNA is coated on gold beads and administered as powder by pressure-driven epidermal delivery, or gene gun. NSCLC patients and prostate cancer patients have been enrolled for this study in New York Presbyterian Hospital and MD Anderson. Attenuated viruses, such as vaccinia or fowlpox, engineered to encode NY-ESO-1 are also currently used, at Nordwest Krankenhaus in Frankfurt and Roswell Park Cancer Institute, to determine safety and optimal dose for inducing immune responses in cancer patients. Preliminary results indicate the potential for antibody, CD4, and CD8 T-cell responses against NY-ESO-1 in a multiepitopic fashion. The alternative use of different poxvirus vectors during vaccination may be of importance. A study at Oxford University showed that vaccination with modified vaccinia virus encoding HLA class I-restricted short epitopes from NY-ESO-1 and other melanoma-derived antigens led to immunodominant responses against vaccinia itself, suggesting that strategies combining vectors for priming and boosting may be most successful to focus the specific immune response on the recombinant antigen (Smith *et al.*, 2005).

It is thus exciting to see a flurry of other recombinant vectors that are or soon will be available for evaluation of NY-ESO-1 delivery: NY-ESO-1-recombinant bacterial vectors including *E. coli*, *Salmonella*, yeast, and *Listeria*. All these construct offer built-in adjuvanticity and have specific modes of immunization. Altogether, the framework for further evaluating integrated immune responses in vaccine settings is in place for eventually optimizing clinical benefit.

F. Efficacy: Processing Requirements for Tumor Recognition by T cells

The major hurdle facing vaccine programs with NY-ESO-1 is to determine the best conditions needed to not only obtain high frequencies of CD8 T cells against the immunogen but also to ensure that they are able to recognize tumor cells, at least when tested *in vitro*. Even though it appears clear that peptide-specific responses can be measured as a result of NY-ESO-1 immunization, either peptidic or full-length, the ability of these vaccine-induced CD8 T cells to consistently recognize tumor cells has been a challenge. Many reasons can be evoked: T cells raised by synthetic peptide or recombinant protein vaccines could differ from naturally primed effectors in their fine specificity to epitopes recognized (Le Gal

et al., 2005). Efforts to map potential posttranslational modifications and to estimate the impact of subtle conformational changes in HLA–peptide interactions may help address this hypothesis (Chen *et al.*, 2005a). It is also possible that regulation of coreceptor expression on vaccine-induced T-cell effectors prevents optimal recognition of poorly immunogenic targets. This is less likely since tumor cells pulsed with peptide can overcome their lack of recognition by vaccine-induced T cells. Partial regulation from other cells, such as regulatory cells, blunting T-cell reactivity in vaccine settings may also be critical and is addressed below (cf. Section II.G). But failure to recognize low amounts of natural HLA–peptide complexes on NY-ESO-1 expressing tumor cells mostly points toward a strict requirement for a minimal threshold affinity and avidity of T cells.

Most of the work on T-cell effector affinity and tumor cell recognition has been with CD8 T cells directed to NY-ESO-1 epitope 157–165 restricted by HLA-A2. When analyzing a patient with a cancer-induced spontaneous integrated immune response to NY-ESO-1, these effectors appear to be able to consistently kill tumor cell lines *in vitro* that express NY-ESO-1 and HLA-A2 (Jäger *et al.*, 1998). The typical affinity of CD8 T-cell clones showing tumor reactivity is measured by quantifying the lowest amount of synthetic peptide epitope required to activate T cells, and it is in the range of 1 nM in the case of tumor reactive cells (Dutoit *et al.*, 2002a). However, in patients vaccinated with peptide 157–165, a majority of CD8 T cells fail to reach a sufficient affinity and only recognize 50 nM to 1 μM of peptide. From various clones and lines directed against NY-ESO-1 157–165 with and without tumor reactivity, the peptide concentration equivalent to naturally expressed peptide complexes on the surface of tumor cells is estimated in the range of only 5–10 nM (Gnjatic *et al.*, unpublished observations). Thus, an efficient vaccination should be able to consistently generate effectors with affinity to their epitope at or below this level.

Many efforts have focused on the use of analog peptides with improved binding and immunogenicity. For peptide 157–165 the cysteine residue in position 165, which serves as an anchor motif for HLA-A2, has been replaced by valine or alanine and were shown to augment the capacity of eliciting strong responses *in vitro* (Chen *et al.*, 2000, 2005a; Dutoit *et al.*, 2002a). It may however not be sufficient, as minor conformational changes may focus the CD8 T-cell response to very high affinity to the modified peptide and distract from the intended cross-reaction on the native peptide, either synthetic or found on tumor cells.

A major factor to consider for increasing the efficacy of vaccine-induced T cells is the role of adjuvant and its capacity to counteract the effect of negative cellular regulation during priming.

G. Precautions and Contraindications: NY-ESO-1 and Regulatory T cells

If the immune system recognizes cancer, why are tumors still there? It has been reported that primary tumor development is affected by $CD4^{+}CD25^{+}$ regulatory T cells, which were originally described as suppressors of autoimmunity (Nishikawa *et al.*, 2005c; Sakaguchi *et al.*, 1995). Our preliminary unpublished data from ovarian cancer patients with NY-ESO-1 antibody (in collaboration with Kunle Odunsi at Roswell Park Cancer Institute) show that there is a high accumulation of CD8 T cells specific for NY-ESO-1 at the tumor site, as evidenced by tetramer staining. Up to 20% of CD8 cells within infiltrating lymphocytes were shown by phenotypic staining to be specific for NY-ESO-1 epitopes, and they were detected directly *ex vivo* without need for expansion (Gnjatic *et al.*, unpublished observations). This is in contrast with frequencies of NY-ESO-1-specific CD8 T lymphocytes from peripheral blood that rarely reach 0.1% in the best cases. However, despite a high frequency, it appears that these cells may be impaired when found locally. They can home to the tumor site, accumulate there but may be in a nonresponsive state since only a fraction of cells are able to respond to direct *in vitro* peptide stimulation. The main candidate for muting the immune response is a high number of $CD4^{+}CD25^{+}$ cells also present in large proportions in most samples analyzed so far. Their simple removal, however, is not sufficient for immediate reversal of nonresponsiveness. Only on culture and cloning do these cells regain their capacity to recognize naturally processed NY-ESO-1 and secrete IFN-γ in response to peptide stimulation. The relation between regulatory T cells and NY-ESO-1–specific effectors is a critical issue for vaccine development and needs to be better understood.

Although T-cell responses to NY-ESO-1 are only found in cancer patients who have antibodies to NY-ESO-1, NY-ESO-1–specific CD4 T-cell precursors actually exist at relatively high frequency in healthy individuals (Danke *et al.*, 2004). They appear to be kept in check by $CD4^{+}CD25^{+}$ regulatory T cells. If these cells are removed, NY-ESO-1 peptide-specific responses are detected after a single *in vitro* stimulation. The repertoire of these regulated T cells specific for NY-ESO-1 is exclusively naïve, including in subsets of cancer patients expressing NY-ESO-1 in their tumor (Nishikawa *et al.*, 2005a). This inhibitory mechanism by regulatory T cells is apparently bypassed when natural immunity occurs, together with the induction of antibodies. In seropositive patients, the majority of CD4 T cells are antigen-experienced and thus also readily detectable even in the presence of $CD4^{+}CD25^{+}$ regulatory T cells. The likely explanation is that a determining priming environment in patients with spontaneous immunity to

NY-ESO-1 led to a full activation and consequently lesser susceptibility to regulatory T-cell action. Understanding the molecular basis of the regulation of NY-ESO-1–specific T cells is a challenge for vaccination strategies and NY-ESO-1 may be a good model to learn how to modulate these cellular interactions.

The specificity of $CD4^{+}CD25^{+}$ regulatory T cells does not appear to be critical in the process. Nevertheless, it is fascinating to note that the first target antigen of regulatory T cells to be reported in humans was LAGE-1, a family member of NY-ESO-1. It was described as a candidate for direct recognition by regulatory T cells from clones derived from TILs of a melanoma patient (Wang *et al.*, 2004a). The generation of CD4 T cells to LAGE-1 with regulatory capacity on the proliferation and activity of other cells is not yet fully understood. Targets of regulatory T cells are generally believed to be self-antigens (Nishikawa *et al.*, 2005b), which may require to be expressed in the thymus (Bensinger *et al.*, 2001; Jordan *et al.*, 2001). Although it is still unclear whether antigen expression in thymus is enough for induction of $CD4^{+}CD25^{+}$ regulatory T cells, the report of NY-ESO-1 and other CT antigens in medullary epithelial cells could offer an explanation for LAGE-1 specific regulatory T cell induction (Gotter *et al.*, 2004). These findings are in stark contrast with the strong immunogenicity of NY-ESO-1 in the subset of NY-ESO-1 seropositive patients. A resulting hypothesis is that a lack of adequate priming during tumor emergence may lead to impaired activation and differentiate antigen-specific CD4 T precursors into cells with regulatory activity.

When looking at the capacity of either LAGE-1 or NY-ESO-1 to induce helper T-cell effectors, the prospect of regulatory T cells could also be influenced by their phylogenetic proximity. The description of LAGE-1–specific T cells with regulatory activity in relation to the frequent coexpression of LAGE-1 with NY-ESO-1 leads to an intriguing hypothesis. Noting the redundancy in sequence homologies between close CT antigen relatives, such as NY-ESO-1 and LAGE-1, one may potentially counteract the other and set a balance in the resulting immunogenicity. If considering a directed evolution of the tumor cell to escape immunosurveillance, LAGE-1 expression could act as the "evil twin" for NY-ESO-1, preventing efficient T-cell antitumor activity to NY-ESO-1 epitopes or even to different regions of LAGE-1 itself. This model would argue that a close relative of an immunogenic antigen may dampen cross-reactive responses. One indirect way to test this theory is to note the apparent lack of LAGE-1-specific antibody response; whereas NY-ESO-1 is occasionally detected by patient sera that do not cross-react with LAGE-1, no responses to LAGE-1 without NY-ESO-1 has yet been observed. Similarly, more detailed analysis of the pattern of NY-ESO-1 and LAGE-1 expression in relation to specific

antibody presence may offer insights in the pathways leading to regulation versus immunosurveillance.

III. CONCLUDING REMARKS

Knowing what to expect is not a luxury when planning immunotherapeutic intervention, and NY-ESO-1 with its strong inherent immunogenicity in a subset of cancer patients has set the standards to reach. With a booming number of clinical trials with NY-ESO-1 as a model antigen, clinical correlations of immunogenicity and patient benefit, if any, should soon be established. Current vaccines still have a lot of room for improvement, both in antigen and in adjuvant formulations. An efficient vaccine for NY-ESO-1 that recognizes tumor cells with high frequency will need to meet the following characteristics: include any potential epitope present within NY-ESO-1, be naturally processed or be available/deliverable to the cytoplasm of antigen-presenting cells, be persistent once injected or repeatedly available for optimal priming (i.e., adjuvant protecting antigen or with depot effect), be primed in an environment preventing the rise of regulatory cells (i.e., usefulness of adjuvants with inflammatory properties). NY-ESO-1 has given us many of its secrets but still has a lot to prove to live up to its full therapeutic potential.

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Order, Disorder, Death: Lessons from a Superorganism

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Animal models contribute to the understanding of molecular mechanism of cancer, revealing complex roles of altered cellular-signaling networks and deficient surveillance systems. Analogous pathologies are documented in an unconventional model organism that receives attention in research on systems theory, evolution, and aging. The honeybee (*Apis mellifera*) colony is an advanced integrative unit, a "superorganism" in which order is controlled via complex signaling cascades and surveillance schemes. A facultatively sterile caste, the workers, regulates patterns of growth, differentiation, homeostasis, and death. Workers differentiate into temporal phenotypes in response to dynamic social cues; chemosensory signals that can translate into dramatic physiological responses, including programmed cell death. Temporal worker forms function together, and effectively identify and terminate abnormal colony members ranging from embryos to adults. As long as this regulatory system is operational at a colony level, the unit survives and propagates. However, if the worker phenotypes that collectively govern order become too few or change into malignant forms that bypass control mechanisms to replicate aberrantly; order is replaced by disorder that ultimately leads to the destruction of the society. In this chapter we describe fundamental properties of honeybee social organization, and explore conditions that lead to states of disorder. Our hope is that this

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chapter will be an inspirational source for ongoing and future work in the field of cancer research. © 2006 Elsevier Inc.

I. ORDER IN LIVING ORGANISMS

Cancer is a disease of genes (Bishop, 1991) that is characterized by loss of intercellular order (Schafer, 1969). Tumor cells emerge through carcinogenic mutations at critical loci that underlie regulation of cell growth and cell death (Ryan *et al.*, 2000; Thompson, 1995). Disorder arises from the uncontrolled behavior of the mutated cell type, which invades neighboring tissues and spreads malignant cells (Karin and Greten, 2005). The tumorigenic process of initiation, promotion, and progression (Hoeijmakers, 2001; Karin and Greten, 2005), thereby, is a complex product of defective control mechanisms that collectively tear down the order of the living organism.

Animal models contribute broadly to insights into the dynamics of cancer (Van Dyke and Jacks, 2002). *Drosophila* researchers have developed tools for rapid characterization of tumor genes (Potter *et al.*, 2000), and forward genetic approaches in the zebra fish have uncovered cancer pathways (Shepard *et al.*, 2005). Studies of the mouse, in particular, have highlighted the intricacy of cancer: unraveling the faceted interface between the tumor cells and their "host" organism, and the subtle genetic interactions that influence individual susceptibility to disease (Balmain, 2002; Van Dyke and Jacks, 2002). Our understanding of the molecular systems that drive the onset and progression of carcinogenesis are thus developing rapidly, while research is uncovering mechanisms that are expected to lead to new paradigms for prevention, diagnostics, and therapy (Hoeijmakers, 2001).

Yet, novel insights into complex phenomena can be constrained by the models that are used also when the accumulation of knowledge in a field is remarkable (Golstein *et al.*, 2003). Work on pending problems can be inspired by findings in distant research areas and the models that turn out to be the most valuable in this context are often the least predictable. Here, we argue that lessons at the intersection between order, disorder, and death can arise from biological systems other than the molecular networks that govern organismal order. Our outline focuses on an unconventional model in medical research—a superorganism.

II. SUPERORGANISMS

A superorganism is defined as "a collection of single creatures that together possess the functional organization implicit in the formal definition of organism" (Wilson and Sober, 1989). The concept emerged from studies of the tightly interlinked colonies of eusocial insects: the ants, wasps,

bees, and termites (Wheeler, 1911, 1928). Societies of eusocial species are characterized by differentiation of individuals into reproductive and facultative or functionally sterile forms called castes. Colonies consist of numerous overlapping generations of such individual forms that function as an integrative whole; a unique biology that in many aspects mimics the basal anatomy of multicellular organisms (Wilson, 1985). By adopting a definition of Metazoan animals as "composed of cells arranged in at least two nonuniform layers and differentiated into reproductive and somatic cells with different functions" (Kaestner, 1969), the reproductive castes become analogous to the germ line, and the sterile forms become analogous to the soma (Wilson, 1971, 1975). Like somatic cells, the sterile individuals further specialize through processes of differentiation, and communicate to produce coordinated patterns of growth, homeostasis, provisioning, and defense before death.

The usefulness of the superorganism analogy has been debated, in particular, with respect to natural selection on the level of social groups (Mitchell, 2003). However, studies of the organizational principles of social insect societies have led to deep insights into the emergent properties of biological systems (Camazine *et al.*, 2001; Fewell, 2003; Hölldobler, 1995; Theraulaz *et al.*, 2002), and research on the underlying modes of interindividual communication has inspired the design of control algorithms that exhibit high degrees of flexibility and robustness (Bonabeau *et al.*, 2000). Thus, although comparisons between the evolution and development of multicellular creatures and superorganisms are controversial (Mitchell, 2003; Wilson, 1975; Wilson and Sober, 1989), the highly integrative societies of insects are recognized systems for understanding how complex order arises from the actions of individual parts (Fewell, 2003).

The remarkably coordinated and manifold activities of ant, wasp, bee, and termite societies have long been subject to curiosity and awe (Mitchell, 2003). This focus may explain why the dynamics of disorder have received less general attention than the principles that govern the exceptional order of these systems. Initiation, promotion, and progression of states that tear down the well-organized structure of superorganisms nevertheless have been examined, and it is possible that deeper knowledge of such states can lead to conceptual insights of heuristic value. In the following, we take a first step toward such insights by reviewing features of the biology and pathology of the honeybee (*Apis mellifera*), the best studied eusocial invertebrate to date.

III. HONEYBEE SOCIETY

The honeybee is one of several social bees of the genus *Apis*. It is widely domesticated as a source of honey and beeswax and a well-known research system for understanding the characteristic differentiation of eusocial

individuals into reproductive and sterile forms (Amdam *et al.*, 2004a; Bloch *et al.*, 2002; Robinson *et al.*, 2005). The bee is an established model in systems theory (Mitchell, 2003; Page and Erber, 2002), behavioral ecology (Seeley, 1995), neurobiology (Farooqui *et al.*, 2004; Humphries *et al.*, 2003; Menzel, 1979; Scheiner *et al.*, 2001), and aging (Amdam and Page, 2005; Amdam *et al.*, 2005; Omholt and Amdam, 2004; Seehuus *et al.*, 2006). It has a rising position in molecular research that is fueled by an emerging availability of functional genomic tools (Aase *et al.*, 2005; Amdam *et al.*, 2003; Beye *et al.*, 2002; Omholt *et al.*, 1995; Yu *et al.*, 1997) and a newly annotated genome sequence (www.hgsc.bcm.tmc.edu).

A. Anatomy of Individuals

Honeybees are haplo-diploid (males are haploid). Their sex is determined by a single gene, *csd*, which has structural similarity to the *tra* genes of dipteran insects (Beye *et al.*, 2003). Females are derived from fertilized eggs and are heterozygous at the *csd* locus, whereas males typically develop from unfertilized eggs and are hemizygous at the *csd* locus. Diploid males occur when the locus is homozygous (Santomauro *et al.*, 2004).

Honeybee females differentiate into two distinct forms: the queen (Fig. 1A) and the worker (Fig. 1B). The queen's anatomy is characterized

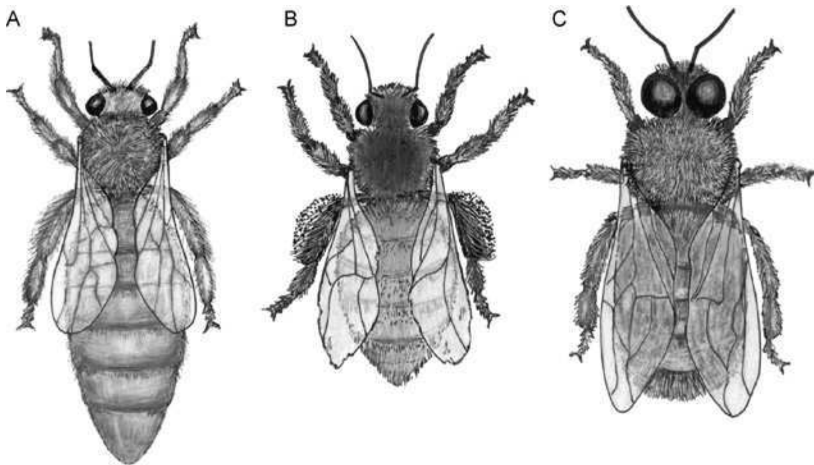

Fig. 1 The castes of the honeybee. There is only one queen (A) in a honeybee colony. She is the dominant reproductive female and can live up to 3–5 years. The worker (B) is a facultatively sterile female with a typical lifespan of 4–6 weeks. A colony normally consists of 10,000–30,000 worker bees. The male caste, or drone (C), is only raised under favorable conditions when there may be 300–3000 drones present in the society. Few drones survive more than 4–5 weeks.

by an enlarged ovary (180–200 ovarioles per ovary) and a sperm-storage organ, the spermatheca, that can store over 5 million spermatozoa (Kraus *et al.*, 2004; Snodgrass, 1956). The end stylet of the queen's stinging apparatus is smooth so it can be retracted after stinging, and the queen produces a specific signature of C21–C33 cuticular hydrocarbons in addition to a pheromone blend from enlarged glands in the head (mandibular gland), abdomen (Dufour and tergite gland), and tarsus (tarsal gland); major components being (*E*)-9-oxodec-2-enoic acid, (*E*)-9-hydroxydec-2-enoic acid, methyl *p*-hydroxybenzoate, and 4-hydroxy-3-methoxyphenylethanol (Free, 1987; Keeling *et al.*, 2003).

The worker (Fig. 1B) has 2–16 ovarioles per ovary and no spermatheca. The end stylet of her stinger has series of recurved barbs that ensures that the stinger and venom sac is anchored in the enemy after stinging. The worker has a specialized third hind-leg segment, called the corbicula or "pollen basket," in which pollen and propolis is carried to the hive. She also has a set of paired head glands, the hypopharyngeal glands, that during the worker's temporal nurse bee stage (Section IV) produces a proteinaceous jelly used for brood rearing (royal jelly). The jelly contains proteins of the MRJP/yellow family, sugars, lipids, vitamins, and free amino acids; and has fractions with disinfectant, antitumor and anti-inflammatory activities (Kamakura and Sakaki, 2005). The worker bee has two specialized abdominal glands: the wax gland and the Nasanov gland. The latter gland releases an attractant aggregation pheromone in response to disturbances; major components being geraniol, nerolic acid, and geranic acid (Winston, 1987). Furthermore, the stinger discharges alarm pheromones (primarily isoamyl acetate) after it has been embedded in the opponent. The worker bee is characterized by a stage-specific profile of C21–C33 cuticular hydrocarbons and pheromones, including eicosenol, ethyl oleate, and 10-hydroxy-decenoic acid that changes as a function of the social role of the animal (Chaline *et al.*, 2005; Dor *et al.*, 2005; Leoncini *et al.*, 2004a,b).

The males, or "drones" (Fig. 1C), do not have a stinger because this character evolved from the female ovipositor. Drones also lack specialized body parts for food collection (Snodgrass, 1956) and are easily recognizable with broad thoraxes, rectangular abdomens, and enlarge compound eyes. They are designed to perform one successful mating flight, and the act of mating is fatal to the drone (Winston, 1987). Diploid drones are normally recognized and selectively killed by workers shortly after hatching from the egg (Section V). *In vitro* rearing studies indicate that these males are characterized by small testes and diploid spermatozoa (Herrmann *et al.*, 2005). For further details on honeybee anatomy, see work by Snodgrass (1956).

B. Anatomy of the Colony Unit

The honeybee colony dwells in a nest cavity reinforced by the resinous substance propolis (Fig. 2). Brood (eggs, larvae, and pupae) and hoarded foods (honey and pollen) are located in the main nest structure, the wax combs. The combs are arranged in sheets and the nest is organized spatially with the brood located centrally (Winston, 1987). A controlled amount of pollen (Section IV) is stored close to the brood. Pollen is the main amino acid source for the honeybees. Honey is stored in surplus at the periphery of the wax combs and is the society's source of carbohydrates. The pollen, honey, and propolis resin of the nest may, in addition to the jelly, be part of a collective antibacterial defense system. Active compounds are, for example, chrysin and cinnamic acid derivatives, such as baccharin and drupanin, that have growth-inhibitor and also antitumor activities (Mishima *et al.*, 2005). The central nest is thermoregulated at 33°C and 80% relative humidity (Section IV), which is optimal for the growth and development of the brood.

The queen is most frequently found in the central nest along with the drones and young worker bees. The workers feed developing larvae, and they are responsible for colony hygiene, comb construction, and food processing (Winston, 1987). Later in life, worker bees make a transition from nest tasks to guarding and foraging outside the colony (Seeley, 1982).

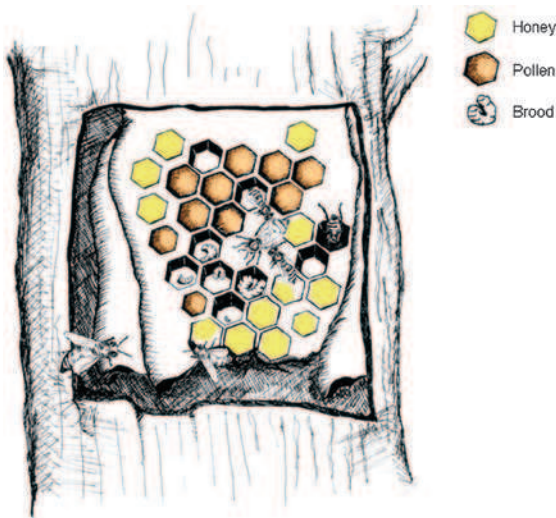

Fig. 2 The honeybee colony—schematic illustration of a feral nest inside a trunk cavity. The cross-section shows the spatial arrangement of wax-cells of honey at the periphery and a pollen storage close to the central brood nest.

After this shift, they are seldom observed performing tasks within the nest other than those directly related to foraging such as unloading pollen and nectar and performing recruitment dances. When agitated by alarm pheromones, though, workers of diverse ages and functions can be recruited to the colony's strong defensive response (aggressive stinging). The overall anatomy of the colony is maintained through the temporal and spatial division of labor between the workers; a pattern of organization that, in principle, arises through the emergence of new adults in the central brood nest (Fig. 2) that replaces the bees that shift to tasks in the periphery and eventually die.

IV. REGULATION OF DIFFERENTIATION, GROWTH, AND HOMEOSTASIS

The female forms (Fig. 1A and B) are the fundamental components that underlie superorganismal order in the bee because the drones do not have colony-level functions other than the act of mating. Note, however, that this phenomenon is specific to the Hymenoptera lineage of social insects. In the termites, Isoptera, males and females are diploid, and both sexes have worker and reproductive castes.

A. Differentiation of Female Forms

Segregation of female phenotypes occurs at two levels of biological organization in the honeybee superorganism. A developmental bifurcation separates the queen caste from the worker caste during the larval stage, and adult regulatory pathways subsequently drive the temporal specialization of the mature worker bees.

The caste trajectories of the queens and workers are decoupled during the third larval instar via a feeding-sensitive endocrine switch (Fig. 3). Differentiation, thereby, is ultimately determined by social nursing regimens that are enforced by the adult worker population (Winston, 1987). Ample feeding of proteinaceous jelly to a larva triggers an increase in the circulating titer of the systemic hormone "juvenile hormone" that is secreted by the corpus allatum complex (Hartfelder and Engels, 1998). The resulting signaling cascade gives rise to a queen phenotype. Poorer dietary conditions that result from reduced feeding suppress juvenile hormone signaling and trigger programmed cell death in the developing ovary (Capella and Hartfelder, 1998). The endocrine shift changes global gene expression patterns (Evans and Wheeler, 1999) and yields a worker

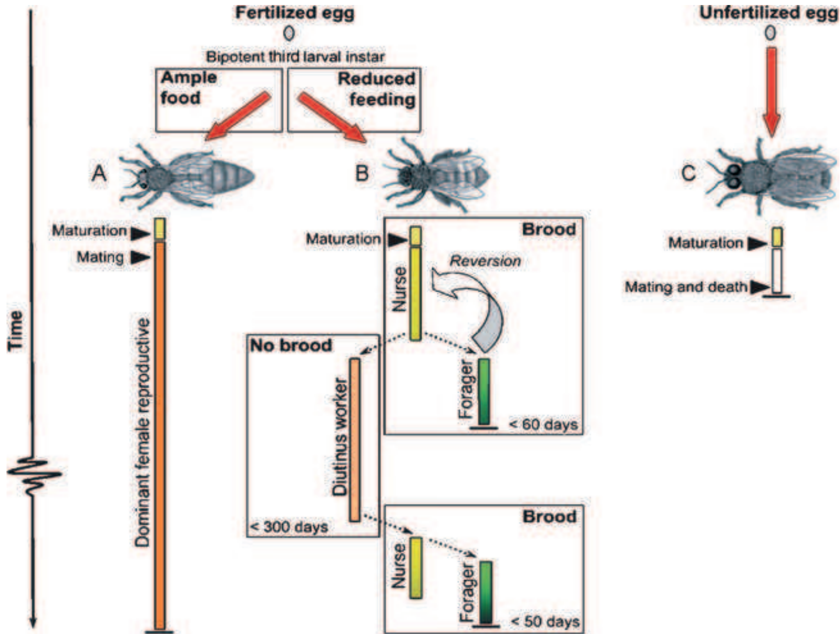

Fig. 3 Differentiation of honeybees castes and further specialization of workers. After hatching, a diploid honeybee larva can either become a worker or a queen. The differentiation depends solely on nourishment provided by adult workers during the first days of larval life. Ample feeding leads to a queen phenotype (A). Newly emerged virgin queens go through a maturation period before they mate and become the dominant reproductive female of a nest. Reduced feeding of larvae leads to a worker phenotype (B). After maturation, the worker enters the temporal nurse bee stage (duration of this stage is typically in the range of 7–40 days). Nurse bees can differentiate into foragers or diutinus workers depending on whether brood is present in the colony. As a forager, the bee collects resources in the field (duration of this stage is about 7–21 days), but may also revert to nest tasks if many nurse bees are removed from the colony. As a diutinus worker, the bee survives for several months (up to 280 days) before she differentiates into a nurse or a forager (Omholt and Amdam, 2004). The males, or drones (C), are haploid and die as soon as they have mated with a virgin queen.

phenotype characterized by half the body weight of a queen (a queen is ~200 mg). The role of feeding in caste differentiation has been linked to a proposed growth factor- or hormone-like activity of the honeybee jelly protein p56kP-4, which has close homology to *Drosophila* Yellow protein (Kamakura and Sakaki, 2005).

The adult workers differentiate into three temporal forms referred to as the "nurse bee," the "forager," and the "diutinus worker" stages, respectively (Amdam and Page, 2005). The nurse bee (Fig. 3) is the phenotype that performs tasks within the nest, and the majority of workers mature into nurse bees within 3–4 days after adult emergence (Naiem *et al.*, 1999).

They become characterized by enlarged jelly-synthesizing hypopharyngeal glands, hypertrophied abdominal fat bodies (analogous to the mammalian liver and adipose tissue), and they also have a high number of circulating hemocytes (immune cells). Like T cells, insect hemocytes have several important immunological functions in nonself recognition, phagocytosis, encapsulation, nodulation, and wound repair (Millar and Ratcliffe, 1994), and the circulating number reflects the organism's capacity to cope with immunogenic challenges (Doums *et al.*, 2002; Kraaijeveld *et al.*, 2001). Severe infections, that is, more than 1000 microbes per microliter hemolymph (blood), thus, rarely occur in nurse bees (Amdam *et al.*, 2005).

Foragers (Fig. 3) typically develop from nurse bees (Seeley, 1982). They work in the nest periphery and are responsible for collecting nectar, pollen, water, and propolis in the field. Workers in this temporal stage secrete low levels of digestive endopeptidases and are unable to obtain amino acids from the colony's pollen store (Crailsheim, 1990). The protein intake of a forager, therefore, is controlled by the nurse bees that feed the forager in proportion to her foraging activity level (Crailsheim *et al.*, 1999). A forager is characterized by atrophy and apoptosis of the hypopharyngeal glands (De Moraes and Bowen, 2000) and the abdominal fat body (Seehuus and Amdam, unpublished data). She has very few circulating hemocytes (Rutz *et al.*, 1974; Wille and Rutz, 1975) as the immune cells also apoptose during the transition from nurse tasks to foraging activities (Amdam *et al.*, 2005), see Section V for mechanisms. The differentiation of nurse bees into foragers is driven by social signals (Section IV). At the physiological level, these signals modulate a positive regulatory feedback loop between the *vitellogenin* gene, which is expressed in the fat body, and juvenile hormone (Amdam and Omholt, 2003; Guidugli *et al.*, 2005). Specifically, *vitellogenin* gene activity suppresses the endogenous juvenile hormone level during the nurse bee stage, whereas reduced signaling from the *vitellogenin* gene releases juvenile hormone activity during the forager differentiation (Guidugli *et al.*, 2005). This endocrine signal feeds back to the regulatory system to further inhibit the synthesis of vitellogenin protein (Pinto *et al.*, 2000), shift the gene expression pattern of the hypopharyngeal glands (Ohashi *et al.*, 1997), and suppress immunity (Amdam *et al.*, 2005; Rutz *et al.*, 1974; Wille and Rutz, 1975).

Diutinus workers (Fig. 3) develop during unfavorable periods without opportunities for brood rearing (Amdam and Page, 2005; Maurizio, 1950; Omholt and Amdam, 2004). They differentiate from nurse bees that no longer take care of brood (Amdam and Omholt, 2002; Huang and Robinson, 1995; Omholt and Amdam, 2004). A diutinus worker is characterized by hypertrophy of the hypopharyngeal glands and the abdominal fat body (Deseyn and Billen, 2005; Koehler, 1921; Maurizio, 1954). She has a high number of circulating hemocytes, an elevated vitellogenin protein

level, and a low juvenile hormone titer (Amdam *et al.*, 2004b; Fluri *et al.*, 1977, 1982). The diutinus stage is described as an oxidative stress-resistant survival form similar to the dauer larva of *Caenorhabditis elegans* (Seehuus *et al.*, 2006). Diutinus workers develop the physiological and behavioral attributes of nurse bees and foragers when the queen starts to lay eggs at the onset of the next favorable season (Maurizio, 1954; Sekiguchi and Sakagami, 1966).

The temporal differentiation of honeybee workers typically follows the trajectory in which the forager stage follows the nurse stage, as described earlier. Yet, worker bees also have the unique plasticity to revert (Huang and Robinson, 1996; Robinson *et al.*, 1992). This implies that differentiated foragers can go through a physiological reversion (Section IV)—an ontogeny (Fig. 3) that is characterized by reduced juvenile hormone signaling, elevated vitellogenin protein activity (Amdam *et al.*, 2005), reactivation of the hypopharyngeal glands (Amdam *et al.*, 2005; Huang and Robinson, 1996), and reversal of immunosenescence (Amdam *et al.*, 2005).

B. Colony Growth

The honeybee superorganism reproduces as a unit through fission (swarming) and the production of drones. The process of swarming is initiated by workers that appear to respond to a queen-specific pheromone signal that diminishes as a function of colony size. Explicitly, circulation of queen pheromones is a continuous process mediated by food exchange (trophallaxis) and physical contact between workers as well as volatile transmission (Winston, 1987). This distribution process becomes less efficient as the colony grows in size. As the signal from the queen weakens, workers start to rear new queen larvae (Baird and Seeley, 1983). One of the resulting virgin queens takes over the maternal nest while the old queen leaves in a swarm with the majority (50–90%) of the workers to establish a colony at a new nest site (Severson, 1984). The virgin queen uses her retractable stinger to kill all other developing queens (Fig. 4A). Subsequently, she engages in one or several mating flights before she takes over the role as the dominant reproductive female in the old nest.

After swarming, the colony grows through the reproductive effort of the queen and the alloparental investment of the workers. The queen may produce up to 1500 eggs daily, and thus if resources for brood rearing are abundant the colony can grow rapidly (Winston, 1987). Growth continues until the society once more is split by swarming or until the onset of the unfavorable season when the rearing of brood ceases and the stress-resistant diutinus workers emerge. The next favorable period triggers a phase characterized by exclusive production of worker bees that strengthen the

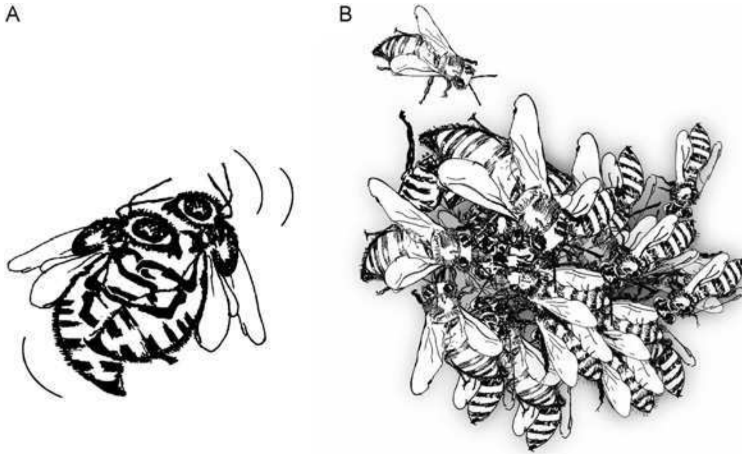

Fig. 4 Regulation of death. The illustrations show the lethal fight between two virgin queens (A), and the excessive coating response of honeybee workers that have targeted an adult individual for termination (B). The undesired individual in the center dies of thermal stress.

colony's work force before drones are reared close to the peak of the colony cycle. Through these simple mechanisms, the development of the superorganism and its offspring societies is regulated.

C. Colony Homeostasis

Over the course of the favorable period, the allocation of workers to the nurse bee stage and the forager stage is not under centralized control. Rather, a dynamic differentiation emerges through social signals produced by the workers themselves. The foragers synthesize a blend of cuticular hydrocarbons and pheromones that are specific to their stage (Leoncini *et al.*, 2004b; Pankiw, 2004a). This signature is transferred by physical contact with the nurse bees (Huang and Robinson, 1992). Nurse bees and foragers antennate, groom, and feed each other (Winston, 1987), and though these social transfers the nurse bees can identify the relative size of the forager population (Huang and Robinson, 1996). As foragers are lost, consequently, nurse bees perceive the decline and some differentiate into foragers to take the place of those that have vanished. Likewise, foragers recognize the presence of the nurse bees, and an experimental removal of the nurse bee population triggers a fraction of the forager population to revert to nurse tasks (Amdam *et al.*, 2005; Huang and Robinson, 1996; Robinson *et al.*, 1992; Toth and Robinson, 2005). Individual response thresholds to social signals probably constitute one factor that determines

if a particular bee differentiates in response to demographic changes (Amdam and Omholt, 2003).

The amount of stored pollen in the colony (Fig. 2) is also under dynamic decentralized control. Returning pollen foragers deposit their loads directly into empty cells or cells containing pollen close to the area of the nest where the young larvae are raised. Stored pollen is consumed by nurse bees that convert it into the proteinaceous secretions of the hypopharyngeal glands (Crailsheim *et al.*, 1992). Stored pollen inhibits pollen foraging, while pheromones produced by larvae (methyl palmitate, methyl stearate, methyl linoleate, methyl linolenate, methyl oleate, ethyl palmitate, ethyl stearate, ethyl linoleate, ethyl linolenate, and ethyl oleate) (Slessor *et al.*, 2005) stimulate pollen foraging (Fewell and Winston, 1992; Pankiw *et al.*, 1998). Colonies, therefore, regulate the amount of stored pollen probably through a combination of the inhibiting effects of pollen and stimulating effects of brood pheromone. This regulatory scheme, per se, is put into operation by the workers that specialize on pollen collection. In response to accumulation of pollen in the nest and reduced pheromone signaling from brood, these foragers collect smaller pollen loads or stop foraging altogether (Fewell and Winston, 1992). Equilibrium pollen intake thus equals the pollen consumption that meets the protein demands of the developing larvae. Remarkably, the workers' sensory perception of this equilibrium is determined genetically, and colonies can be selected for high and low levels of pollen hoarding (Page and Fondrk, 1995).

The temperature and humidity of the central brood nest is similarly regulated through the behavior of individual worker bees (Jones *et al.*, 2004). In response to environmental variability (Schmickl and Crailsheim, 2004), workers in the nest fan their wings (cooling), vibrate their thorax muscles (warming), or collect water that is deposited on the combs (cooling and humidifying). Individual response thresholds to environmental fluctuations also have a strong genetic component (Jones *et al.*, 2004). In this connection it has been proposed that the polyandrous mating biology of the honeybee queen promotes environmental homeostasis because it generates a diversity of worker genotypes that produces a fine-tuned rather than an excessive colony-level response to variations. As a whole, the social unit is a homeothermic superorganism (Grodzicki and Caputa, 2005).

V. REGULATION OF DEATH

As in multicellular organisms, order emerges in the honeybee society at the intersection between growth, differentiation, and death. The colony can regulate the lifespan of its members in two different ways. One mechanism

is the programmed life termination that is built into the physiology of the temporal forager stage. The other approach is the targeted killing of abnormal and disposable colony members, which is a mechanism that is under social decentralized control.

A. Programmed Disposal of Soma

Reduced rates of tactile interactions between nurse bees and foragers cause the hemocytes, or immune cells, of the nurse bees to terminate through programmed cell death (Amdam *et al.*, 2005). This response is one component of the workers' transition from nest tasks to foraging activities (Section IV). The loss of hemocytes is associated with a complete deterioration of the nodulation immune response (Bedick *et al.*, 2001), a predominant defense reaction to infection in insects (Franssens *et al.*, 2005). Severe infections are more frequently observed in foragers than in nurse bees (Amdam *et al.*, 2005), and in general terms increased susceptibility to pathogens and toxins is a characteristic of the forager stage (Meled *et al.*, 1998). This frailty is nonintuitive from the perspective that foragers, to a much larger extent than other workers, encounter dangerous substances as they engage in numerous foraging trips outside the protected nest: flowers have rich faunas of bacteria and fungi, and some pollens and nectars are poisonous to bees (Maurizio, 1950, 1954).

Having a robust forager caste may not be advantageous for the society, however. If resistant foragers repeatedly brought virulent strains of bacteria and fungi as well as loads of poisonous nectars to the nest, the situation could soon become disastrous for the superorganism. The increased frailty of honeybee foragers, therefore, can be interpreted to reflect a colony-level selection pressure for disease control. Foragers typically return to the same source of pollen or nectar over the course of days (Seeley, 1995) and in being strongly susceptible to substances that potentially could harm the colony unit, an individual forager dies before she can bring substantial amounts of contaminants to the hive. A wide range of physiological stressors, such as cooling, oxygen deprivation, mechanical wounding, and parasite exposure, cause nurse bees to differentiate into foragers (Bühler *et al.*, 1983; Ebadi *et al.*, 1980; Kovac and Crailsheim, 1988; Tustain and Faulke, 1979). Thereby, individuals in poor condition can also be driven into a physiological state in which they rapidly perish (Amdam *et al.*, 2004b).

From a regulatory perspective, the programmed termination of the cellular immune system of the bee is astonishing because it in essence implies that an apoptosis pathway can be conditionally activated upon loss of a social signal, that is, the forager signature of hydrocarbons and pheromones. The response can be provoked in the lab by placing a nurse bee in an isolated

cage, and although the bee is provided with ample pollen and nectar she will not survive for many days (Maurizio, 1950). Unfortunately, knowledge on the underlying molecular cascade is limited. What has been determined, however, is that the immune cells of the honeybee are sensitive to the level of zinc in the hemolymph. Programmed cell death is triggered *in vitro* if hemocytes are cultured in a zinc-deprived medium (Amdam *et al.*, 2004b). Remarkably, the zinc concentration of worker bee hemolymph is a function of the bee's social role, so that foragers have much lower levels (0–4 ppm) than nurse bees and diutinus workers (up to 28 ppm). The vitellogenin molecule appears to be the major circulating zinc ligand in the bee (Amdam *et al.*, 2004b), and thus the dramatic decline in the hemolymph zinc concentration of new foragers can be linked to the feedback suppression of vitellogenin synthesis that occurs as part of the workers' transition from nest tasks to foraging activities (Section IV). This scenario suggests that hemocyte apoptosis, and possibly also patterns of programmed cell death in the hypopharyngeal glands (De Moraes and Bowen, 2000) and abdominal fat body (Seehuus and Amdam, unpublished data), are downstream of a causal chain in which social deprivation triggers a decline in *vitellogenin* gene activity. At a mechanistic level, the inhibitory effect of vitellogenin-derived zinc availability on hemocyte apoptosis may relate to the finding that zinc also inhibits activation of apoptotic caspases 9 and 3 in cultured cancer cells (Zhao *et al.*, 2004).

B. Collective Termination of Colony Members

Adult worker honeybees have an astonishing ability to detect abnormal character states in the developing brood and their sister workers. Under certain conditions the worker bees also determine that the drones and the queen are no longer productive members of the colony unit. These collective insights unfold as swift responses in which one or several workers target an individual for immediate termination.

1. KILLING OF DEVELOPING BROOD

The ovary of the honeybee worker is typically inactive due to suppression of oogenesis by pheromones from the brood (Winston and Slessor, 1998) and the queen's mandibular gland (Hoover *et al.*, 2003). Yet, a worker bee will occasionally produce an egg and deposit it in the nest. This egg is unfertilized and can develop into a normal haploid drone. Few if any such drones reach adulthood, however, because the worker population has the collective ability to identify and destroy the worker-laid eggs (Ratnieks, 1993). Numerous nurse bees inspect the nest daily by inserting their head

and thorax into brood cells, and if they determine that an egg is of worker origin, they eat it. Workers probably establish the source of an egg on the basis of substances that are deposited on the egg's surface when it is laid (Katzav-Gozansky *et al.*, 2001). The queen's eggs have a more complex hydrocarbon coating than worker-laid eggs, and acetates of fatty alcohols, alkenes, and monomethylalkanes are characteristic to eggs that are laid by a queen (Katzav-Gozansky *et al.*, 2003).

Diploid males, which occur when the sex-determining *csd* locus of the bee is homozygous (Section III), constitute another group that is targeted for termination. Diploid male larvae are viable but are recognized and killed as soon as they hatch from the egg (Woyke, 1980). Adult worker bees identify these drones based on their cuticular hydrocarbon profile. The cuticular profile of newly hatched honeybee larvae is mainly composed of alkanes and squalene, but the diploid drones, the diploid females, and the haploid drones produce these compounds in different amounts (Santomauro *et al.*, 2004). The signature of the diploid females and the haploid drones are accepted by the workers whereas the profile of the diploid males is not.

Odors are also used by the worker bees to detect brood that is infested with pathogens. Sensitivity to these olfactory cues are enhanced by the neuromodulator octopamine (Spivak *et al.*, 2003), and amazingly workers can recognize odors specific to infested brood though the wax capping that is laid down over the comb when bee larvae pupate (Gramacho and Spivak, 2003; Spivak, 1996). When a diseased individual is detected, the workers collectively uncap the brood cell, pull the pupae out of the brood nest, and dispose it of outside the colony (Boecking and Spivak, 1999). The bee's ability to discriminate between olfactory signatures may be of relevance to research on the use of odors in tumor diagnostics (Yamazaki *et al.*, 2002). Honeybee olfactory sensitivity is remarkable, and bees can easily be trained to discriminate between odors that are presented to them (Masterman *et al.*, 2001). Simplified, a bee sticks out her tongue in a "proboscis extension reflex" (PER) when she perceives a specific odor that previously has been paired with a sugar reward. Most bees respond with PER to the odor alone after as little as two to five conditioning trials where the odor and the sugar reward are presented together (Gerber *et al.*, 1998).

2. STINGING, "COATING," AND EVICTION OF ADULTS

Worker bees are wary of other adults, and if a worker encounters an individual that is not acceptable to her, she takes action (Winston, 1987). The probability of launching such attacks is stage dependent, and nurse bees and diutinus workers are less hostile than bees that typically work in the periphery of the nest (Pearce *et al.*, 2001). The victim is usually a worker in poor condition, one with developed ovaries (a "laying worker") or one that

has lost her way while foraging and entered a colony that is not her own (Pearce *et al.*, 2001; Ratnieks, 1988; Winston, 1987). Such individuals are most likely singled out based on deviations in the hydrocarbon blend of their cuticle (Dani *et al.*, 2005).

The assault unfolds as the attacker bites and tries to sting the worker that is perceived as undesired or foreign. The attacker will attempt to maneuver the bee toward the colony entrance, but the victim will try to flee from its attacker. During this initial phase, however, the target is marked by alarm pheromones that are secreted by the attacker (Pettis *et al.*, 1998). Analogous to effects of cytokines secreted by T cells, the pheromones agitate other workers and attract them to the scene. Thereby, a "coat" forms around the victim that is stung to death or bitten until she is defenseless and can be disposed of. Sometimes the coat grows so large that it takes the shape of a ball of bees that surrounds the target (Fig. 4B). In this case, the attackers do not sting but instead collectively increase the temperature inside the coat until the undesired individual in the core dies of thermal stress (Ken *et al.*, 2005). This defense strategy is also used when predators, such as hornets, attempt to enter the honeybee nest.

Another well-described phenomenon is the massacre of the drones (Maeterlinck, 1901). As the favorable season comes to an end and there are no more virgin queens to be mated, the males are no longer tolerated by the colony. The massacre starts suddenly, and the situation escalates rapidly over the course of a day until all the males in the colony unit are under forceful attack. Without a stinger the drones are defenseless, and they are chased around and bitten before they are dumped on the ground outside the hive. Those that are able to make it back are rejected by the worker bees that guard the entrance of the nest, and soon these last males starve or freeze to death.

Likewise, the queen is not protected from the collective surveillance schemes that are enforced by the workers. The life of the queen is inevitably over when her storage of sperm becomes depleted or when her pheromone blend no longer is perceived to be an acceptable signal of fertility and health. Under these conditions, the worker bees rear a replacement virgin without swarming. The old queen may continue to lay eggs while the new queen is developing, but she is eliminated when the virgin queen has mated and begun laying eggs (Winston, 1987).

VI. ORDER, DISORDER, AND DISEASE

Through the mechanisms that are described in the previous section, superorganismal order arises in the honeybee society. The living parts of the colony are constantly replaced through differentiation and death.

Homeostasis is maintained, growth and reproduction is regulated, colony defense systems are in operation, and unproductive individuals are not permitted to become a burden on the society. As in multicellular life forms, however, these states of order can be replaced by states of disorder that ultimately lead to the destruction of the superorganism.

A. Growth Disorder

The size of the worker population is a critical variable that underlies the dynamic organization of the honeybee society (Jeffree and Allen, 1956). Order can be replaced by disorder, consequently, if the number of viable and diverse worker phenotypes declines. Irreversible failure to keep up the production of new worker bees can occur as a result of insufficient sperm transfer to the queen's spermatheca after the completion of her mating flights, and may also arise because of inbreeding.

Postmating sperm migration from the queen's lateral oviducts into the spermatheca is a wasteful process, and the polyandrous mating strategy of the honeybee may be a means of which to ensure that the spermatheca becomes adequately filled (Kraus *et al.*, 2004). If this filling is incomplete, it is probable that the queen's sperm store is depleted prematurely. Once the availability of sperm decreases to critical levels, the nest will soon contain an increasing proportion of unfertilized eggs that develop into haploid drones. It is unlikely that the worker population will attempt to replace the queen with a new virgin when this situation arises during the queen's first year of life (Collins, 2004), see also Section V. Consequently, colony growth is repressed dramatically.

Growth is also inhibited in inbreed populations, which are characterized by reduced brood viability (Beye *et al.*, 2003). Homozygosis at the *csd* locus is one factor that contributes to reduced amounts of worker brood in inbred groups. Yet, in addition to the constraints that emerge from production of diploid drones (Section V), reduced viability is also found for immature females of inbred strains (Fuchs and Schade, 1994). These individuals develop growth abnormalities (Simões, personal communication) that in many cases resemble tumorous outgrowths (Fig. 5). Due to the surveillance systems of the colony (Masterman *et al.*, 2001), however, these embryos that die within the egg membrane or hatch as abnormal larvae are efficiently removed. Larvae with growth abnormalities, therefore, are typically only observed in experiments that involve *in vitro* rearing (Bergem and Kaftanoglu, personal communication). These setups have further shown that embryos exposed to UV light develop morphological characteristics similar to some of those observed in inbred strains (Figs. 5A vs 6B). UV light is a key environmental carcinogen (Maglio *et al.*, 2005). Therefore, it may

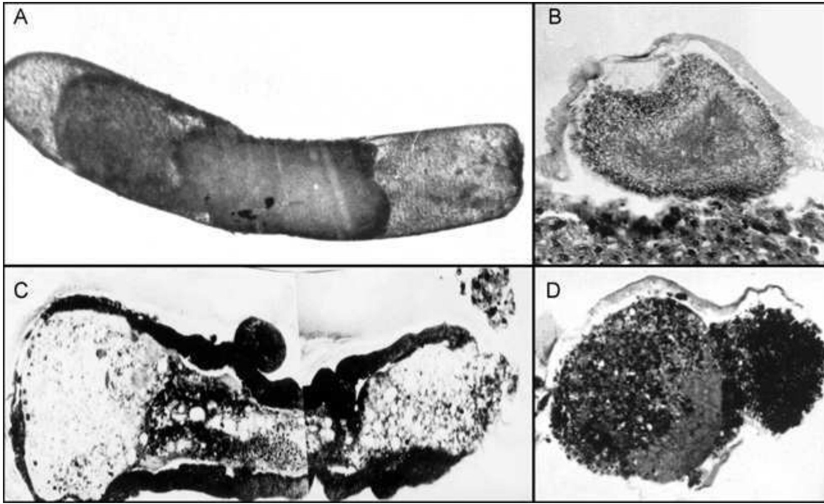

Fig. 5 Growth abnormalities in embryos of an inbred strain. Embryos are stained with hematoxylin/eosin. Abnormal development within the egg membrane (A) (photo at 10×) can lead to death of the embryo and the egg never hatches. An older embryo shows severe growth abnormalities (B) (two superimposed photos at 16×) with tumor-like outgrowths (C and D) (close ups at 40×). Resulting larvae may survive for a limited time *in vitro* (M. Bergem, personal communication). In the colony setting, similar individuals appear to be rapidly killed by adult workers. Image courtesy of Z. L. P. Simões.

deserve further attention that UV exposure probably induces mutations (Fig. 6) that worker bees can detect by use of olfaction.

Several mechanisms fundamental to the emergent order of the honeybee society fail in the small units that develop as the number of worker bees declines. The colonies' defense systems are destabilized (Schneider and McNally, 1992), the workers become more accepting of abnormal individuals including diploid drones (Polaczek *et al.*, 2000), the colonies' foraging efforts are less efficient (Eckert *et al.*, 1994), and the ability to regulate the nest environment can collapse (Omholt, 1987). The proximate mechanisms responsible for the collective loss of surveillance functions are not well understood compared to the principles that underlie the foraging and thermal properties of healthy honeybee colonies (Anderson and Ratnieks, 1999; Bourke, 1999; Eckert *et al.*, 1994; Free and Racey, 1968; Fukuda, 1983; Seeley, 1995; Wilson *et al.*, 1999). Yet, it is clear that features of the social connectivity of the honeybee society are compromised in units that are very small (Villumstad, 1977). Associated weakening of colony-level functions, such as the defensive response toward abnormal or foreign individuals, increase susceptibility to diseases and raids against the colony's food stores (Atkins *et al.*, 1975; Morse and Nowogrodzki, 1990; Winston, 1987).

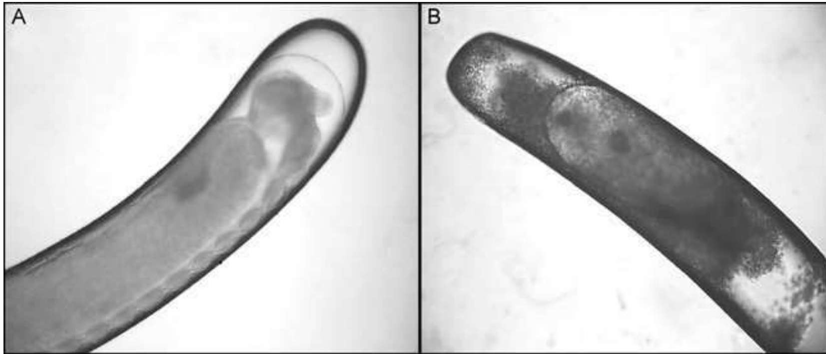

Fig. 6 Embryos that were left untreated or UV irradiated (0.1 second at 1 J/cm^2) when 4-hour old (photos at 10×). Untreated honeybee embryos (A) show normal development 54 hours after collection, whereas irradiated embryos (B) after 54 hours develop abnormally. Image courtesy of M. Bergem.

Loss of homeostatic mechanisms further enhances the risk of superorganismal death in response to changes in ambient conditions (Lee and Winston, 1987).

B. Social Cancer

The reproductive capability of individual worker bees is normally suppressed by pheromone-driven inhibition of oogenesis and nest surveillance schemes enforced by the worker population (Section V). This system can be compromised, however (Martin *et al.*, 2002). The order of the honeybee society is subsequently torn apart by uncontrolled replication of a malignant worker phenotype, a situation that is comparable to a lethal social cancer (Oldroyd, 2002).

Uncontrolled worker replication is initiated if an *Apis mellifera capensis* (Cape honeybee) worker is able to enter an *Apis mellifera scutellata* (African honeybee) host colony. No special mechanisms exist that allow *A. m. capensis* workers to enter the nest, but a key difference between *A. m. capensis* and all other honeybee subspecies is that the worker caste can reproduce via thelytokous parthenogenesis (Ratnieks, 1988). Thereby, if an unmated worker lays an egg, it can develop into a fully viable diploid replicate of its mother or, if sufficiently nourished, the offspring can grow to become a queen. The ovary of *A. m. capensis* workers develop rapidly inside the host society, probably implying that these bees do not respond to the *A. m. scutellata* queen and larval pheromones that normally suppress

worker oogenesis (Martin *et al.*, 2002). Additionally, *A. m. capensis* worker bees provide their eggs with a pheromone coating that mimics that of eggs laid by the *A. m. scutellata* queen (Section V). Thereby, their offspring is readily accepted by the *A. m. scutellata* host. This ability to escape detection is in some ways analogous to tumor immune evasion by expression of self-antigens by the tumor cells (Houghton and Guevara-Patino, 2004; Kortylewski *et al.*, 2005).

When reared by *A. m. scutellata* nurse bees, *A. m. capensis* brood elicits more food than the host's own larvae (Calis *et al.*, 2002). The resulting *A. m. capensis* adults often have enlarged ovaries and other morphological and pheromonal characteristics similar to that of queens (Section III). This class of offspring does not engage in collective division of labor, but instead the workers feed off the host society's food stores and start laying their own eggs after as little as 6 days (Martin *et al.*, 2002). The *A. m. scutellata* queen is killed by the worker bees within 5–6 weeks, and the society subsequently breaks down when the remaining population of *A. m. scutellata* workers becomes so small that the social connectivity of the colony collapses (Fig. 7). As the host's brood rearing, foraging, and defensive efforts thereby come to an end, the *A. m. capensis* workers disperse in search of new *A. m. scutellata* colonies.

Note that in the native maternal nest, replication of *A. m. capensis* worker bees does not come out of control—although the worker caste can lay eggs by thelytokous parthenogenesis as it does in *A. m. scutellata* host societies. This is probably because all worker offsprings are nourished normally (Beekman and Oldroyd, 2003; Calis *et al.*, 2002). The resulting adults, therefore, are not queen-like and display normal social phenotypes that engage in collective division of labor. Genetic analysis has further shown that all workers with the malignant *A. m. capensis* phenotype, although collected from different *A. m. scutellata* colonies, are the parthenogenetic descendants of a single bee (Moritz, 2002). It has been proposed that this clonal population emerged from an extraordinary *A. m. scutellata* nest infestation; one *A. m. capensis* worker genotype suited to evade the full array of control mechanisms inherent to the host society.

Within European honeybee populations (*A. m. mellifera*), a more benign dynamic arises through the behavior of so called "anarchistic workers" (Beekman and Oldroyd, 2003; Oldroyd *et al.*, 1999). Anarchistic bees activate their ovaries despite the presence of inhibitory pheromones from the queen and brood, and they lay unfertilized eggs that are not disposed of by nestmates (Hoover *et al.*, 2003, 2005). Although a considerable number of the drones in such colonies may stem from eggs laid by workers, the queen continues to be the dominant female reproductive. Thus, the society does not progress into the destructive state that emerges under *A. m. capensis* infestation. Some colony-level functionality may still be lost,

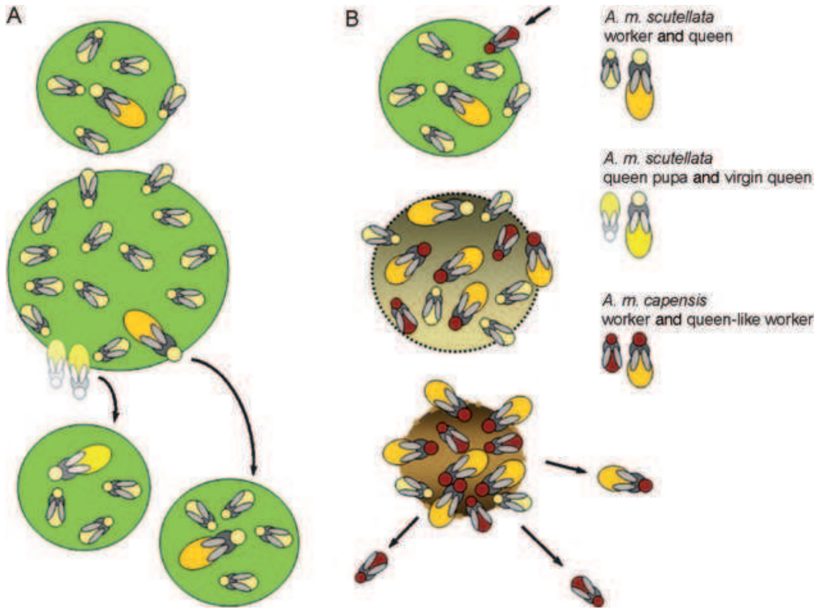

Fig. 7 Normal colony development versus social cancer. The propagation unit for a honeybee colony is the swarm (A). Swarming (splitting) occurs after the colony unit has grown to a certain size. Worker bees produce new queen larvae and prior to the adult emergence of the first virgin, the old queen leaves to find a new colony together with a fraction of the worker population. The malignant *A. m. capensis* phenotype enters a host nest (B), overrides the control mechanisms of the society, and starts laying diploid eggs by thelytokous parthenogenesis. Gradually the resulting female clones, which do work, break down the social integrity of the society and the host colony perishes. *A. m. capensis* subsequently disperse in search of new host colonies. In many ways, this process is comparable to metastasis.

however, because anarchistic bees do not work as hard as normal worker bees (Dampney *et al.*, 2004).

VII. CONCLUSIONS

The amazing order of social insect societies is a mass phenomenon that emerges from integration of much simpler individual patterns by means of signal transduction or social communication. We have described how the honeybee superorganism uses tactile, thermal, and chemosensory cues to control growth, differentiation, homeostasis, and death. Individual bees perceive these signals through sensory receptors on their antennae, feet,

and proboscis (tongue) (Gould and Gould, 1988; Snodgrass, 1956), and the subsequent integration of peripheral stimuli in the honeybee brain is an area that receives much attention (Belzunces *et al.*, 1996; Dacher *et al.*, 2005; Farooqui *et al.*, 2004; Scheiner *et al.*, 2002).

Chemosensory cues, in particular, play an important role in honeybee social organization (Sections IV–VI), having powerful effects on individual gene expression patterns (Grozinger *et al.*, 2003), behavior (Leoncini *et al.*, 2004b; Pankiw, 2004a; Pankiw and Page, 2003), and physiology (Hoover *et al.*, 2003; Huang and Robinson, 1992, 1996). A unique phenomenon that emerges from integration of social chemosensory cues is the behavioral and physiological differentiation of temporal worker forms (Section IV). The shift from nurse tasks to foraging duties is intriguing because it demonstrates that programmed cell death can be released by signals transferred between individuals (Amdam *et al.*, 2005). Programmed cell death in addition is observed in the ovary of workers inhibited by pheromones from queen and brood (Capella and Hartfelder, 1998), possibly explaining how worker reproduction is controlled. The idea that worker propagation is conditionally suppressed through an apoptosis pathway that is governed by pheromones raises interesting questions relative to the casual basis of anarchistic workers and malignant *A. m. capensis* phenotypes. We believe that the honeybee in this connection can provide valuable insights into molecular mechanisms that enable interindividual signals to translate into apoptotic responses in target tissues. Such insights may be of general interest since apoptosis contributes to the antitumor activity of many chemotherapeutic drugs (Fisher, 1994; Johnstone *et al.*, 2002).

Insights into honeybee chemosensory signaling are fundamental also for understanding how the biological order of the society can fail. Specifically, destructive dynamics that turn order into disorder are intimately linked to a progressive loss of integration of chemosensory cues—at the level of individual bees and the colony unit. We have described outcomes that emerge when workers responsible for social connectivity become few or when malignant phenotypes override the regulatory machinery of the colony and subsequently replicate in an uncontrollable manner. The latter is the social cancer of *A. m. scutellata* that is regarded as lethal. Similar infestations by malignant *A. m. capensis* phenotypes occurred in 1928 and 1977 but eventually petered out (Oldroyd, 2002). The causes of these remissions are not understood but suggest that the dynamics between the current clone and its host may change in future—creating possibilities for insights into how aberrant replication can be restrained and order restored at the level of a superorganism.

Approximately 50 substances synthesized by honeybee queens, workers, and brood have been identified as having functional roles in social organization (Pankiw, 2004b). Remarkable physiological effects of such

compounds have been a driving force of studies exploring if interindividual dynamics can translate into patterns of cell growth and cell death (Amdam *et al.*, 2005). Such findings, along with the availability of the honeybee genome sequence, open up for new insights into how emergent biological order arises through control of individual molecular pathways (Robinson *et al.*, 2005). In this context, our chapter is a first initiative to underline that the honeybee, in addition, can become a model for understanding the frailty of underlying regulatory systems—thereby increasing the knowledge of principles of disorder.

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Control of Differentiation in Progression of Epithelial Tumors

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The central concept of this chapter is that derangement of microenvironment, which takes place in tumor progression, leads to the partial or full dedifferentiation of epithelial tumors. The review considers the role of intercellular communications and interaction of cells with extracellular matrix (ECM) in differentiation and tumor progression. To illustrate this point, we consider the main characteristics of normal hepatocyte differentiation and its alterations in the course of hepatocellular carcinoma progression and epithelial-to-mesenchymal transition. The control of liver differentiation is mainly implemented by hepatocyte nuclear factors (HNFs). Derangement of HNF regulatory network is clearly associated with hepatocarcinogenesis and progression. We suppose that tissue-specific factors, playing the most important role in the differentiation of particular epithelial cell types, are the preferential targets for inactivation in the progression of corresponding tumors. Moreover, these transcriptional regulators may mediate the interaction of epithelial cells with the microenvironment.
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I. INTRODUCTION

Differentiation state of malignant tumors is an important characteristic, which helps to establish their histological origin and understand the degree of deviation from normal biology, as well as the stage in tumor progression and peculiarities in clinical behavior. It offers the basis for search of tumor markers and investigation of their nature. Differentiation antigens are absolutely necessary for precise classification of hemoblastoses and, hence, for their diagnosis and prognosis. The immunophenotyping is a routine procedure in hematological oncology (Van Dongen *et al.*, 2002). Little is known about epithelial tumors, and this chapter discusses about this problem.

Malignant growth can be considered as a result of two successive events—cell transformation and tumor progression. Here, we define transformation as autonomous proliferation of an immortal cell clone, while the progression as the process, leading the immortal transformed clone to invasion and metastasis.

Progression proceeds through continuing selection of the most autonomous cell variants from a genetically unstable population of transformed cells (Nowell, 2002). Progression is a stepwise endless process started *in vivo* in the transformed clone. Acquisition of malignancy is the result of tumor progression rather than transformation.

Transformation itself is not necessarily associated with the loss of differentiation. Certain well-differentiated hemoblastoses, such as plasmocytomas continuing immunoglobulins (Ig) production throughout their growth in primary host or during serial transplantations, might serve as an illustration of compatibility of transformation and malignancy with persistence of differentiation (Stevenson and Cragg, 1999). Another example is chronic myeloid leukemia associated with overproduction of mature granulocytes lacking the ability to enter apoptosis (Deininger *et al.*, 2000). Maintenance of differentiation state in the hemoblastosis progenitor cell might be regarded as a rule for hemopoietic neoplasms (Abelev, 2000).

In epithelial tumors, the progenitors (transformed cells) evolve during tumor progression and become more and more autonomous. In this process, first, the tumor cells change their morphology and behavior. Second, they lose cuboidal shape and polarity, and become more independent from neighboring tissues. Finally, they acquire the capacity to invade the underlying tissue and form distant metastases. Hence, tumor progression is usually associated with partial or complete loss of morphological and biochemical features of the original tissue, that is, with dedifferentiation.

We assume that the tumor microenvironment plays an especially important role in maintenance of the differentiation state, and independence from the microenvironment is a crucial factor in dedifferentiation of tumor cells. We would try to substantiate this opinion in this chapter.

It looks highly plausible that epithelial cell interactions with microenvironment are responsible for the differentiation state maintenance as well as for the control of behavior of transformed cells. The capacity to invasive growth and metastasis is acquired by transformed cells in the process of tumor progression, which leads simultaneously to gradual or stepwise liberation from microenvironment control and, hence, to partial or complete loss of their differentiation state.

II. NORMAL EPITHELIOCYTE

A. Differentiation Markers

Epitheliocyte is a very peculiar cell with remarkable morphophysiological characteristics. It is cuboidal, or columnar in shape, polygonal, and polar with distinct domains: apical and basolateral (Fig. 1). Polarized cells form acini with lumens inside the acini. The apical domain faces the lumen and is covered by glycoproteins, which belong to Ig superfamily: biliary glycoprotein 1 (Bgp1) in the apex of hepatocyte (Daniels *et al.*, 1996; Kuprina *et al.*, 1990), or carcinoembryonic antigen (CEA) along the apical part of intestinal cells (Hammarström, 1999). These glycoproteins are very clearly recognized by specific antibodies and are distinct markers of epitheliocyte (especially hepatocyte) polarity.

The basal part of epitheliocyte is rich in integrins recognizing ECM, particularly proteins of basal membrane and fibronectin. The basal membrane is a common part of all epithelial tissues. It serves as a mechanical support of any epithelia and delimits the territory of epithelial tissue. The basal membrane supports the epithelial layer and determines the polarity of epitheliocytes (Brill *et al.*, 2001). The basal membrane of different epithelial organs has different chemical composition, for example, type I collagen in the tendons and type IV collagen in the liver, gastrointestinal tract, and kidney.

The lateral surfaces of epitheliocyte are rich in characteristic adhesion protein, E-cadherin, an essential component of adherence junctions and desmosomes, responsible for specific recognition of homologous neighboring cells and for creating continuous intercellular network of tissue specific intermediate filaments, responsible for epithelial layer elasticity.

It is very important that cytoplasmic domains of E-cadherin are associated with α-, β-, and γ-catenin molecules. When E-cadherin is downregulated or disrupted, the catenin complex is dissociated—β-catenin migrates into the

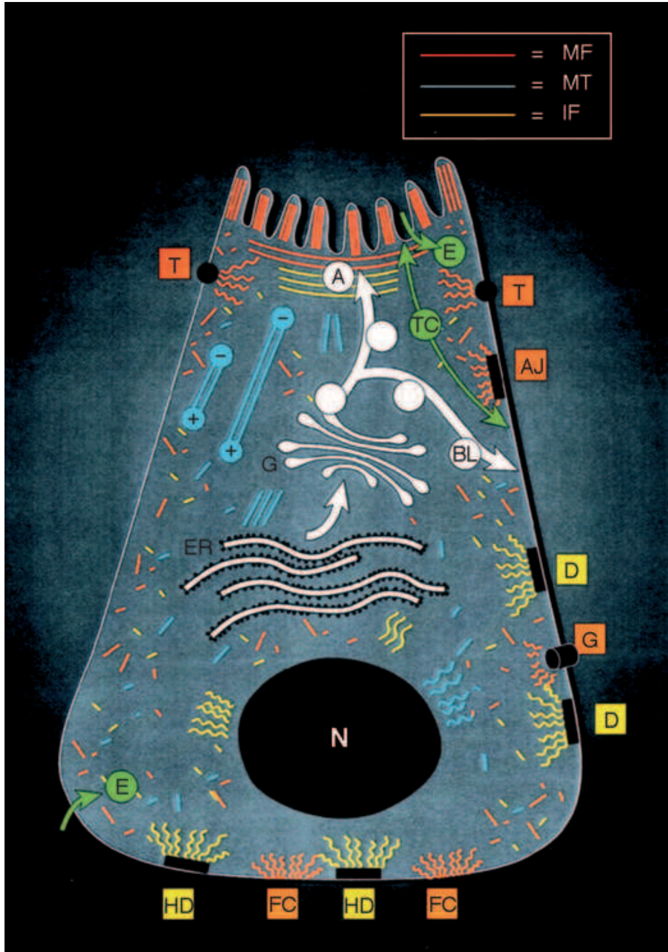

Fig. 1 Schematic diagram of epithelial cell polarity, cell–cell, and cell–substratum junctions. Polarized epithelia, as found in the intestine, contain an apical domain with specialized features, such as microvilli and a basolateral domain, which are separated by tight junctions. Plasma membrane proteins reach their ultimate target domain by a direct or an indirect (transcytotic) pathway involving microfilaments (MFs) and microtubules (MTs). The apical and basolateral domains have distinct organization of underlying cytoskeleton. For example, the MT organizing center underneath the apical membrane generates a uniform polarity of MT with the apical minus and basal plus ends, allowing vesicle transport in two directions. Tight, gap, and adherens junctions and focal contacts link with actin filaments (attachment of MF with gap junctions is not well defined, which is indicated by the close proximity but not attachment to MF), whereas desmosomes and hemidesmosomes connect with intermediate filaments (IFs). A, apical; BL, basolateral; T, tight junction; AJ, adherens junction; D, desmosome; G (on cell surface), gap junction; HD, hemidesmosome; FC, focal contacts; E, endocytosis; TC, transcytosis; N, nucleus; ER, endoplasmic reticulum; G (in cytoplasm), Golgi apparatus (Ku *et al.*, 1999).

nucleus and functions as a transcription factor (Cavallaro and Christofori, 2004). E-cadherin has a very peculiar localization between the lateral surfaces of epitheliocytes in the epithelial layer and can be detected by the corresponding antibodies (Ku *et al.*, 1999). Specific proteins of gap junctions' connexins (Cx) are also localized on the lateral surfaces on epithelial cells (Fig. 1). The network of intermediate filaments is tissue specific and serves as a specific marker for epithelial classification, and detection of remote metastases. Intermediate filaments localize in the cytoplasm and are connected with desmosomes and hemidesmosomes, located on basal membrane (Fig. 1). Ig-like cell adhesion molecules are additional markers, which could also be visualized immunohistochemically.

Integrins are molecules of great importance; they recognize specifically ECM components such as collagen of different types, fibronectin, and proteoglycans (Giancotti and Ruoslahti, 1999; Ruoslahti, 1999).

Additional markers of epitheliocytes are proteins synthesized by them, such as serum albumin, alpha-fetoprotein (AFP), transferrin, α_1-antitrypsin or cytochrome P450 in hepatocytes, specific proteases and amylases in the pancreas, or casein in mammary gland epithelium (Ben-Ze'ev *et al.*, 1988; Di Persio *et al.*, 1991; Guillouzo *et al.*, 1993; Schmeichel and Bissell, 2003).

Epitheliocytes (especially hepatocytes) possess a remarkable property to quickly and reversibly dedifferentiate after isolation and explantation on plastic, but maintain differentiated state in proper ECM. This property allows studying the dynamics of epitheliocyte markers' expression during de- or redifferentiation of hepatocytes (Section III.B).

Studies of the expression patterns of epitheliocyte markers in transformation and progression can disclose elementary events associated with the evolution from transformation to malignancy.

B. Tissue-Specific Transcription Factors

Transcription factors, essential for expression of the majority of functional epithelial proteins, are invaluable markers of tissue differentiation and its changes in the process of progression.

Tissue-specific gene regulation is best studied in the liver. The extensive studies of the last 15 years led to detailed characterization of liver-specific transcriptional network. Here we review the main properties of this regulatory cascade in hepatocytes and then discuss its relation to other epithelial organs.

1. HEPATOCYTE NUCLEAR FACTORS IN LIVER DEVELOPMENT AND DIFFERENTIATION

The fine regulation of liver-specific gene expression and the maintenance of hepatic differentiation are mainly implemented by combinatorial action of hepatocyte nuclear factors (HNFs) (Locker, 2001; Tronche and Yaniv, 1992). This class of proteins includes five families of transcriptional regulators (Table I) with binding sites located in the majority of regulatory modules of liver-specific genes and required for their proper expression (Cereghini, 1996; Lazarevich, 2000; Schrem *et al.*, 2002). The tissue specificity of the expression of each hepatic gene is achieved by simultaneous participation of several HNFs in the regulation of this process.

HNF1 family includes factors HNF1α (Cereghini *et al.*, 1988) and HNF1β (De Simone *et al.*, 1991; Rey-Campos *et al.*, 1991), binding the same DNA consensus as homo- or heterodimers with different transactivation properties (Tronche and Yaniv, 1992). HNF1 binding sites are abundant in the promoters of liver-specific genes (Tronche *et al.*, 1997). While interacting with multiple coactivators that possess histone acetyltransferase activity, HNF1 proteins are able to alter the local chromatin structure and activate transcription (Soutoglou *et al.*, 2000).

HNF1β is first detected in early embryonic development and is essential for visceral endoderm formation in mouse embryo as indicated by early embryonic lethality of HNF1β knockout mice (Barbacci *et al.*, 1999; Coffinier *et al.*, 1999). Liver-specific inactivation of the HNF1β induces defects in intrahepatic bile ducts and gallbladder formation (Coffinier *et al.*, 2002). HNF1β expression is also detected at the onset of pancreatic exocrine ducts and kidney tubules formation (Coffinier *et al.*, 1999).

The expression of HNF1α starts later, during liver specification, and its level is significantly lower than that of HNF1β (Ott *et al.*, 1991). In the adult liver, this balance is changed and HNF1α predominates. Targeted disruption of *HNF1*α gene in mice does not generate considerable abnormalities in liver development, but causes progressive wasting syndrome, defects in insulin secretion, renal dysfunction, increase of liver mass, and downregulation of some liver-specific genes during the first weeks after birth (Pontoglio *et al.*, 1996, 1998). HNF1β may play a key role in organogenesis, while HNF1α is mainly involved in the maintenance of epithelial differentiation.

Forkhead proteins **HNF3**α, -β, and -γ (Costa *et al.*, 1989; Lai *et al.*, 1990) interact with the corresponding sites as monomers (Clark *et al.*, 1993). A significant structure similarity of HNF3 "winged helix" DNA-binding domain to histones provides these factors with the ability to bind the compacted chromatin and alter its nucleosomal organization during gene activation (McPherson *et al.*, 1993). This property has been proposed to

Table I Hepatocyte Nuclear Factors

Gene (synonym)	Structure of DNA-binding domain	Embryonic expression (days of gestation)	Representative target genes	Pattern of expression
HNF1α (HNF1 LFB1, TCF1)	Variant homeodomain	10.5	AFP, albumin, α_1-antitrypsin, tyrosine aminotransferase, transthyretin, aldolase B, apolipoproteins A2, B	Liver, intestine, kidney, pancreas
HNF1β (vHNF1, LFB3, TCF2)		4.5		Liver (low), kidney, intestine, pancreas, esophagus, thyroid
HNF3α (FoxA1, TCF3A)	Winged helix	7.5	Albumin, AFP, transthyretin, α_1-antitrypsin, transferrin, apolipoprotein B, tyrosine aminotransferase, HNF1α, HNF3α, HNF3β	Liver, intestine, stomach, lung, pancreas, prostate
HNF3β (FoxA2, TCF3B)		6.5		Liver, intestine, stomach, lung, pancreas
HNF3γ (FoxA3, TCF3G)		8.5		Liver, intestine, stomach, testes
HNF4α (NR2A1)	Zink finger	4.5	HNF1α, transferrin, α_1-antitrypsin, transthyretin, albumin, apolipoproteins A1, A2, B, C2, C3, aldolase B	Liver, intestine, pancreas, kidney
HNF6 (Onecut1)	Onecut	9.0	HNF1β, HNF3β, HNF4α, transthyretin, α_1-antitrypsin, glucokinase	Liver, pancreas, spleen, brain
C/EBPα	Basic region leucine zipper	13.0	AFP, albumin, apolipoproteins A1, A2, B, transthyretin, transferrin, tyrosine aminotransferase, type 1 collagen, metalloproteinases	Liver, intestine, lung, adipose, ovary, mammary gland, skin, skeletal muscle, placenta
C/EBPβ (LAP, NF-IL6, TCF5)		12.0		Ubiquitous, predominates in liver and lung

play the decisive role in hepatogenesis. Embryonic specification of liver is determined by signals from the cardiac mesoderm cells, inducing alterations in gene expression and morphology of endodermal epithelial cells (Zaret, 2002). The competence of these cells to enter hepatic differentiation is attributed to the activities of HNF3 and GATA transcription factors, recognizing the corresponding binding sites and opening the compacted chromatin structure of hepatic genes (Cirillo *et al.*, 2002).

During embryonic development HNF3β, -α, and -γ are activated successively in the definitive endoderm with HNF3β being detected at the onset of gastrulation (Ang *et al.*, 1993; Monaghan *et al.*, 1993). Even HNF3β inactivation leads to early embryonic lethality associated with abnormal development of the foregut endoderm that gives rise to the liver and pancreas (Ang and Rossant, 1994; Dufort *et al.*, 1998; Weinstein *et al.*, 1994). Targeted disruption of *HNF3*α gene causes postnatal growth retardation and death within the first week of life due to glucose homeostasis failure (Kaestner *et al.*, 1999). Mice with inactivated HNF3γ have no developmental abnormalities and differ from the normal animals by altered transcription of several liver-specific genes (Kaestner *et al.*, 1998). Based on these data, HNF3β has been proposed to play the decisive role in early hepatic specification.

Note that HNF3β inactivation in the adult liver does not entail any significant changes in hepatic function and morphology (Sund *et al.*, 2000), suggesting essential alterations in HNF's function and regulation at different stages of development.

HNF4α is nuclear hormone receptor (Sladek *et al.*, 1990), which binds DNA exclusively as homodimer (Jiang *et al.*, 1997). HNF4α is the main activator of HNF1α expression, which in turn regulates a wide range of liver-specific genes (Kuo *et al.*, 1992). Also, HNF4α directly regulates the transcription of numerous genes essential for hepatocyte differentiation and function.

Two groups of HNF4α isoforms originating by different promoter usage have been identified (Sladek and Seidel, 2001). The variants transcribed from P1 promoter are predominant in the adult liver and differentiated hepatomas (Nakhei *et al.*, 1998; Torres-Padilla *et al.*, 2001). The isoforms regulated by an alternative promoter P2 are mainly expressed in stem cells, embryonic liver, pancreatic β-cells, and dedifferentiated hepatoma cell lines (Nakhei *et al.*, 1998; Thomas *et al.*, 2001; Torres-Padilla *et al.*, 2001). Two groups of isoforms have different impacts on target genes expression (Torres-Padilla *et al.*, 2001) apparently due to different patterns of interaction with coactivators (Torres-Padilla *et al.*, 2002). The "embryonic" forms more effectively activate the promoters of early hepatic genes, *AFP* and *transthyretin*, while "adult" variants have a more significant impact on the transcription of mature hepatic markers (Torres-Padilla *et al.*, 2001).

HNF4α transcription from P1 and P2 promoters is driven by distinct mechanisms, and the ratio of resulting two groups of isoforms (referred hereafter as $\alpha 1$ and $\alpha 7$) influence the maintenance of hepatic differentiation.

During mouse embryonic development, HNF4α mRNA is first identified on day 4.5 in the primitive endoderm (Chen *et al.*, 1994; Taraviras *et al.*, 1994). Inactivation of the *HNF4α* gene leads to embryonic lethality on day 10 due to a block in visceral endoderm differentiation (Chen *et al.*, 1994; Duncan *et al.*, 1997). This defect can be rescued by complementation of HNF4$\alpha^{-/-}$ embryos with a tetraploid embryo-derived visceral endoderm (Duncan *et al.*, 1997). While HNF4α appeared to be dispensable for early specification of hepatic lineage, it was found to be essential for the subsequent steps of hepatic differentiation and for metabolic regulation and liver function (Li *et al.*, 2000). Loss of HNF4α expression in midgestation induces dramatic abnormalities of liver morphology linked with disruption of cell adhesion and cell junction contacts and downregulation of the number of genes critical for epithelial morphology maintenance (Parviz *et al.*, 2003). Conditional knockout of HNF4α in the adult liver suggests that this factor is the central regulator of genes involved in lipid and urea homeostasis (Hayhurst *et al.*, 2001; Inoue *et al.*, 2002). Thus, the experiments on knockout mice have clearly demonstrated the essential role of HNF4α in liver differentiation and morphogenesis at different stages of development.

HNF6 binding the same sites as HNF3 is expressed in the adult liver and pancreas (Lemaigre *et al.*, 1996; Samadani and Costa, 1996).

In the liver, HNF6 is expressed both in hepatocytes and in cholangiocytes from the initial steps of their development. The HNF6 knockout in mice causes the abnormal development of intra- and extrahepatic bile ducts, absence of gallbladder, hepatic artery malformations, and cholestasis (Clotman *et al.*, 2002, 2003). Defects in the development of the biliary tract are similar to those observed in liver-specific HNF1$\beta^{-/-}$ mice (Clotman *et al.*, 2003; Coffinier *et al.*, 2002). Moreover, HNF6 was shown to control the expression of HNF1β in the embryonic biliary epithelial cells (Clotman *et al.*, 2002). HNF6 inactivation is also associated with pancreatic abnormalities and diabetes mellitus due to defects in endocrine cells differentiation (Jacquemin *et al.*, 2000). These data suggest that HNF6 is a key regulator of hepatoblasts differentiation into biliary epithelial cells and endocrine lineage differentiation in pancreas.

CCAAT/enhancer-binding proteins (**C/EBPs**) comprise the most widespread family of transcriptional regulators binding CCAAT box as homo- or heterodimers (Landschulz *et al.*, 1988; Ramji and Foka, 2002; Schrem *et al.*, 2004). Being expressed in different combinations in a wide range of tissues and cell types, C/EBPs regulate the variety of essential physiological processes such as organogenesis, differentiation, apoptosis, inflammatory

response, metabolism, and some others. Transcriptional activity of C/EBP family members is controlled tissue- and stage-specifically at multiple levels and is responsive to hormonal, mitogenic, nutrition, and stress signals (Ramji and Foka, 2002).

C/EBPα and -*β* genes predominantly expressed in the liver produce multiple protein isoforms with different transactivation properties (Descombes and Schibler, 1991; Ossipow *et al.*, 1993). Specifically, one of C/EBPβ isoforms, liver-enriched transcriptional inhibitory protein (LIP), lacks the functional transactivation domain but preserves the ability for dimerization with other family members, acting as a dominant negative regulator of C/EBP-dependent transcription (Descombes and Schibler, 1991). The multiple control of C/EBP transcriptional activities do ensure the strict regulation of their impact in diverse biological processes.

In the liver C/EBP factors regulate the expression of functional hepatic genes and mediate the acute phase response. Moreover, C/EBPα is associated with terminal differentiation and inhibits hepatocyte proliferation, while C/EBPβ exerts the opposite effect. The balance of C/EBPα and -β reflects the proliferative state of hepatocytes: during regeneration, the level of C/EBPβ significantly increases while that of C/EBPα rapidly falls (Greenbaum *et al.*, 1995; Rana *et al.*, 1995). C/EBPα transcription is strongly enhanced after the birth when hepatocytes differentiate and convert to the quiescent state (Wang *et al.*, 1995).

Mice lacking C/EBPα die shortly after birth because of impaired glycogen synthesis and storage (Wang *et al.*, 1995). These animals have defects in lung development, increased hepatic proliferation, and distortion of liver architecture with acinar formation (Flodby *et al.*, 1996). The primary hepatocyte cultures obtained from C/EBP$\alpha^{-/-}$ neonatal livers exhibit rapid growth, chromosomal instability, and increased transformation frequency, but maintain the hepatocyte-like morphology with cell–cell contacts and albumin expression (Soriano *et al.*, 1998).

Mice with inactivated C/EBPβ are viable but display defective differentiation of adipose, ovarian, and mammary gland (Ramji and Foka, 2002). C/EBP$\beta^{-/-}$ mice demonstrate an impaired hepatocyte proliferation in response to partial hepatectomy (Greenbaum *et al.*, 1998).

Note that C/EBPβ is able to induce pancreas to hepatic transdifferentiation. Enforced expression of C/EBPβ in a pancreatic cell line, AR42J-B13, causes downregulation of pancreatic marker amylase, translocation of HNF4α to nucleus, and activation of a set of liver-specific genes (Shen *et al.*, 2000). This example shows that differentiation and functional program of epithelial cells can be critically modified by altered activities of very few tissue-specific transcriptional regulators.

The transcriptional pattern of each HNF is not restricted to the liver alone but all transcription factors of that class are expressed only in this

organ. The impact of each transcription factor on liver differentiation is not static and significantly alters in the course of development (Jochheim *et al.*, 2004). The transcriptional hierarchy of different HNFs is highly complex (Cereghini, 1996; Lazarevich, 2000; Locker, 2001) and not completely solved yet.

There is growing evidence that the balance of HNFs determines to a great extent the fate of different cell lineages in organogenesis. All the critical steps of liver formation are preceded or accompanied by modulation of HNF's network. HNFs may be responsible for the competence to inductive signals coming from different mesodermal cell types (Lemaigre and Zaret, 2004; Zaret, 2002) and reprogramming of gene transcription induced by these signals. Sequential activation of HNF3β, HNF6, and HNF4α accompanies *in vitro* differentiation of murine embryonic stem cells toward hepatic phenotype (Jochheim *et al.*, 2004).

HNF3 factors are essential for acquisition of the endoderm competency to adopt a hepatic fate (Zaret, 2002). HNF1β and HNF6 mediate hepatoblasts differentiation into cholangiocytes and bile ducts formation (Clotman *et al.*, 2002; Coffinier *et al.*, 2002). HNF4α (Parviz *et al.*, 2003) and C/EBPα (Wang *et al.*, 1995) are essential for differentiation of embryonic hepatocytes and HNF1α plays an important role in the functional maturation of hepatocytes during the postnatal period (Akiyama *et al.*, 2000; Pontoglio *et al.*, 1996).

Thus, specification of hepatocytes and cholangiocytes, endodermal components of the liver originated from the common progenitors, depends on differential expression of HNF4α, HNF1α, C/EBPα and HNF6, HNF1β, respectively. Importantly, reversible dedifferentiation of normal hepatocytes in primary culture is accompanied by decrease in activity of HNF4α, HNF1α, C/EBPα, and activation of HNF3 proteins, indicating that these factors can mediate the alteration of hepatocyte transcriptional program in response to disturbing normal liver structure (Section III.F).

In summary, having been primarily identified as transcriptional regulators of functional hepato-specific genes, HNFs were then clearly shown to influence a variety of cell and tissue characteristics, particularly proliferation, morphology, organogenesis, apoptosis, stress response, and so on. The wide range of processes modulated by this class of factors and the profile of HNFs expression suggests their involvement in similar regulatory processes taking place in other epithelial structures.

2. HNFs IN NONHEPATIC EPITHELIA

While HNFs were first discovered in the liver, they are in different combinations expressed in the epithelia of other organs like pancreas, kidney, lung, intestine, stomach, mammary gland, and skin (Cereghini, 1996;

Lazarevich, 2000; Locker, 2001; Tronche and Yaniv, 1992), and clearly participate in gene regulation and tissue differentiation. The tissue-specific regulatory network in these organs is formed by cooperative action of HNFs and additional transcriptional regulators specific for definite cell lineage. In a later section, we will focus on the place of HNFs in the regulatory cascade that defines the development of pancreas and then briefly summarize the impact of liver-enriched transcriptional factors on the differentiation of other organs.

Being derived from the common ontogenetic precursor, the primitive foregut, the liver and pancreas demonstrate very similar patterns of HNFs expression (Locker, 2001). Notably, the most significant difference in HNF set between the liver and pancreas are substitution of "adult" isoforms of HNF4α by "embryonic" ones and expression of HNF4γ absent in hepatocytes. Activation of the P2 promoter, driving the expression of embryonic isoforms of HNF4α, can be attributed to the activity of Pdx1 and HNF1 factors which bind the P2 promoter (Thomas *et al.*, 2001), and to the absence of adult HNF4α isoforms reported to suppress α7 promoter activity (Briancon *et al.*, 2004). Perhaps these differences are not sufficient to define the divergence of the developmental fate of the liver and pancreas; moreover, the regulatory links revealed in the liver transcriptional network are altered in pancreas by the addition of new players. Still, the significance of HNFs in pancreas development is apparent.

Pancreas arises from the fusion of the ventral and dorsal buds of primitive gut epithelium. It undergoes a complex set of morphological transitions giving rise to several highly specialized cellular lineages (Habener *et al.*, 2005; Kim and Hebrok, 2001; Slack, 1995). The mature pancreas consists of two functional compartments: (1) Exocrine acinar and duct cells responsible for secreting enzymes into the digestive tract and (2) Endocrine hormone-secreting cells located in the islets of Langerhans. Endocrine population includes four functionally specialized cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP-cells. Specification and differentiation of this complex structure are driven by signaling from different patterns of the overlying mesoderm and are accompanied by induction of a branching cascade of transcription factors.

The early stages of pancreatic development are defined by the activity of homeodomain and basic helix-loop-helix factors Pdx1, Ptf1a, Hlxb9, Isl1, or Hex, while additional set of transcriptional regulators, namely, Neurogenin-3, Pax4, Pax6, NeuroD/β2, and Nkx2.2, is essential for pancreatic lineage specification and function (Edlund, 2001; Habener *et al.*, 2005). HNFs widely cooperate with these factors in pancreatic organogenesis and lineage specification. Liver-enriched transcription factors were found to regulate transcription of *Pdx1*, *Pax4*, *Neurogenin-3*, and *Nkx2.2* genes.

Cereghini laboratory has reported that HNF1β deficiency in mice causes pancreas agenesis due to the failure of ventral pancreas bud specification and reduction of the dorsal pancreas, caused by diminished cell proliferation (Haumaitre *et al.*, 2005). HNF1β inactivation induced a dramatic deregulation of the pancreatic transcriptional network including the repression of transcription factor Ptf1a, essential for the acquisition of pancreatic fate, loss of early endocrine pancreatic markers, and ectopic expression of Sonic hedgehog and Indian hedgehog signaling molecules, which regulate regional specification of embryonic gut endoderm. These complex abnormalities result in defective developmental patterning of the primitive gut. These findings allow placing HNF1β to one of the top positions of the identified hierarchy of transcription factors defining early pancreatic morphogenesis.

Importantly, transcription factors may influence pancreatic differentiation not only by direct regulation of transcriptional programs but also morphogenetically, as was shown in Zaret laboratory for the homeobox gene *Hex*, required for the onset of hepatogenesis (Bort *et al.*, 2004). *Hex* regulates proliferation of cells at the leading edge of the ventral endoderm and thus their positioning relative to the cardiogenic mesoderm, which can trigger the pancreatic differentiation program. Similar mechanisms may also mediate the morphogenic properties of some other transcriptional regulators.

HNFs play a key role in the function of mature pancreatic β-cells, particularly, in regulation of insulin secretion. While HNF1 family members can regulate insulin gene activity by direct binding to the promoter sites, HNF4α, HNF3β, and HNF6 may affect insulin transcription indirectly through modulation of HNF1, Pdx, or NeuroD/β2 expression (Habener *et al.*, 2005). Mutations of the human *HNF4*α, *HNF1*α, or *HNF1*β genes cause an autosomal dominant form of noninsulin-dependent diabetes mellitus called maturity-onset diabetes of the young (MODY), types 1, 3, and 5, respectively, while types 4 and 6 are attributed to *Pdx1* and *NeuroD/*β*2* mutations (Habener *et al.*, 2005; Ryffel, 2001).

Studies on animal models confirm the importance of HNF1α, HNF1β, and HNF4α for pancreatic β-cell differentiation (Gupta *et al.*, 2005; Haumaitre *et al.*, 2005; Pontoglio *et al.*, 1998; Wang *et al.*, 2004). An abnormal pancreatic phenotype with perturbed differentiation of endocrine cells was also revealed in HNF6 $^{-/-}$ mice (Jacquemin *et al.*, 2000). Disruption of HNF3β in pancreatic β-cells results in deregulation of insulin and glucagon secretion (Sund *et al.*, 2001). Importantly, HNF3 family members are also involved in pancreatic α-cells differentiation and regulation of proglucagon transcription (Kaestner *et al.*, 1999; Lee *et al.*, 2005).

The disturbance of normal HNF function may influence not only functional but also morphological properties of pancreatic cells. For example,

expression of a dominant-negative form of human HNF1α in pancreatic β-cells (Yamagata *et al.*, 2002) impaired both insulin secretion and tissue morphology, which was due to the disruption of E-cadherin–dependent cell adhesion in the islets. The impairment of HNF1 function also diminished the expression of E-cadherin. The islet structural abnormalities were similar to those observed in transgenic mice, expressing dominant-negative E-cadherin (Dahl *et al.*, 1996). Taking into account that due to dimerization with endogenously expressed forms a dominant-negative HNF1α can block the function of both family members, these findings indicate that *E-cadherin* gene is a potential target for HNF1-responsive regulation in the pancreas.

Both members of HNF1 family are expressed in the kidney, but exhibit distinct patterns of expression (Lazzaro *et al.*, 1992; Pontoglio *et al.*, 1996). Apparently, these factors are essential for kidney morphogenesis and regulate the number of genes defining renal function and morphology. Importantly, inherited mutations of *HNF1*α (MODY3) cause reduced tubular reabsorption of glucose (Pontoglio *et al.*, 2000), while *HNF1*β mutations (MODY5) are associated with renal cystic abnormalities and/or genitourinary defects (Nishigori *et al.*, 1998). HNF1α knockout induced the renal Fanconi syndrome characterized by urinary glucose loss in mice (Pontoglio *et al.*, 1996). HNF1α was shown to regulate proximal tubule-specific gene expression, while HNF1β regulates the number of genes responsible for renal cystogenesis and kidney-specific Ksp-cadherin (Gresh *et al.*, 2004; Igarashi, 2003). Renal-specific inactivation of HNF1β causes the derangement of kidney tubular differentiation and renal cystic abnormalities (Gresh *et al.*, 2004).

Members of the HNF3 family were also proposed to mediate epithelial–mesenchymal interactions in embryogenesis. Together with thyroid transcription factor 1 and other forkhead proteins, HNF3α and HNF3β regulate signaling and transcriptional programs required for morphogenesis and cell differentiation during formation of the lung (Costa *et al.*, 2001). As indicated by mice knockout studies, HNF3α and HNF3β control cell proliferation, regulate transcription of lung epithelial cell markers, and control branching morphogenesis, possibly, through regulation of Sonic hedgehog transcription (Wan *et al.*, 2005). HNF3α was also reported to regulate prostate-specific expression of prostate-specific antigen (*PSA*) gene (Gao *et al.*, 2003).

In concordance with their wide tissue distribution, C/EBP proteins are involved in differentiation and/or proliferation control in a variety of organs, including skin, intestine, lung, adipose tissue, and mammary gland.

For example, C/EBPα, -β, and -δ are differentially expressed throughout the mammary gland postnatal development. The differentiation stages of the mammary gland during pregnancy and lactation are clearly associated with regulating the balance between differentiation, proliferation, and

apoptosis (Hennighausen and Robinson, 1998). C/EBPβ is essential for normal growth and functional differentiation of the mammary gland (Robinson *et al.*, 1998; Seagroves *et al.*, 1998). C/EBPβ-deficient mammary epithelium was shown to be defective in both proliferation and differentiation during pregnancy and failed to express the milk protein genes. In transplantation experiments, the C/EBP$\beta^{-/-}$ mammary gland fat pad was able to support the normal development of wild-type epithelial cells, which indicates that this effect is not influenced by C/EBPβ activity in the stromal component. Thus, the abnormal development of mammary gland in the absence of functional C/EBPβ is likely a consequence of disturbed hormonal regulation or other extracellular signaling cascades in mammary gland epithelial cells.

C/EBPα and -β have discrete expression patterns in epidermis and are implicated in keratinocyte differentiation (Zhu *et al.*, 1999). In accordance with previously pointed role of CEBPα as a terminal differentiation factor, it is involved in lineage-specific maturation in lung (Flodby *et al.*, 1996) and intestinal epithelium (Chandrasekaran and Gordon, 1993). C/EBP family members are also involved in the regulation of tissue-specific gene expression and in the acute phase response in certain epithelial cell types.

Taking into account that the early development of certain epithelial organs, like the pancreas, lung, or intestine, is defined by strict continuity of growth factor signaling, cell–cell and cell–ECM interactions, it is not surprising that some liver-enriched factors, which transduce these signals into alterations of the transcriptional program during liver organogenesis, conserve similar function in other endodermal lineages. Importantly, transcription factors may realize the developmental programs not only by direct regulation of transcription but also by modulation of proliferative and morphological properties of cells, which in turn define their proper positioning during tissue specification in the embryo (Bort *et al.*, 2004). Although the impact of liver-enriched factors on cell differentiation is essentially modified by cooperation with other tissue-specific regulators, some basic principles of HNFs' influence on physiologic properties, proliferation, and maintenance of epithelial morphology remain conserved.

III. MICROENVIRONMENTAL CONTROL OF TRANSFORMED CELLS

A. Control of Transformed Cells by Normal Surrounding

It is well known that the epithelial cell layer separating different tissues from each other and extracellular fluids contains several kinds of impermeable

contacts, both for cells and soluble macromolecules—intercellular adhesion contacts. They include different types of junctional contacts, such as tight junctions, gap junctions, and intercellular contacts through adhesion molecules as well as contacts with ECM by means of integrin receptors. These contacts determine the epithelial cell polarity, integrate cells into uniform tissue, and participate in their functioning.

On their way to overt neoplasms, transformed cells should be liberated from limitations overlaid by the surrounding tissue, that is, from influence of intercellular and cell-matrix contacts, including interaction with basal membrane.

There is no doubt that epithelial tumors have some "memories" about the differentiated state of their progenitor cells. For example, primary liver tumors induced by a "mild" carcinogen always retain tissue-specific antigens, at least some of them (Abelev, 1965; Guelstein and Khramkova, 1965; Khramkova and Guelstein, 1965). Primary and even subcutaneously transplanted carcinogen-induced hepatomas look like pieces of liver after staining with liver-specific antibodies (Engelhardt *et al.*, 2000). However, long-term transplanted hepatomas lose a significant part of tissue-specific antigens in the course of progression.

What barriers should be overcome, at least at the initial steps of progression? The first barrier is the control of microenvironment exerted by the normal tissue. This is formulated by Weinberg (1989), while the first experimental evidence for this statement was offered much earlier by demonstration of *initiation* and *promotion* in carcinogen action (Berenblum, 1954). *Initiation* is induced by a subcarcinogenic dose of chemical carcinogen, while *promotion* "develops" the initiated tissue by noncarcinogenic substances. Initiation is maintained for long time and initiated cells are able to produce neoplasms within months and years after initiation. Initiation is similar to transformation or to some critical step leading to transformation, while promotion leads to the development of overt neoplasm from initiated cells. Initiation could be a transforming mutation, while promotion is apparently a condition favoring selection of a transformed clone from targeted cells. One of the strongest promoters, phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), stimulates anchorage independent growth of an initiated clone (Dotto *et al.*, 1985; Karen *et al.*, 1999). This property is typical for growth of malignant clones rather than normal cells. Promoters have many different activities but among them there is a property common for various promoters, that is, block of gap junctional communications (Krutovskikh, 2002; Murray and Fitzgerald, 1979; Yamasaki, 1990).

Weinberg (1989) demonstrated that individual *ras*-transformed cells dispersed with low frequency in a monolayer of normal cells were effectively controlled by the latter ones. A massive infection of cells with oncogenic virus bearing the same oncogene (*ras*) developed regular transformation

focuses. Mixture of transformed and normal cells forms tumor nodules *in vivo* only when transformed cells highly predominate. This means that there is a universal system controlling the behavior of individual cells in the tissues (metabolic cooperation). It is unknown how such control is performed, but there are reasons to believe that disruption of gap junctional communications leads to impairment of this control. All these data suggest that transformation and malignancy could be separated in carcinogenesis and that malignant properties arise during progression of initially transformed cells.

A new step along this way has been made by Laconi *et al.* (2001a). They found that normal or initially carcinogen-transformed rat hepatocytes, injected intravenously to normal partially hepatectomized rats, were integrated into the structure of regenerating normal liver without signs of tumor growth. However, poisoning of recipient animals with retrorsine, alkaloid which inhibits hepatocyte division, led to 80–90% replacement of recipient liver with donor cells (Laconi *et al.*, 2001a). Transplantation of carcinogen-initiated liver cells into retrorsine-treated rats resulted in development of overt neoplasms during the process of recipient liver replacement (Laconi *et al.*, 2001b). These data, once again, confirmed the existence of normal cellular barrier on the way of initiated cells to malignancy, but the nature of this barrier is still unknown.

There is one more system, which allowed not only to demonstrate the "normal" control of tumor progression but also disclosed a little bit of the underlying events. The system included a 3D model of human skin keratinocytes seeded on basal membrane covered with skin fibroblasts. Intraepithelial nodules consisting of premalignant (initiated) keratinocytes could be reversed into normal keratinocytes when seeded in large excess of normal cells (25:1), but at 4:1 ratio the cell culture behaved as an initial step of tumor progression. It was promoted by basal membrane, which stimulated outgrowth of progressed keratinocytes and created a significantly increased tumor-promoting effect (Andriani *et al.*, 2003; Javaherian *et al.*, 1998; Vaccariello *et al.*, 1999).

Connective tissue elements associated with keratinocyte outgrowth became components of a future stroma, whose role is decisive at least at the early stage of tumor progression. The role of stroma in tumor development changes in the process of progression from induction of a tumor clone due to independence from growth factors to its invasive growth, loss of tissue architecture, and, finally, to its metastatic growth (Cunha *et al.*, 2003). Increasing understanding of the role of stroma in tumor development is reflected in a number of analytical reviews (Cunha *et al.*, 2003; De Wever and Mareel, 2000; Pupa *et al.*, 2002).

Thus, there is no doubt that initiated cells should overcome restraining limitations of the normal cellular microenvironment, that is, interrelations

with normal neighbors (Radisky and Bissell, 2004). Such initiated cells were found not only in carcinogen-treated but also in the normal breast tissue (Holst *et al.*, 2003). For review of early literature see Rubin (2001).

We know only some elements of these interactions and gap junctions are among them. It is known that disruption of these communications (metabolic cooperation) is always associated with the promoter action (Krutovskikh, 2002; Yamasaki, 1990) and is, probably, necessary, at least for *maintaining the epitheliocyte differentiation state*. Hence, this first step of progression is most probably associated with the initial steps of epithelia dedifferentiation. Further steps are also associated with continuing progressive loss of epithelial differentiation.

B. From Intercellular Interactions to Interaction with ECM

Studies of differentiation markers of hepatocytes and AFP led us to the establishment of a crucial role of intercellular relations in liver cell differentiation and then to the most important factor of these relations, cell–ECM interactions.

AFP is expressed in the yolk sac endoderm, fetal liver, and, transiently, in the regenerating liver. AFP synthesis is resumed in hepatocellular carcinoma (HCC) (Abelev, 1971; Abelev and Eraiser, 1999). Immunohistochemical analysis of CCl_4-induced liver regeneration in mice revealed a clear-cut localization of AFP, strictly in the perinecrotic area of damaged liver, more precisely *in one layer* of cells surrounding this area (Engelhardt *et al.*, 1984; Gleiberman and Abelev, 1985). Detailed immunohistochemical analysis of cells in the perinecrotic layer revealed that these cells gradually lost their polarity with the disappearance of apical bile canaliculi antigen, Bgp1 (Kuprina *et al.*, 1990), and behaved like cells isolated from the liver plate structure. Simultaneously they reexpress AFP.

Immunohistochemical study of AFP fading in early ontogenesis showed that the decrease of AFP expression is gradient-like starting in the portal area and ending around the central veins (Gleiberman and Abelev, 1985; Spear, 1999). The "rings" around the central veins are the latest sites of expression AFP before the complete disappearance of this protein. This process coincided with the liver plate formation, which starts in the region of portal tract and spreads toward the central veins. The appearance of apical marker Bgp1 and its concentration at the bile capillaries is an excellent marker of hepatocytes in the liver plate, where it borders bile capillaries. Thus, AFP synthesis suppression is associated with hepatocytes incorporation into the structure of liver plate. Drop out of liver plate leads

to reexpression of AFP, as clearly seen in the liver during regeneration after treatment with different hepatotoxins (Kuprina *et al.*, 1985). The association of AFP suppression with the liver plate formation was defined as "structural repression" (Abelev, 1978) or "architectural regulation" (Notenboom *et al.*, 1996)—the terms stressing the association of AFP suppression with cell–cell interactions in morphogenetic process. In the latter work, a suspension of fetal rat hepatocytes was introduced into the spleen of young recipient rats. When transplanted cells build liver plate structures, they cease to synthesize AFP. This process was associated with establishment of the lobular pattern of liver-specific enzymes expression and AFP transcription (Moorman *et al.*, 1990; Notenboom *et al.*, 1996).

Direct evidence of the significance of cell–cell contacts for hepatocyte differentiation was obtained in experiments with a suspension of isolated mature hepatocytes. Perfusion of the liver with collagenase led to dissociation of the liver tissue into a suspension of single hepatocytes. Transfer of the hepatocyte suspension onto plastic dishes led to adhesion of the cells to plastic, where they formed a sparse monolayer without intercellular junction communications. Individual cells in the monolayer lost their polarity and Bgp1 antigen. In a few days, they began to synthesize AFP very actively. They showed dedifferentiated phenotype, typical to the hepatocytes liberated out of plate structure. (The distribution of HNFs, typical for dedifferentiated hepatocytes is described in Section II.B.) As a result of gentle rotation of culture dishes, cells were gathered around the center, and dense center and sparse periphery were formed. AFP synthesis was visualized as a ring only in the periphery, and gap junction communications were seen only in AFP-negative dense central part of the monolayer (Gleiberman *et al.*, 1989a). Thus, clear association of gap junctions with AFP suppression, which served as a marker of mature hepatocyte, took place.

But it was only a part of the whole picture. According to earlier data on the role of ECM in maintaining hepatocyte shape and function (Ben-Ze'ev *et al.*, 1988; Guguen-Guillouzo *et al.,* 1983), the effect of 3D ECM on differentiation markers synthesis was investigated.

When hepatocytes were placed on collagen (type I) dried in the plastic dish, they formed a sparse monolayer similar to that formed on plastic alone. But if cells were inserted between two layers of fresh collagen gel they formed liver-like islands of highly organized hepatocytes. Cells in these islands were cuboidal and polygonal, connected by gap junction communications, forming bile canaliculi, and expressing Bgp1 antigen at the border of bile capillaries. The cells produced serum albumin, while AFP synthesis was completely suppressed, that is, they were typical mature hepatocytes (Gleiberman *et al.*, 1989b). Thus, 3D ECM was necessary for maintenance of the mature type differentiation of hepatocytes. This effect required the

presence of 3D ECM but was independent from the collagen nature: type I instead of type IV present in the liver.

The same effect was obtained in a mixed culture of hepatocytes with nonparenchymal liver cells as was earlier suggested by Guguen-Guillouzo *et al.* (1983) and reproduced later by Gleiberman *et al.* (1989b). In this system, not only gap junction communications were clearly seen, but also abundant 3D ECM was produced. Under such conditions, hepatocytes performed their physiological functions, that is, drug detoxication and serum protein synthesis. Principally similar results were obtained by Di Persio *et al.* (1991) with serum albumin synthesis by hepatocytes. These authors showed that serum albumin enhancer was activated significantly in 3D ECM, which determined cuboidal shape of hepatocytes and their differentiation. Thus, these experiments were among the first indications to the role of 3D ECM in epithelial differentiation.

Kudryavtseva and Engelhardt (2003) showed that *Ras*-transformed variant of IAR (nonparenchymal liver cell line) in a mixed culture with hepatocytes produced defective ECM, which neither suppressed AFP synthesis nor supported the liver-like cellular organization. The same result was observed with RSV-transformed IAR cells. Normal IAR in control experiments supported hepatocyte maturation with AFP suppression.

These observations have something in common with the data demonstrating that radical stromal changes or even stromal cell transformation are necessary for epithelial cell transformation leading to prostate carcinoma (Cunha *et al.*, 2003) or breast carcinoma (Bissell *et al.*, 2002; Kuperwasser *et al.*, 2004).

Thus, cell–cell communications and cell–matrix interactions appeared to be necessary for the initiation of hepatocyte differentiation or at least for its maintenance. It was shown that embryonic hepatocytes more actively express embryonic liver enzymes while growing on embryonic liver basal membrane ECM than on adult liver basal membrane ECM and vice versa (Brill *et al.*, 2004).

Now we would show that the regularities found in liver development are of general significance for the epithelial organs. We will discuss the mammary gland formation and functioning. An attempt to construct a *mouse* model for *human* mammary gland studies was made by transplantation of human breast epitheliocytes into *nude* or *scid* mice. But transplantation failed unless the human fat pad and stromal fibroblasts were preliminarily transplanted. Only transplantation of human breast epithelium together with tissue (or cells) creating the mammary gland microenvironment led to the formation of functioning (casein synthesizing) mammary gland in mice (Kuperwasser *et al.*, 2004).

Normal differentiation of mammary gland epithelium, as was shown on the breast tumor tissue, was restored by interaction of integrin system

of this tissue with the blocking antibody to β1-integrin in 3D ECM (Chrenek *et al.*, 2001; Weaver *et al.*, 1997). These data, though obtained on tumor models, clearly show that at least maintaining mammary gland differentiation requires 3D ECM and is realized through integrins (Weaver *et al.*, 2002).

Very interesting experiments on cell–ECM relations were carried out by Cukierman and her collaborators with the use of fibroblasts (Cukierman *et al.*, 2001, 2002; Yamada *et al.*, 2003). They used the elongated shape of fibroblasts and their ability to synthesize collagen and fibronectin as criteria of fibroblast differentiation. These properties of mature fibroblasts were observed only in 3D collagen gel of homologous ECM. The same ECM pressed to 2D plate did not induce fibroblast maturation, like heterologous ECM (Cukierman *et al.*, 2001). These data emphasized the importance of ECM origin (chemical composition?) as well as its 3D structure.

Cell–ECM interactions are realized by integrins, which specifically recognize ECM molecules and influence cell microenvironment "from inside" the cell (Hynes, 2002). Wide variability of integrins composition and their conformational liability enable these molecules to recognize different components of ECM, as well as to transduce different signals, produced by ECM alone or in association with growth factors, into cells (Faraldo *et al.*, 2002; Yamada *et al.*, 2003).

All those indications to the importance of 3D ECM for cell differentiation raised a problem: how does spatial arrangement of cell–matrix interaction lead to activation of tissue-specific genes? There were only few attempts to understand the mechanisms underlying these interactions, when it was shown that nuclear shape and nuclear matrix configuration depended on the 3D structure of ECM (Lelievre *et al.*, 1998).

Anyway, it is clear that rearrangement or disruption of such interactions taking place in tumor progression lead to derangement or loss of differentiation in epithelial tumors.

C. Dedifferentiation in Carcinomas as Consequence of Tumor Progression

This chapter considers transformation and progression as distinct events in the origin and evolution of malignant growth. As was shown in an earlier section, transformation is a process not necessarily associated with the loss of differentiation state of tumor *progenitor* cell. It can be blocked, or "frozen," at certain stages of differentiation with the inhibition of later stages, but with retaining of the preceding ones (Abelev, 2000; Greaves, 1982; Potter, 1978; Tenen, 2003). These properties are typical especially for hemoblastoses and are routinely used for their classification based on

immunophenotyping. Rare cases of "mixed" phenotype, typical for different hemopoietic cell lines, are usually observed in acute leukemia, derived from the earliest stages of progenitor-cell differentiation, expressing markers of various lines of differentiation (Giles *et al.*, 2002; Greaves, 1986; Van Dongen *et al.*, 2002).

Highly differentiated (minimal) Morris hepatomas illustrate carcinomas, often demonstrating high degrees of differentiation, as well as antigen retaining in mouse hepatomas (Section III.A). Morphological similarity of epithelial tumors to tumor progenitor tissue is one of the features widely used in pathomorphological tumor diagnosis.

Intermediate filaments (Ku *et al.*, 1999) of epithelial tissue (cytokeratins) are the most conservative markers of different types of epithelia and are widely used in carcinoma classification and diagnostics as well as in micrometastases detection (Braun *et al.*, 2000).

So-called tumor markers, such as AFP, CEA, PSA, and ovarian cancer serum marker (CA-125), are tissue specific antigens, specific to fetal or adult stages of certain tissues development (Abelev and Sell, 1999).

Thus, a more or less differentiated status is maintained in epithelial tumors but it changes in the process of tumor progression, most probably due to altered tumor cell interactions with the microenvironment.

1. LOSS OF GAP JUNCTIONAL COMMUNICATIONS

The first barrier, which should be overcome in the course of tumor progression, is the normalizing effect of surrounding normal cells. It has been extensively studied with chemical carcinogens, which allowed discrimination between initiation and promotion. Promoter effect leads, among others, to gap junctional communication disruption or blockage, as discussed in Section III.A.

It is not yet clear how this blockage induces the loss of differentiation, but it does lead to it since downregulation of differentiation in sparse monolayer of isolated hepatocytes is always associated with a block of gap junctional communications (Section III.B). Conversely, induction of gap junctional communication by ECM enhances mammary epithelial cell differentiation marked by β-casein upregulation (El-Sabban *et al.*, 2003).

2. CADHERINS AND ADHERENCE MOLECULES

The next barrier includes cell junctions through cadherins and adherence molecules. In the epithelia, the most abundant member cadherin family is E-cadherin, which is responsible for immediate contact of neighboring cells (Cavallaro and Christofori, 2004; Peinado *et al.*, 2004, Section II.A).

E-cadherin is the central component of intercellular contacts: it connects the network of intermediate filaments of different cells with each other and enters the occlusion bodies. Hence, it creates a continuous network between homologous cells and integrates individual cells into the entire tissue (Cavallaro and Christofori, 2004). Downregulation of E-cadherin leads to tissue dissociation into separate cells and is a general component of tumor progression. Conversely, transfection of *E-cadherin* gene stops tumor progression and restores the ability of dissociated cells to form tissue-like aggregates (Arias, 2001; Christofori and Semb, 1999; Thiery, 2002). E-cadherin has not only structural functions, but is linked to several signaling pathways (Behrens *et al.*, 1993; Matsumura *et al.*, 2001). The cytoplasmic domain of E-cadherin is associated with α-, β-, and γ-catenins, while β-catenin can act as nuclear transcription factor responsible for regulation of proliferation and differentiation. *E-cadherin* gene expression may be affected by mutation, methylation, or repressed by transcription factor, particularly, *Snail or Slug* (Peinado *et al.*, 2004). When *E-cadherin* gene is downregulated or disrupted, β-catenin is liberated, migrates into the nucleus, and stimulates proliferation and tissue-specific gene transcription.

One of the main signaling cascades, *Wnt*, participating in carcinogenesis is linked to β-catenin, which can modify the activity of this pathway (Cavallaro and Christofori, 2004).

Both functions of E-cadherin, structural and signaling, are associated with cell differentiation, that is, expression of tissue-specific genes, but precise ways of realization of this function are not yet clear.

Impermeability of epithelial cell layer for migrating cells and for fluids is mainly due to E-cadherin presence. Downregulation or loss of E-cadherin is a very characteristic step in epithelial tumor progression (Perl *et al.*, 1998; Strathdee, 2002). One of the consequences of E-cadherin downregulation is disruption of gap junctional communications, which are necessary for normal cell functioning (Krutovskikh, 2002). It is an additional step in dedifferentiation associated with tumor progression.

3. INTEGRINS–ECM RELATIONS IN TUMOR PROGRESSION

Tissue-specific integrins are partially lost during progression of many epithelial tumors. In parallel, these tumors lose the ability to recognize matrix components, that is, fibronectin, the main component of ECM (Ruoslahti, 1999). Loss of recognition of ECM is probably accompanied by the gain of *anchorage independent proliferation,* which is the most characteristic feature of tumor cells.

Role of integrin derangement in tumor progression seems to be very important, since integrins specifically and variably recognize amorphous ECM as well as basal membrane. Partial redifferentiation or reversion of epithelial

tumors through specific action on their integrin system is the evidence of the impact of integrin downregulation or derangement in the dedifferentiation of corresponding epithelial tumors (Danen and Sonnenberg, 2003; Kenny and Bissell, 2003).

Partial reversion was observed in the breast cancer cell lines grown in tissue culture as a disorganized cell mass. Treatment of the 3D culture with an antibody to $\beta 1$ integrin results in the formation of acini by polarized epithelium and to secretion of milk proteins in response to hormonal stimulation (Schmeichel and Bissell, 2003). Cell proliferation in the acini was significantly inhibited as compared to that in the original culture.

Loss of adhesion of epithelial cells to plastic or basement membrane, realized by integrins, leads to downregulation of epithelial differentiation (Danen and Sonnenberg, 2003).

Identification of transcription factors, which mediate redifferentiation through individual integrin transfection, is a key problem in understanding of concrete function and possible reversion via integrins.

4. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are tissue enzymes with proteolytic activity, which act in tissue remodeling in ontogenesis and destruction of microenvironment during tumor progression. ECM, including basal membranes, is a primary target of MMPs in tumor progression (Stamenkovic, 2003). ECM components are destructed by MMPs localized on the tumor cell membranes or secreted into the extracellular fluid (Hernandez-Barrantes *et al.*, 2002). Overproduction of MMPs is a very important factor in invasion and metastasis, since they destroy the natural barriers for dissemination of tumor cells into foreign territories as well as amorphous ECM (Stetler-Stevenson and Yu, 2001).

Destruction of amorphous ECM and basement membrane probably can play another and specific role: MMPs destroy factors necessary for maintaining epithelial differentiation. Moreover, MMPs undoubtedly play an important role in epithelial–mesenchymal transdifferentiation (Section III.E).

Taken together, the data on alterations of cell adhesion in tumor progression show that it is realized through:

1. Destruction of gap junctional communication and, hence, loss of metabolic cooperation of target tissue
2. Disruption (or weakening) of cell–cell contacts
3. Disruption or weakening of cell–ECM interactions with loss of epithelial cell polarity and specific functions
4. Significant changes in cell–stroma interrelations.

D. Tissue "Architecture" in Progression: Key to Tumor Markers Origin

Tumor markers constitute a special field of oncology, both fundamental and clinical. They include oncofetal antigens, normal products of the embryo reappeared in some tumors, such as AFP, or are the result of significant derangement of excretion pathways, as is in the cases of CEA and PSA (Abelev and Sell, 1999).

Causes of the appearance of a certain marker or significant increase in its blood serum level may be different, but the common event is the change in "tissue architecture" typical for different epithelial tumors.

Earlier it was shown that the formation and integrity of liver plate is necessary for suppression of AFP synthesis in hepatocytes. Liver plate derangement when the hepatocytes are "liberated" from the plate is a necessary condition for *AFP* gene expression. The presence of 3D ECM and interaction of liver cells with ECM lead to reestablishment of *AFP* gene suppression (Abelev and Eraiser, 1999).

There must be initial distinct stages in liver tumor progression. At the initial stage, the tumor tissue retains the morphology of adult liver with polygonal hepatocytes, with characteristic surface domain distribution and retaining cell–cell interactions, overproduction of intercellular ECM, and with obligatory suppression of AFP synthesis. Chain of further stages leads step-by-step to the loss of epithelial characteristics. Destruction of regular liver plates and cell–ECM interactions as well as "architectural" derangements in general are obligatory events at the advanced stages of progression. Thus, reexpression of AFP looks like the most expected event. Of course, additional experimental evidence is required to accept this quite logical concept. Anyway, AFP is a widely used serum marker of hepatocellular and germ cell carcinomas (Abelev and Elgort, 1982).

Another system, where "architectural changes" look most plausible, is CEA increase in the blood of intestinal carcinoma patients (Hammarström, 1999). CEA is a component of normal intestinal glycocalyx and is localized strictly on the apical part of intestinal epithelium in the borderbrush. It is normally excreted into the intestinal lumen. When the epithelial layer of intestine is destroyed, CEA loses its strictly apical localization and can be seen throughout the cytoplasm. It can diffuse into circulation through basolateral surfaces of depolarized epitheliocytes, and that is, most probably, why the blood level of CEA is raised in colorectal cancer patients.

The third marker is PSA. Its increased blood level is a very probable indication of prostate cancer. And again, the most plausible reason is disruption of the normal way of its secretion (Stenman *et al.*, 1999). PSA is kallikrein protease normally secreted to the seminal fluid. In malignant

prostatic tissue, the content of dead cells may get into the blood and lead to increased PSA level.

Thus, these three most popular markers appear in blood as a consequence of tumor progression rather than of expression due to transformation.

E. Epithelial–Mesenchymal Transition as the Late Step in Progression of Epithelial Tumors

The most impressive phenomenon in tumor progression is the phenotypic change of malignant epithelium, when cells lose their epithelial polarity, specific epithelial functions, their need in basement membrane, become motile, and begin to express vimentin, typical for motile mesenchymal cells, instead of cytokeratins, typical for epithelium (Gotzmann *et al.*, 2004; Thiery, 2002). This transition looks like genetically determined transdifferentiation, but it is rather a morphogenetic event based on changes in the relations of tumor cells with microenvironment.

Epithelial–mesenchymal transition (EMT) is relevant to and uses mechanisms similar to those involved in epithelial remodeling in early ontogenesis, which suggests so-called epithelial plasticity. However, EMT most probably leads to invasion and metastases, which are usually associated with the late stages of tumor progression.

It is likely that EMT is associated with the considerable changes in expression of tissue-specific transcription factors and as a consequence leads to significant or complete epithelial dedifferentiation (Lazarevich *et al.*, 2004). Is it reversible? Is it determined on "one-gene–expression basis" or does it have more complicated nature?

EMT in *ras*-transformed cell lines can be induced by transforming growth factor (TGF)-β, which clearly shows that it is not necessarily caused by genetic changes (Fischer *et al.*, 2005; Gotzmann *et al.*, 2002; Stahl and Felsen, 2003; Zavadil *et al.*, 2001). Implication of large polyclonal communities of cells in EMT is also in line with physiological (morphogenetic) nature of this phenomenon rather than gene mutations.

F. Tissue-Specific Transcription Factors in Carcinoma Progression

1. DYSFUNCTION OF HNFs IN TUMOR PROGRESSION

While the general steps of cellular transformation have been postulated (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000), each tumor type retains the distinctive features that arise from characteristics of its

tissue of origin. The loss of differentiation observed in the majority of epithelial tumors can be due to disbalanced tissue-specific transcriptional network. In this section we will consider the alterations in HNF signaling during epithelial carcinogenesis and/or tumor progression.

Since HNFs are critical for the specification and differentiation of various epithelial cell lineages, it is reasonable to suppose that the dysfunction of these factors is an important determinant of transformation and/or progression of epithelial tumors. Studies of the last decade demonstrated the correlation of alterations in HNF expression and function with epithelial carcinogenesis (Table II). Remarkably, the factors playing the most important role in the differentiation of particular tissues are the preferential targets for inactivation in the corresponding tumor types.

This point can be illustrated by the investigations of HCC progression mechanisms performed in our laboratory in the last few years. HCC is the prevalent liver tumor and one of the world's most common cancers. The major risk factors for the development of HCC are chronic infection with hepatitis B or C viruses and prolonged exposure to hepatocarcinogenes (Bosch *et al.*, 1999). HCC development is a continuous multistep process that results from the accumulation of genetic alterations leading to the acquisition of more aggressive tumor phenotype. The analysis of genetic abnormalities and gene expression alterations in HCC revealed some candidate genes and signaling pathways possibly implicated in hepatocarcinogenesis (Thorgeirsson and Grisham, 2002). These genes encode growth factors (TGF-α and -β, hepatocyte growth factor (HGF) and their receptors), tumor suppressors (Rb and p53), components of the Wnt/catenin pathway, and other cell communication and adhesion molecules (Buendia, 2000). However, the molecular basis of hepatic tumor progression and its relationship to normal cell differentiation remain obscure.

To elucidate the role of liver-enriched transcription factors in HCC progression, we have developed an experimental model in which a chemically induced slow-growing transplantable mouse HCC (sgHCC) rapidly gave rise *in vivo* to a highly invasive dedifferentiated fast-growing variant (fgHCC) (Lazarevich *et al.*, 2004).

sgHCC is a highly differentiated tumor with a pattern of gene expression that closely resembles normal hepatocytes. The progression of sgHCC was accompanied by loss of epithelial morphology, activation of telomerase, extinction of liver-specific gene expression, invasion, and ultimately metastasis (Lazarevich *et al.*, 2004; Varga *et al.*, 2001).

The loss of epithelial morphology in fgHCC comprises a complete loss of cell polarity and a decrease in cell–cell and cell–matrix adhesion. sgHCC has a typical basal membrane which consists of type IV collagen, laminin, entactin, and fibronectin; each cell interacts with the basal membrane. In contrast, in fgHCC, all ECM components are synthesized but not associated

Table II HNFs Dysfunction in Epithelial Tumors

HNFs dysfunction in epithelial tumors				
Transcription factor	**Tumor type**	**Alteration described**	**Reversion**	**Reference**
HNF1α	Hepatocellular adenoma	Mutation	ND	Bluteau *et al.*, 2002
HNF1α/HNF1β	HCC	Decrease of HNF1α/HNF1β ratio	ND	Ninomiya *et al.*, 1996
HNF1α	Dedifferentiated HCC	Reduced expression	ND	Wang *et al.*, 1998
HNF1α	Colorectal cancer	Mutation	ND	Laurent-Puig *et al.*, 2003
HNF1α	Renal cell carcinoma	Reduced expression; diminished binding activity	ND	Anastasiadis *et al.*, 1999; Sel *et al.*, 1996
HNF1α and -β	Renal cell carcinoma	Germline mutations	ND	Rebouissou *et al.*, 2005
HNF1β	Clear cell carcinoma of the ovary	Upregulation	Apoptotic cell death	Tsuchiya *et al.*, 2003
HNF3β	Lung cancer	Downregulation, mutations, promoter methylation	Growth arrest	Halmos *et al.*, 2004
C/EBPα	HCC	Reduced expression	Growth inhibition, suppression of tumorigenicity	Flodby *et al.*, 1995; Watkins *et al.*, 1996; Xu *et al.*, 2001

C/EBPα	Breast cancer	Reduced expression, cytoplasmic localization	Growth inhibition, differentiation	Gery *et al.*, 2005
C/EBPα	Lung cancer	Reduced expression	Proliferation arrest, apoptosis, morphological changes	Halmos *et al.*, 2002
C/EBPα	Skin squamous cell carcinoma	Reduced expression	Growth arrest	Shim *et al.*, 2005
C/EBPβ-LIP	Breast cancer	Upregulation	ND	Zahnov *et al.*, 1997
C/EBPβ-2 LAP	Breast cancer	Upregulation	ND	Bundy and Sealy, 2003
C/EBPβ	Ovarian epithelial tumor	Upregulation	ND	Sundfeldt *et al.*, 1999
C/EBPβ	Colorectal cancer	Upregulation	ND	Rask *et al.*, 2000
C/EBPβ	Renal cell carcinoma	Increased activity	ND	Oya *et al.*, 2003
C/EBPβ	Wilms tumor	Upregulation	Apoptosis	Li *et al.*, 2005
HNF4α	Hepatoid adenocarcinoma of stomach	Upregulation	ND	Yano *et al.*, 2003
HNF4α	HCC	Reduced expression	Growth arrest, restoration of epithelial morphology, gene reexpression	Lazarevich *et al.*, 2004; Spath and Weiss, 1998
HNF4α	Renal cell carcinoma	Diminished binding activity	ND	Sel *et al.*, 1996

with the membranes, thus, cell–matrix interactions are completely disarranged. The tight junction marker protein ZO-1 is present on the membrane of sgHCC, while fgHCC cells exhibit no membrane staining of ZO-1. This fact together with the redistribution of domain-specific markers indicates the loss of epithelial polarity in fgHCC (Engelhardt *et al.*, 2000; Kudryavtseva *et al.*, 2001).

The morphological changes observed during a one-step progression meet the criteria of the EMT (Thiery, 2002). It is accompanied by alterations in the expression of several genes coding for the cell–cell and cell–ECM communication components (Lazarevich *et al.*, 2004). Noteworthily, sgHCC, characterized by extremely strong cell–cell and cell–matrix contacts, overexpresses E-cadherin and β-catenin mRNAs as compared to normal liver. fgHCC shows significantly decreased steady state mRNA levels of the major liver gap junctional proteins Cx32 and E-cadherin, and an overexpressed $\alpha 3$ integrin subunit, which is expressed in immature or transformed hepatocytes and in biliary cells and mediates ECM-induced differentiation (Lora *et al.*, 1998). Downregulation of E-cadherin transcription is accompanied by a significant increase of mRNA steady state level of *Snail*, a zinc finger transcription factor involved in the epithelial-to-mesenchymal transition through repression of the E-cadherin promoter (Peinado *et al.*, 2004). In the mouse HCC model, EMT was also mediated by increased levels of the mesenchymal cell marker vimentin and splice form of fibronectin containing extradomain A (EDA). EDA form of fibronectin promotes the increase of cell spreading and migration (Manabe *et al.*, 1997).

In addition to the loss of epithelial morphology, fgHCC cells demonstrate reduced expression of most markers of mature hepatocytes (albumin, α_1-antitrypsin, transthyretin, phenylalanine hydroxylase, apolipoproteins, L-type pyruvate kinase, glucokinase, and so on) as well as the expression of AFP normally restricted to fetal hepatoblasts and induced in sgHCC as compared to normal liver. Thus, according to the expression pattern, the one-step progression caused severe dedifferentiation of fgHCC as compared to sgHCC. These alterations were coupled with the reduced expression of the entire block of liver transcription factors that are essential for hepatocyte differentiation, namely, HNF1α, HNF1β, HNF3γ, C/EBPα, HNF4α (both $\alpha 1$ and $\alpha 7$ isoforms), and HNF6 (Lazarevich *et al.*, 2004). The retained expression of several liver-specific genes in fgHCC confirms the hepatic origin of these cells (Lazarevich *et al.*, 2004).

Multiple phenotypic changes defining the development of a highly malignant phenotype occur rapidly implying that HCC progression in this model is a consequence of a limited set of molecular events. We suppose that HCC progression can result from dysfunction of liver-specific transcription factors, which control hepatic gene expression and differentiation. Extensive investigations on animal models indicate that the key liver-specific

transcription factors providing for mature hepatic phenotype are HNF4α1, HNF1α, and C/EBPα (Section II.B.1).

The relevance of these factors for the maintenance of hepatic differentiation is confirmed for hepatoma cell cultures. The expression of HNF4α1 as well as of its direct transcriptional target HNF1α is generally restricted to differentiated hepatomas, while HNF1β and HNF4α7 are mainly expressed in dedifferentiated cells (Cereghini *et al.*, 1988; Nakhei *et al.*, 1998; Spath and Weiss, 1998; Torres-Padilla *et al.*, 2001). In concordance with these data, a significant decrease of HNF1α expression was revealed in poorly differentiated human HCCs relative to well-differentiated tumors (Wang *et al.*, 1998).

Similarly, the expression of C/EBPα, associated with the maintenance of a quiescent differentiated state of hepatocytes, is very low or undetectable in liver nodules and HCC tumor samples (Flodby *et al.*, 1995). Forced expression of C/EBPα in human hepatoma cell lines results in impaired proliferation and suppressed tumorigenicity (Watkins *et al.*, 1996).

The expression of exogenous HNF4α1 in a dedifferentiated hepatoma cell culture H5 induces the expression of several hepatic genes and reestablishment of epithelial cell morphology. This transition is associated with reexpression of cytokeratins and E-cadherin production. These findings indicate that HNF4α may be a key regulator of hepatic differentiation, which integrates tissue-specific gene expression and epithelial morphogenesis (Spath and Weiss, 1998).

The studies on the one-step model of HCC progression in mice confirmed the essential role of HNF4α dysfunction in hepatocarcinogenesis (Lazarevich *et al.*, 2004). Forced expression of HNF4α1 in cultured fgHCC cells partially restored epithelial morphology. fgHCC cells expressing HNF4α1 formed epithelial sheets with tight junctions marked by ZO-1 plasma membrane staining. In addition, fibrils of ECM components were located along cell–cell interfaces indicating the formation of cell–matrix contacts. At the same time, E-cadherin was not revealed on the cell membranes of epithelial islands suggesting that epithelial conversion in this case is an E-cadherin independent process.

HNF4α1 reexpression in fgHCC cell culture also restored the expression of several functional hepatic proteins (apolipoproteins, α_1-antitrypsin, Cx32, and so on) and transcription factors (HNF1α, HNF6, HNF4α7, and HNF3γ), reduced the proliferation rate *in vitro,* and dramatically inhibited tumor formation and lung metastases in congenic recipient mice. The multiple effects of HNF4α1 on tumor cell characteristics can be mediated by the activation of other HNF4α-regulated liver-enriched transcription factors. Nevertheless, the reexpression of individual transcription factors that were HNF4α-inducible did not result in obvious changes in fgHCC cell proliferation and morphology (Lazarevich, unpublished data).

Thus, the profound changes in the differentiation state and cell proliferation in this model of tumor progression are at least partially defined by HNF4α1 suppression. The restoration of HNF4α1 function can promote the reversion of invasive HCC toward a less aggressive phenotype by inducing hepatic redifferentiation.

The central role of HNF4α1 in the control of morphological, proliferative, and functional properties that define hepatic differentiation was confirmed in other models. Studies of gene expression profiles on the collection of chemically induced mouse HCCs of independent origin revealed a strict correlation between HNF4α1 transcription and tumor differentiation status. Downregulation of HNF4α1 transcription in the fast-growing poorly differentiated tumors coincided with the extinction of transcription factors that previously proved to be HNF4-inducible (HNF1α, HNF4α7, and HNF3γ). We also detected downregulation of HNF4α1 expression in 13 of 16 human HCCs not associated with hepatitis viruses infection (Fleishman and Lazarevich, in preparation). Moreover, a high incidence of dysfunction of HNF4α1 and its transcriptional target HNF1α was revealed in human renal cell carcinomas (Anastasiadis *et al.*, 1999; Sel *et al.*, 1996).

Thus, these factors may be considered as potential tumor suppressor genes at least in the kidney and liver, where HNF4α1/HNF1α regulatory pathway clearly defines the lineage-specific differentiation. These data support the hypothesis that dysfunction of tissue-specific transcription factors essential for definite tissue specification can be a critical step in the dedifferentiation and progression of the corresponding epithelial tumors.

This point can be illustrated by dissecting the role of C/EBP factors in epithelial carcinogenesis. Reduced expression of C/EBPα was described in breast (Gery *et al.*, 2005), lung (Halmos *et al.*, 2002), and skin (Shim *et al.*, 2005) tumors. In lung cancers, the decrease of C/EBPα expression correlates with the histological subtype and tumor grade (Halmos *et al.*, 2002). Restoration of C/EBPα expression in nonsmall cell lung cancer cell lines induces apoptosis, proliferation arrest, and morphological changes toward a more differentiated phenotype.

mRNA and protein levels of C/EBPα are downregulated in mouse skin squamous cell carcinomas (SCCs) and cell lines with activated Ras (Shim *et al.*, 2005). Transduction of keratinocytes with oncogenic *ras* diminishes the expression and DNA binding of C/EBPα, indicating the involvement of activated Ras in negative regulation of C/EBPα transcription. Forced expression of C/EBPα in SCC cell lines inhibits the proliferation of SCC cells containing oncogenic Ras indicating that the antiproliferative activity of C/EBPα is dominant over the Ras effects.

In humans, the normal mammary tissue expresses only C/EBPβ activatory isoform 1 (Bundy and Sealy, 2003). The overexpression of the dominant

negative isoform of C/EBPβ induces proliferation and hyperplasia of the mammary gland (Zahnow *et al.*, 2001). In addition, the overexpression of C/EBPβ activatory isoform 2 in mammary epithelial cell line induces EMT and development of invasive phenotype characterized by the loss of E-cadherin junctional localization, acquisition of a spindle-shape morphology, actin stress fibers, and the appearance of the mesenchymal intermediate filament vimentin (Bundy and Sealy, 2003). C/EBPβ regulates the expression of cyclooxygenase 2, the key enzyme of prostaglandin biosynthesis that induces mammary tumorigenesis in transgenic mice and is frequently overexpressed in invasive breast cancers. There is increasing evidence that C/EBPβ acts as downstream effector of Ras-signaling. C/EBPβ also plays an important role in Ras-mediated skin tumorigenesis. C/EBP$\beta^{-/-}$ mice are refractory to chemically induced skin carcinogenesis (Zhu *et al.*, 2002). All these findings support the idea that dysfunction of tissue-specific transcriptional regulators is an important event in the development of malignant phenotype of different epithelial cell types.

Certainly it is unlikely that the function of the HNF transcriptional network is implemented autonomously from the ubiquitous pathways controlling the growth, morphological, and housekeeping properties of cells. While a significant progress in understanding the impact of HNFs in epithelia differentiation and function was achieved in the last decade, the mutual influence of this tissue-specific transcriptional network and the components of the main signal transduction cascades are still underestimated. In this context, we discuss the presumable mechanisms of HNF4α influence on hepatic differentiation.

What mechanisms define the HNF4-dependent control of hepatic function, epithelial morphology, and proliferation? This question might be addressed by identification of new transcriptional targets of tissue-specific regulatory factors. HNF4α effects on gene regulation can be realized both directly and by regulation of transcription of other HNFs. However, the inactivation of HNFs in mice (Section II.B.1) and their reexpression in dedifferentiated tumors have less dramatic consequences suggesting that the most critical regulation of cell properties is realized by HNF4α per se.

Obviously the major part of experimentally proved HNF4α targets is functional genes encoding blood coagulation factors, enzymes of lipid (Hayhurst *et al.*, 2001), amino acid, glucose (Parviz *et al.*, 2003), and urea (Inoue *et al.*, 2002) metabolism. Importantly, HNF4α regulates the expression of cytochromes P450 and some other enzymes and transporters involved in drug metabolism (Tirona and Kim, 2005). Thus, the loss of HNF4α during HCC progression can have an additional significance—HNF4-negative tumors can have altered responsiveness to therapy. In this case, reexpression of HNF4α can be considered as a possible approach to sensitization of HCCs to therapy.

The morphogenic properties of HNF4α may be realized by transcriptional regulation of several cell adhesion and junction proteins like E-cadherin (Parviz *et al.*, 2003; Spath and Weiss, 1998), Bgp1, gap junctional proteins Cx32 (presumably, through HNF1α regulation) (Piechocki *et al.*, 2000) and Cx26 (Parviz *et al.*, 2003), and tight-junction components occludin and claudins 6 and 7 (Chiba *et al.*, 2003). There is also an evidence for the existence of HNF4-dependent posttranslational mechanisms of ZO-1 dysfunction (Parviz *et al.*, 2003).

HNF4α putative transcriptional targets that can mediate the antiproliferative effects of this factor include p21 (Chiba *et al.*, 2005) and Fas ligand (Osanai *et al.*, 2002). HNF4α is also essential for the hypoxic induction of the *erythropoietin* gene in the kidney and liver, thus, it is likely involved in the cellular oxidative stress response in normal and tumor tissues (Galson *et al.*, 1995).

Additional candidate *HNF4*α target genes were identified using microarray hybridization (Naiki *et al.*, 2002). Chromatin immunoprecipitation analyses combined with promoter microarrays with Hu13K human DNA chip identified about 1500 putative *HNF4*α target genes in human liver (Odom *et al.*, 2004). The experimental verification of this data will help to disclose the new mechanisms defining the key role of HNF4α in hepatic differentiation.

Studies on the one-step HCC progression model allowed us to demonstrate that the regulatory region of HNF4α1 is inactive in dedifferentiated fgHCC, indicating HNF4α1 repression at the transcriptional level as oppose to mutation, DNA rearrangement, or other damage of the HNF4α1 coding or regulatory regions. This result provides a strong evidence for the existence of HNF4α upstream mechanisms responsible for tumor progression.

2. HNFs MEDIATE MICROENVIRONMENTAL EFFECTS ON HEPATIC DIFFERENTIATION

It has been clearly demonstrated that the maintenance of epithelial morphology is an important determinant influencing hepatic differentiation. Isolation and cultivation of hepatocytes associated with disruption of normal interaction between cells and ECM leads to drastic changes in the gene expression program. Freshly isolated adult liver hepatocytes, cultivated on dried collagen substrate in the presence of growth factors, actively proliferate, express cytoskeletal components (actin, tubulin, cytokeratins, and vinculin), but exhibit low levels of liver-specific gene expression. Cultivation of hepatocytes on reconstituted ECM gel from Engelbreth-Holm–Swarm mouse sarcoma (EHS gel), primarily composed of laminin, type IV collagen,

heparin sulfate proteoglycan matrix, and several growth factors, diminishes DNA synthesis, decreases the transcription of cytoskeletal components, and prevents the loss of liver-specific (albumin, transferrin, and α_1-antitrypsin) gene expression (Ben-Ze'ev *et al.*, 1988). What molecular mechanisms translate the extracellular signals into alterations of liver-specific gene expression?

Cultivation of hepatocytes on collagen I decreases HNF4α and C/EBPα expression, while growth on EHS gel maintains the transcriptional level of HNF4α similar to the *in vivo* level (Oda *et al.*, 1995; Runge *et al.*, 1997). Moreover, dedifferentiated hepatocytes grown on dried collagen I matrix can redifferentiate after the addition of EHS gel. This transition is accompanied by induction of C/EBPα DNA-binding activity (Runge *et al.*, 1997) and HNF4α transcription (Runge *et al.*, 1999). These data suggest that the key factors of hepatic maturation, HNF4α and C/EBPα, can mediate ECM-dependent regulation of liver-specific gene expression. However, the absence of C/EBPα does not prevent ECM-dependent upregulation of albumin gene in primary hepatocytes isolated from C/EBP$\alpha^{-/-}$ mice (Soriano *et al.*, 1998). It is likely that C/EBPα alone is not responsible for this effect.

HNF3 factors are essential for early liver development and activation of liver-specific genes (Lemaigre and Zaret, 2004). HNF3α expression and HNF3 DNA-binding activity are upregulated by ECM in the hepatic-derived cell lines H2.35 and HepG2 (DiPersio *et al.*, 1991). In these cell lines, the activation of HNF3 is critical for upregulation of albumin transcription induced by ECM assuming that HNF3 factors can mediate ECM-dependent differentiation. However, the dedifferentiation of primary hepatocytes in culture leads to the upregulation of HNF3α (Oda *et al.*, 1995; Runge *et al.*, 1998), while EHS gel-induced redifferentiation is accompanied by the reduction of HNF3 DNA-binding activity (Runge *et al.*, 1998). Thus, it is likely that HNF3 cannot provide ECM-responsive activation of the differentiation program in normal adult hepatocytes. These data are consistent with the observation that the activity of HNF3 proteins has the most profound impact on hepatic differentiation at the early stages of liver development (Sund *et al.*, 2000).

The results supporting the key role of HNFs in ECM-mediated differentiation were obtained on the model of "small hepatocytes" differentiation in culture (Sugimoto *et al.*, 2002). Small hepatocytes are proliferating cells with hepatic characteristics isolated from adult liver. Cultivation in EHS gel induces the changes of the cell shape typical for mature hepatocytes, formation of bile canaliculi-like structures, and production of liver-specific proteins, which correlate with the activation of HNF4α, HNF6, C/EBPα, and C/EBPβ transcription. Addition of individual growth factors, ECM components of the EHS, or collagen gel failed to induce similar alterations in cell morphology and gene expression. It is likely that hepatic differentiation

depends on the formation of complex basal membrane-like structures and is modulated by cytokines signaling.

From the first steps of hepatic organogenesis, the paracrine cytokine signaling from mesodermal cells substantially determines the hepatic differentiation into functional hepatocytes (Lemaigre and Zaret, 2004). The liver bud formation is induced by fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) produced by the cardiogenic mesoderm and septum transversum, respectively. Further stages of liver development are also mediated by the action of various cytokines produced by stromal cells, which control both morphogenesis and proliferation of hepatocytes. Besides, tumor cells, especially those undergoing EMT, gain the ability to secrete growth factors in an autocrine fashion, thus gaining additional independence from the microenvironment (Gotzmann *et al.*, 2002).

The complex interplay between growth factor signaling and ECM effects on the expression of HNFs can be illustrated by studies of *in vitro* differentiation of fetal mouse hepatocytes. Expression of mature hepatic genes and upregulation of HNF4α in fetal hepatocytes can be induced by EHS and interleukin-6–related hepatic maturation factor oncostatin M (Kamiya *et al.*, 2003). In fetal hepatocytes, this factor directly activates K-Ras and promotes the formation of E-cadherin–based adherence junctions by the Ras-dependent mechanism (Matsui *et al.*, 2002). Hepatic differentiation induced by oncostatin M and EHS can be suppressed by tumor necrosis factor (TNF)-α, which inhibits the induction of HNF4α and several liver-specific genes (Kamiya and Gonzalez, 2004).

There is growing evidence that the activity of TGF-β signaling pathway can modulate HNF4α-dependent regulation of hepatic differentiation. HNF4α was shown to physically interact and functionally cooperate with Smad 3 and 4 transcription factors that mediate TGF-β signaling (Chou *et al.*, 2003).

In primary rat hepatocytes, TGF-β was shown to repress HNF4α transcription through activation of Wilm's tumor suppressor WT1, transcription factor referred to as dedifferentiation marker in hepatocytes (Berasain *et al.*, 2003). De Lucas *et al.* (2004) reported the decrease of HNF4α DNA-binding activity in human hepatoma HepG2 treated with TGF-β and proposed that TGF-β induces HNF4α degradation in the proteasome.

Downregulation of HNF4α expression was also revealed during TGF-β induced EMT in cultured fetal hepatocytes (Sanchez *et al.*, 1999; Valdes *et al.*, 2002) and immortalized hepatocytes transformed with oncogenic Ha-Ras (Gotzmann *et al.*, 2002). These findings suggest that the loss of HNF4α can at least partially mediate the alterations of the functional and morphological properties of hepatocytes during this process.

C/EBP proteins, important mediators of both proliferation and differentiation, are regulated by injury-related cytokines such as TNF-α and interleukin-6 (Diehl, 1998). Suzuki *et al.* (2003) showed that a gradual

differentiation of hepatic stem cells to albumin-producing hepatoblasts and finally to mature hepatocytes induced by HGF and oncostatin M is mediated by C/EBPα activation. Lack of C/EBP proteins inhibits hepatic differentiation of stem cells. In this model, several ECM components also induce differentiation by C/EBP expression control, although this effect was less pronounced. Modulation of C/EBP signaling can not only directly influence liver-specific gene expression but also modify the production of proteins directly involved in the maintenance of epithelial cell morphology. Functional C/EBP sites are identified in the promoters of genes coding ECM proteins, MMPs, and protease inhibitors (Boffa *et al.*, 2002; Doyle *et al.*, 1997; Greenwel *et al.*, 2000).

We illustrated the role of ECM in tissue-specific gene regulation on various models of hepatic differentiation; however, it seems suitable for other epithelial tissues. For example, ECM-dependent regulation of tissue-specific *αs1*- and *β-casein* genes in mammary cells is mediated by alterations in activity of C/EBP proteins, which are critical regulators of mammary gland differentiation (Jolivet *et al.*, 2005; Myers *et al.*, 1998).

Thus, it is likely that tissue-specific transcription factors essential for the specification of definite cell lineages mediate the interaction of epithelial cells with the microenvironment. ECM remodeling, which can regulate signal transduction pathways through interactions with integrins or by releasing growth factors bound to the matrix, together with paracrine signaling from stroma cells can modulate expression or activity of transcription factors. On the other hand, alteration of the transcription factors activity can regulate various genes essential for the maintenance of epithelial morphology. Interrelations between HNF signaling and microenvironment are highly complex and need further investigations, while the importance of tissue-specific transcriptional regulators for the transmission of extracellular signals into alterations of cell transcriptional program is already clear. Consequently, the impairment of tissue-specific transcriptional network seems a very probable cause of epithelial dedifferentiation and carcinoma progression.

IV. CONCLUDING REMARKS AND FURTHER PERSPECTIVE

A. Different Ways of Progression in Hemoblastoses and Carcinomas

In conclusion, we would like to stress on some not trivial features of progression in relation to differentiation in tumors. These features become most clear when epithelial tumors are compared with hemoblastoses.

The first and the most crucial difference lies in interrelations between the mechanisms of normal differentiation and development of malignant phenotype in both types of neoplasia. Transformation itself is most probably similar in both systems. It includes constitutive activation of protooncogene or inactivation of tumor suppressor gene and immortalization, that is, events leading to autonomous and infinite cell proliferation. These processes take place both in hemopoietic and in epithelial tissues through the similar mechanisms: protooncogene mutations, activation of protooncogene by its translocation next to functional transcription regulatory elements, or formation of fused genes driven by the promoters active in target tissue (Barr, 1998).

Principal differences appear at the next steps of tumor evolution—in tumor progression—in selection of the most autonomous variants from a genetically unstable population. In epithelial tumors it leads to invasion and metastases, that is, to ultimate independence on homologous microenvironment permitting the tissue growth in heterologous territories. Dedifferentiation is associated with either process. Thus, partial or complete loss of tissue-specific proteins and antigens is a natural consequence of epithelial tumor evolution.

On the contrary, progression of hemoblastoses tends to maintain the ability of hemopoietic cells of invading normal tissue (diapedesis) and to expand their ability of forming extramedullar islands of clonal growth. Both properties are inherent in the normal hemopoietic tissue. The fatal feature of progression in this system is overproduction of leukemic cells, their accumulation in the bone marrow and blood, extramedullar hemopoesis, and suppression of normal hemopoeisis. However, autonomous leukemic progenitor cells unlike the normal progenitors do not sense the excess of blood cells in leukemia, while normal progenitors are suppressed by this excess.

It should be kept in mind that after differentiation of hemopoietic cells, which takes place in bone marrow microenvironment, committed or mature cells *no more require specific microenvironment for maintaining their differentiation state*. This is the principal difference between hemopoietic and epithelial tumors, especially in their progression. Progression of hemoblastoses is not only compatible with the normal properties of hemopoietic cells but is also based on their normal physiological functions, thus keeping differentiation antigens unchanged. Rare cases of "promiscuous" hemopoietic line markers expression are due, most probably, to the existence of corresponding transitional stages in development of certain blood cells (Greaves *et al.,* 1986).

B. Future Investigations

We are trying to formulate several questions, which remain unresolved on the way of development of a unifying concept, binding microenvironment to epitheliocyte differentiation, and its dedifferentiation in the process of tumor progression due to disruption of microenvironment.

The first problem includes clarification of the microenvironment function in *induction* versus *maintenance* of epithelial differentiation. The known elements of this process include obligatory participation of heterologous tissue, most typically mesenchymal tissue, in the specification of epithelial organs such as liver, pancreas, kidney, mammary gland, skin epidermis, and so on. This problem raises the underlying one as to: how tissue interactions induce (and maintain?) a network of tissue-specific transcription factors determining epithelial tissue differentiation. It is obviously a key point of the whole concept, including disruption of these mechanisms in tumor progression. The investigations of Kenneth Zaret and his colleagues as well as George Michalopoulos' team on the regulation of tissue-specific HNFs by ECM gel opened a way in this direction.

The most enigmatic but clearly formulated problem is *why* and *how* 3D ECM induces a specific set of transcription factors operating in tissue-specific differentiation? Undoubtedly, this is a key problem relating ECM structure and epitheliocytes differentiation. Its solution is in the air, but the way to solve it is not yet found. Only few publications to our knowledge showed that the nuclear shape and organization of nuclear matrix associated with gene expression depend on ECM structure (Lelievre *et al.*, 1998; Maniotis *et al.*, 1997).

Another very important problem is identification of tissue-specific transcription factors essential for epithelial differentiation. Does epitheliocytes interaction with ECM lead to independent mosaic-like induction of individual transcription factors or implement through a single crucial step, which determines the further chain of events resulting in the formation of a specific transcriptional network? Evidence of the critical role of HNF4α in hepatocyte differentiation favors the latter possibility (Lazarevich *et al.*, 2004).

How should EMT be regarded: as a regular change in epitheliocytes development, including remodeling of epitheliocytes and natural evolution of tissue structure, or as a result of pathological development of epithelial–mesenchymal interrelations?

And finally, to what extent can tumor progression be reversed? Is the normalization of malignant phenotype limited to some intermediate stages, or can the whole process be turned back? In this respect the identification of definitive events of tumor reversion is extremely important (Kenny and Bissell, 2003; Mintz and Illmensee, 1975).

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Cancer Vaccines: Preclinical Studies and Novel Strategies

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The development of cancer vaccines, aimed to enhance the immune response against a tumor, is a promising area of research. A better understanding of both the molecular mechanisms that govern the generation of an effective immune response and the biology of a tumor has contributed to substantial progress in the field. Areas of intense investigation in cancer immunotherapy will be discussed here, including: (1) the discovery and characterization of novel tumor antigens to be used as targets for vaccination; (2) the investigation of different vaccine-delivery modalities such as cellular-based vaccines, protein- and peptide-based vaccines, and vector-based vaccines; (3) the characterization of biological adjuvants to further improve the immunogenicity of a vaccine; and (4) the investigation of multimodal therapies where vaccines are being combined with other

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oncological treatments such as radiation and chemotherapy. A compilation of data from preclinical studies conducted *in vitro* as well as in animal models is presented here. The results from these studies would certainly support the development of new vaccination strategies toward cancer vaccines with enhanced clinical efficacy. © 2006 Elsevier Inc.

I. INTRODUCTION

Tumor-associated antigens (TAAs) are by definition weakly immunogenic or functionally nonimmunogenic if tumors are not spontaneously rejected by the host. The field of cancer immunotherapy has, therefore, focused on the development of vaccine strategies in which the presentation of TAAs to the immune system results in significantly greater activation of T cells than that which occurs normally in a host. The use of cancer vaccines for the potential treatment of human malignancies has reached several new milestones in scientific discovery. Areas of intense investigations include (1) the discovery, development, and characterization of TAAs or tumor-specific antigens (TSAs) that are selectively expressed or overexpressed by malignant cells as compared with normal adult tissues; (2) novel vaccine-delivery systems for the induction of endogenous host antitumor immune responses; and (3) cytokines and other biological adjuvants to further augment immunogenic properties of vaccine preparations.

The review material presented here has been taken from previously published papers where indicated. Specific goals of this chapter are to (1) provide an overview of various vaccine targets, (2) examine different types of vaccine modalities, (3) evaluate cytokines and other biological adjuvants, and (4) review innovations in cancer vaccine development. The use of the described preclinical models and strategies should facilitate the translation of these findings to clinical applications for active immunotherapy of a range of human carcinomas.

II. TYPES OF TARGETS FOR VACCINE THERAPY

Many potential targets for cancer immunotherapy have been identified. These targets can be grouped into two major categories: (1) TSAs and (2) TAAs. TSAs comprise gene products that are uniquely expressed in tumors such as point-mutated *ras* oncogenes (Abrams *et al.*, 1996a), mutated *p53* suppressor genes (Nikitina *et al.*, 2002), anti-idiotype antibodies (Bendandi *et al.*, 1999; Hsu *et al.*, 1996; Ruffini and Kwak, 2001), and viral antigens. Several viruses have been linked with some cancers such as human papilloma virus (HPV) and cervical cancer. TAAs can be subdivided into oncofetal

antigens, nonmutated oncogene products, and tissue-lineage antigens. Oncofetal antigens, which include carcinoembryonic antigen (CEA), the breast cancer mucin MUC-1, and prostate-specific membrane antigen (PSMA), can be overexpressed in tumors and may be expressed at low levels in normal tissues. These antigens are also found in fetal tissues. Oncogene products, such as nonmutated HER-2/*neu* and p53, are analogous to oncofetal antigens in that they can be overexpressed in tumors and may be expressed in some fetal and/or normal tissues. Tissue-lineage antigens, such as prostate-specific antigen (PSA) and melanocyte antigens, MART-1/Melan A, tyrosinase, gp100, and TRP-1 (gp75), are expressed in a tumor of a given type and the normal tissue from which it is derived and are potentially useful targets for immunotherapy.

III. VACCINE STRATEGIES

Selection of an adequate vaccine-delivery system is fundamental in the design of immune strategies for cancer therapy. Each of the various types of vaccine that will be discussed in this section (Table I) has been analyzed in experimental models and, in some cases, in the clinic. A conclusion from

Table I Vaccine Modalities

Vaccine Modalities
Cellular-based vaccines
Whole-tumor cells
Gene-modified tumor cells (GM-CSF, costimulatory molecules)
Dendritic cells (various strategies for antigen loading)
Protein-based vaccines
Proteins
Peptides
Agonist peptides
Anti-idiotype mAb
mAb fusion proteins
Vector-based vaccines
a. Viral vectors
Adenovirus
Vaccinia
MVA
Avipox: Fowlpox, canarypox
b. Bacterial vectors
Listeria
Salmonella
BCG
c. Yeast vectors
d. Plasmid DNA

these studies is that there is no optimal modality for vaccine delivery as of yet, and a combination of some of the strategies has proven to increase the outcome of the intervention against the tumor.

A. Cellular-Based Vaccines

Cellular-based vaccines, which include whole-tumor cell vaccines and dendritic cells (DCs), have been extensively explored in both preclinical and clinical settings.

1. WHOLE-TUMOR CELL VACCINES

Whole-tumor cell vaccines are divided into two categories: (1) autologous vaccines in which tumor cells from a patient are used as a vaccine for the same patient, and (2) allogeneic vaccines in which tumor cells from other patients, usually from established tumor cell lines, are used as a vaccine. In general, a major advantage of whole-tumor cell vaccines is that a broad range of TSAs or TAAs could be presented to the T cells, including antigens that are yet not known. High costs of production and quality control, however, are usually required for the preparation of this type of vaccines. This is an important consideration for autologous tumor cell vaccines, which involve a major effort in obtaining fresh tumor cells at surgery followed by a customized vaccine-preparation process. Allogeneic vaccines, which usually employ one or more tumor cell lines, are relatively easy to prepare when compared with autologous vaccines.

Several approaches, mostly genetic modification of the tumor cells, have been taken to increase the ability of whole-tumor cell vaccines to stimulate antitumor immunity. Tumor cell modification has primarily focused on cytokines and costimulatory molecules. Among the cytokines, transduction of weakly immunogenic murine tumor cells to express IL-2 has shown to reduce tumorigenicity and to induce long-lasting protective immunity against the parental tumor (Fearon *et al.*, 1990; Gansbacher *et al.*, 1990). In different mouse tumor models, irradiated tumor cells expressing murine granulocyte-macrophage colony-stimulating factor (GM-CSF) in a paracrine fashion have shown to stimulate a long-lasting systemic antitumor immunity (Couch *et al.*, 2003; Dranoff *et al.*, 1993). Modification of tumor cells to express high levels of costimulatory molecules has also been used to enhance tumor immunogenicity. Murine adenocarcinoma cells modified via infection with a recombinant vaccinia virus expressing B7-1 have shown to deliver protective immunity against the parental tumor (Hodge *et al.*, 1994). In a murine B-cell lymphoma model, mice vaccinated with tumor cells engineered to express high levels of the costimulatory molecules B7-1,

intercellular adhesion molecule (ICAM)-1, and lymphocyte function-associated antigen (LFA)-3 (designated TRICOM) showed enhanced antitumor responses (Briones *et al.*, 2003). Chronic lymphocytic leukemia (CLL) cells, characterized by a very low expression of costimulatory molecules on their cell surface, have been successfully modified by using a modified vaccinia virus strain Ankara (MVA) vector encoding for the human B7-1, ICAM-1, and LFA-3 molecules (TRICOM) (Palena *et al.*, 2005). When MVA-TRICOM-infected CLL cells were used as stimulators in proliferation assays, proliferation of allogeneic and autologous T cells was demonstrated. Moreover, cytotoxic T lymphocytes (CTLs) that were generated *in vitro* by stimulation of autologous T cells with MVA-TRICOM-infected CLL cells mediated lysis of uninfected CLL target cells, thus, supporting the potential of the approach for immunotherapy of CLL.

2. DENDRITIC CELL VACCINES

DCs are considered to be the most potent antigen-presenting cells (APCs) and, therefore, one of the most powerful tools for immunization (Banchereau *et al.*, 2001a; Steinman and Dhodapkar, 2001). The antigen loading techniques are numerous and include: (1) pulsing with major histocompatibility complex (MHC)-binding peptides; (2) pulsing with protein; (3) loading with anti-idiotype Ab; (4) loading with tumor lysates or apoptotic tumor cells; (5) DNA or RNA transfection; and (6) infection with a viral vector.

In experimental murine models, DCs pulsed with peptides from TAAs have generated antigen-specific immune responses (Banchereau *et al.*, 2001b). It was also demonstrated that the ability of DCs to activate both naïve and effector T cells could be further enhanced by addition of costimulation on their cell surface. By using poxvirus vectors that encode for a triad of murine costimulatory molecules (B7-1, ICAM-1, and LFA-3; TRICOM), the efficacy of DCs in priming specific immune responses has been improved (Hodge *et al.*, 2000). Similarly, human DCs have been modified by infection with a fowlpox virus encoding for the human TRICOM molecules, which resulted in enhanced activation of peptide-specific immune responses (Zhu *et al.*, 2001). Among novel immunization strategies, preclinical studies are being conducted on the use of DC-tumor cell hybrid vaccines (Koido *et al.*, 2002). The use of exosomes, that is, substructures of DCs, may also prove to be a valuable vaccine-delivery vehicle (Taieb *et al.*, 2005; Vincent-Schneider *et al.*, 2002). Finally, the disadvantage of this type of vaccines is that clinical grade DCs are difficult to generate. Large amounts of peripheral blood mononuclear cells (PBMCs) are required, and the PBMCs must be cultured for several days in the presence of cytokines such as GM-CSF, IL-4, and/or tumor necrosis factor (TNF)-α.

B. Directed Intratumoral Expression of Genes Encoding Costimulatory Molecules, Cytokines, or Chemokines

For the efficient activation of T cells, two signals are required. The first signal involves the interaction of the T-cell receptor (TCR) on the surface of the T cell with a peptide–MHC complex on the surface of the APC. The second signal involves the interaction of a T-cell costimulatory molecule on the surface of the APC with its ligand on the surface of the T cell. To date, the most studied of the T-cell costimulatory molecules is B7-1, which interacts with CD28 on the T-cell surface to mediate upregulation of T-cell function. Preclinical studies have shown that the addition of B7-1 to a weakly immunogenic tumor will make it more immunogenic (Hodge *et al.*, 1994). This phenomenon has also occurred when other costimulatory molecules, such as ICAM-1 and LFA-3, have been added to tumors. The direct injection of a vector containing one or more costimulatory molecules into a tumor mass may facilitate an antitumor immune response. Kudo-Saito *et al.* (2004) showed that in an advanced subcutaneous tumor, the antitumor activity induced by intratumoral (i.t.) vaccination with recombinant fowlpox virus expressing a triad of costimulatory molecules, B7-1, ICAM-1, and LFA-3 (designated TRICOM), was superior to either subcutaneous (s.c.) or i.t. vaccination alone. The advantage of this direct-injection approach is that the "vaccine" is now the patient's own tumor, which may express a unique TSA or TAA profile.

Cytokines can also be introduced into the tumor mass using vectors as delivery vehicles, such as a recombinant vaccinia virus (Yang *et al.*, 2003) or a recombinant fowlpox virus (Kudo-Saito *et al.*, 2004), that expressed GM-CSF that was directly injected into mouse tumor lesions to mediate antitumor activity. Kudo-Saito *et al.* (2004) also noted a requirement for a recombinant fowlpox virus expressing GM-CSF in the intratumoral environment. In other studies, Triozzi *et al.* (2005) demonstrated in a mouse model of mesothelioma that complete tumor regressions were observed in mice receiving intratumoral injection with a recombinant fowlpox virus expressing the triad of costimulatory molecules, B7-1, ICAM-1, and LFA-3 (TRICOM), and a recombinant fowlpox virus expressing GM-CSF.

Secondary lymphoid chemokine (SLC) attracts mature DCs and naïve T cells. Colocalization of these cells within local tumor environments may enhance the induction of tumor-specific T cells. Flanagan *et al.* (2004) demonstrated that the local injection of a recombinant vaccinia virus expressing SLC into established CT26 murine colon tumors led to the infiltration of $CD4^+$ T cells, which correlated with inhibition of tumor growth.

C. Protein- and Peptide-Based Vaccines

1. PROTEINS

Protein-based vaccines, which consist of a single protein or combinations of proteins (Bystryn, 1998; Johnston and Bystryn, 2005), have been used against cancer in both preclinical and clinical studies. Proteins are usually given in combination with adjuvant molecules, such us liposomes (Harris *et al.*, 1999; Johnston *et al.*, 2005), or GM-CSF (Samanci *et al.*, 1998). A different type of protein-based approach for cancer immunotherapy is the use of heat-shock proteins (HSPs) purified from tumor cells, which have demonstrated to induce tumor-specific immunity when injected into animals. The immunogenicity of HSPs is not dependent in the protein itself but on tumor-derived peptides that are associated to HSPs along the purification process (Liu *et al.*, 2002).

2. PEPTIDES

The use of peptides as immunogens has many advantages: (1) it minimizes the potential for induction of autoimmunity when compared to the use of a whole protein, which might share fragments with normal cellular proteins; (2) the preparation of peptides is relatively easy and cost-affordable; (3) peptides can be modified to increase their immunogenicity by generating peptide agonists; and (4) it is possible to generate a tetramer specific for a given peptide in order to isolate peptide-specific T cells that have been induced and amplified in the host. Immune responses can be induced via two types of peptides after they interact with the appropriate MHC molecule on the surface of an APC. Short peptides of 8–10 amino acids are capable of binding to MHC class I molecules, while peptides of 11–15 amino acids will bind to MHC class II molecules (provided that, in both cases, they have the appropriate binding residues). The peptide–MHC class I and class II complexes will then interact with the TCR on $CD8^+$ and $CD4^+$ T cells, respectively. This interaction, in concert with costimulation, will induce T-cell activation. Therefore, the ability of a peptide to elicit an immune response depends on both its ability to bind the MHC molecule and the subsequent binding of the peptide–MHC complex to the TCR. $CD8^+$ T cells, designated as CTLs, are responsible for destruction of tumors; activated $CD4^+$ or "helper" T cells, on the other hand, will promote production of cytokines that, in turn, will help in the activation of $CD8^+$ T cells (Bjorkman, 1997).

Several preclinical studies in animal models have utilized single epitope peptides in combination with various types of vaccine adjuvants for the therapy of tumors (Mandelboim *et al.*, 1995; Noguchi *et al.*, 1994). Vaccines containing only epitopes reactive with MHC class I molecules,

however, might induce an adequate but short-lived CTL response because of the lack of "help" typically provided by MHC class II reactive peptides (Valmori *et al.*, 2003). Identification of T-helper epitopes for various TAAs has been achieved, such as HER2/neu (Kobayashi *et al.*, 2000), NY-ESO-1 (Zeng *et al.*, 2000), gp100 (Kobayashi *et al.*, 2001), mutated *ras* p21 (Abrams *et al.*, 1995), and CEA (Kobayashi *et al.*, 2002), among others.

It has been shown that both $CD8^+$ and $CD4^+$ T cells are usually activated for a vigorous antitumor effect (Pardoll and Topalian, 1998; Surman *et al.*, 2000). In order to stimulate CTLs as well as helper T cells, a vaccine should contain both classes of peptides. Oligopeptides that contain both MHC class I and class II reactive epitopes on the same molecule have been constructed (Abrams *et al.*, 1996b; Finn *et al.*, 1995; Roth *et al.*, 2005; Widmann *et al.*, 1992).

3. AGONIST PEPTIDES

The immunogenicity of a peptide can be increased to generate an agonist peptide by modification of specific amino acid residues involved in (1) MHC binding or (2) TCR interaction. The first group is characterized by enhanced binding to the MHC molecule and higher stability of the MHC–peptide complex on the surface of the APC. Examples of this category of agonist peptides are those for gp100, PSA, and MUC-1 protein (Parkhurst *et al.*, 1996; Terasawa *et al.*, 2002; Tsang *et al.*, 2004), which induced stronger activation of T cells *in vitro* than that achieved with unmodified peptides. In the category of agonists with modifications in amino acid residues that bind to the TCR, named as TCR agonists, an agonist for CEA has been identified which is able to induce stronger CTL responses against tumor targets (Salazar *et al.*, 2000; Zaremba *et al.*, 1997).

D. Anti-Idiotype Antibodies and Fusion Proteins

1. ANTI-IDIOTYPE ANTIBODIES

B-cell lymphoma cells express a surface immunoglobulin that contains unique antigenic determinants or idiotypes. These idiotypic determinants can serve as clonal tumor-specific markers that may be targeted with anti-idiotype mAb vaccines (Bendandi *et al.*, 1999; Hsu *et al.*, 1996; Kwak *et al.*, 1992, 1999; Liso *et al.*, 2000; Reichardt *et al.*, 1999). The concept of exploiting the idiotypic network has also been transferred to the treatment of carcinomas; studies in animal tumor models have demonstrated the efficacy of anti-idiotype mAb vaccines in preventing tumor growth and curing mice with established tumors (Bhattacharya-Chatterjee *et al.*, 2000;

Nelson *et al.*, 1987; Sugai *et al.*, 1974). In particular, an anti-CEA anti-idiotype mAb has been shown to induce generation of anti-CEA Ab responses and protective CEA-specific antitumor immunity in an animal tumor model (Bhattacharya-Chatterjee *et al.*, 1990; Pervin *et al.*, 1997).

2. FUSION PROTEINS

Immunocytokines are tumor-specific Ab genetically linked to a cytokine, such as IL-2, that have been extensively tested in experimental models of cancer. Administration of Ab-IL-2 fusion proteins has resulted in long-term resolution of established tumors and protective T-cell antitumor memory, and has proven to be superior over the effect of the combination Ab plus cytokine (Gillies *et al.*, 1998; Imboden *et al.*, 2001; Lode *et al.*, 1999; Neal *et al.*, 2004; Xiang *et al.*, 1998).

E. Vectors for Cancer Vaccines

Vectors are one of the more flexible means of vaccine delivery and several have been examined (Table I). Review articles have been written on the potential merits of these vectors (Carroll and Moss, 1997; Kaufmann and Hess, 1999; Moss, 1996; Paoletti, 1996; Rolph and Ramshaw, 1997; Weiskirch and Paterson, 1997). In general, the advantages of a vector-based vaccine are that (1) the entire tumor antigen gene or parts of that gene can be inserted and (2) multiple genes (including genes for costimulatory molecules and cytokines) can be inserted into some types of vectors. Adenovirus has also been proposed as a vector for application in recombinant vaccine design because its viral genome can be altered to accept foreign genes that are stably integrated, and the construction of the recombinant adenovirus results in an attenuated form of the virus with potentially improved safety. In preclinical studies, immunization of mice with a recombinant adenovirus expressing a model TAA led to the induction of an antigen-specific CTL response and regression of established pulmonary metastases.

One of the most studied groups of all vaccine vectors is the poxvirus group. Vaccinia virus, which was derived from a benign pox disease in cows, has been administered to more than 1 billion people and is responsible for the worldwide eradication of smallpox (Fenner *et al.*, 1988). This, however, means that most cancer patients have some level of a preexisting immunity to vaccinia virus and, thus, likely cannot be given multiple doses in vaccine protocols. Preclinical studies (Abrams *et al.*, 1997; Hodge *et al.*, 1997; Irvine *et al.*, 1997) showed that optimal use of recombinant vaccinia viruses may be to prime the immune response, followed by booster vaccinations with other vectors (such as replication-defective avipox vectors).

A major advantage to using vaccinia virus or the replication-incompetent poxviruses is that large amounts of foreign DNA can be inserted into the vector. Other advantages of poxviruses, such as vaccinia virus, MVA, and avipox viruses (see below), include wide host range, stable recombinants, accurate replication, and efficient posttranslational processing of the inserted gene. The poxvirus family contains the replication-competent vaccinia virus and a derivative of vaccinia virus termed MVA (Moss, 1996). A major attribute of this vector is its potential lack of toxicity because it can infect cells but cannot replicate. Other replication-defective members of the poxvirus family are the avipox vectors (fowlpox and canarypox/ALVAC) (Paoletti, 1996). These avipox vectors infect mammalian cells and express their transgenes for 2–3 weeks followed by cell death; they are incapable of reinfecting cells.

F. Bacterial and Yeast Vectors

Several live, attenuated pathogenic bacteria, such as *Salmonella* (Curtiss *et al.*, 1994; Schodel and Curtiss, 1995) and *Listeria* (Kaufmann and Hess, 1999), are being explored as vectors for vaccine delivery. They have the advantage of (1) an oral route of administration (Darji *et al.*, 1997; Paglia *et al.*, 1998); (2) a positive tropism for professional APCs such as macrophages; and (3) the bacteria itself is a natural adjuvant for the vaccine since bacterial infection is known to induce release of several proimmflamatory cytokines that will enhance the immune response (Wilson *et al.*, 1998). *Listeria monocytogenes*-based vectors are known to be able to activate both MHC class I–restricted $CD8^+$ T-cell responses and MHC class II–restricted $CD4^+$ T-cell responses (Kaufmann and Hess, 1999).

Preclinical studies have shown regression of established *ras* mutation-bearing tumors in mice immunized with whole recombinant yeast expressing mammalian mutant Ras proteins (Lu *et al.*, 2004).

G. Plasmid DNA

Plasmid DNA or "genetic" vaccines represent a potentially powerful and relatively easy to prepare vaccine-delivery system (Pardoll and Beckerleg, 1995). Initial preclinical studies have demonstrated the ability of intramuscularly delivered DNA vaccines encoding for viral antigens to elicit a $CD8^+$ T-cell response (Ulmer *et al.*, 1993). These studies formed the basis for the potential use of DNA vaccines in tumor immunotherapy. Preclinical studies in mouse tumor models have shown the ability of plasmid DNA vaccines to induce effector CTLs able to mediate tumor rejection (Ross *et al.*, 1997).

However, the mechanism for induction of an immune response following plasmid DNA immunization is still not entirely understood. The encoded antigen appears to be presented in the context of both MHC class I and class II molecules, and it is probable that DNA, for example, transfects myocytes after intramuscular delivery and resident APCs as well. To date, however, few clinical trials using polynucleotide vaccines in cancer have been reported (Conry *et al.*, 1994, 1996; Restifo and Rosenberg, 1999).

IV. BIOLOGICAL ADJUVANTS

The term biological adjuvant refers to a group of agents of varied nature that improves the immunogenicity of an antigen. A list of adjuvants is presented in Table II. Their mechanisms of action are different among the various types. For example, classic adjuvants, such as incomplete Freund's adjuvant (IFA), will allow the antigen to be maintained at the injection site so that infiltrating APCs and effector cells can initiate a stronger immune response. This type of action is relevant for peptides or protein-based vaccines that tend to rapidly diffuse from the site of injection.

Bacterial products, such as those present in IFA and BCG, have strong immunostimulatory activity since they contain agonists of Toll-like receptors (TLRs) and therefore can activate the host immune response (Krieg, 2002; Medzhitov, 2001). TLRs are a family of receptors that are expressed by DCs and other innate immune cell types and bind to a number of different microbial components such as lipopolysaccharide (LPS), RNA species, and CpG DNA motifs (Iwasaki and Medzhitov, 2004; Okamoto and Sato, 2003). The most studied of the TLR agonists are CpG motifs (Jahrsdorfer and Weiner, 2003; Krieg, 2000; Prud'homme, 2005; Wooldridge and Weiner, 2003), which admixed with peptide immunogens have shown to enhance

Table II Biological Adjuvants

Type	Example(s)
Classic adjuvants	Incomplete Freund's adjuvant, BCG
"Danger signal"	TLR-agonists—CpGs
	Viral or bacterial vectors
Cytokines	GM-CSF (local)
	IL-2 (systemic)
Chemokines	MIP-1α
Fusion proteins	Antitumor mAb-IL-2
T-cell costimulatory molecules	B7-1, ICAM-1, LFA-3
	Anti-CTLA-4 mAb
Cyclophosphamide	

immune responses in preclinical studies. Clinical studies have also begun to explore the use of CpG motifs in cancer immunotherapy (Wooldridge and Weiner, 2003).

Certain cytokines and chemokines are able to enhance immune responses by promoting the differentiation, activation, or recruitment of APCs, therefore, enhancing antigen presentation and activation of antigen-specific T cells. Within this group, GM-CSF has been reported to enhance antigen-specific T-cell responses (Borrello and Pardoll, 2002; Disis *et al.*, 1996; Dranoff, 2002; Hurwitz *et al.*, 1998a; Kass *et al.*, 2001; Schneeberger *et al.*, 2003; Wolpoe *et al.*, 2003; Yang *et al.*, 2003) by modulating the recruitment and activation of APC populations such as macrophages and DCs (Emens and Jaffee, 2003; Pardoll, 1998). It has been shown that subcutaneous injections of GM-CSF at the vaccination site can significantly increase the infiltration of DCs in regional nodes that drain the injection site (Kass *et al.*, 2001). In murine models of cancer, whole-tumor cell vaccines engineered to secrete GM-CSF in a paracrine fashion have shown to elicit systemic immune responses against the tumor (Thomas *et al.*, 1998).

Several other cytokines and chemokines have also been shown to play a critical role in enhancing T-cell function (Crittenden *et al.*, 2003; Flanagan *et al.*, 2004; Klebanoff *et al.*, 2004; Pertl *et al.*, 2001; Wigginton *et al.*, 2001). IL-2, a cytokine with a wide range of immunologic effects, is perhaps the most studied as a vaccine adjuvant (Dudley and Rosenberg, 2003; Rosenberg, 2001). Cytokines, such as IL-7, IL-12, and IL-15, have also been shown to enhance T-cell responses in experimental models and may also have clinical benefit (Dranoff, 2004; Fry *et al.*, 2003; Waldmann *et al.*, 2001; Wigginton *et al.*, 2001).

V. MULTIMODAL THERAPIES

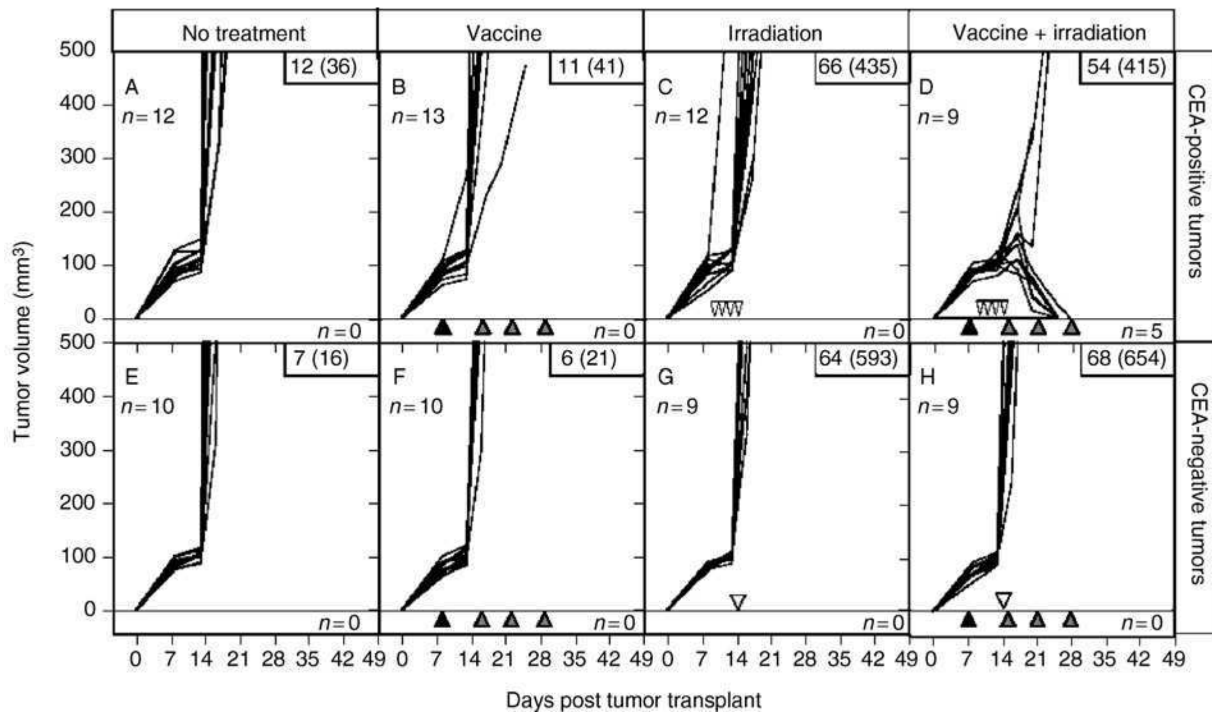
Antitumor effects induced by cancer vaccines may be difficult to obtain with large tumor masses for a variety of reasons, including (1) large tumor masses are difficult to penetrate by T cells; (2) the quantity of T cells generated by the host immune system would be far outnumbered by the cells in a large tumor mass; and (3) tumor cells produce immunoregulatory molecules that have the ability to suppress or anergize T-cell function. Cancer vaccines utilized as a monotherapy would most likely have greatest efficacy in the adjuvant or neoadjuvant setting, where tumor burden is minimal. In situations of advanced or metastatic cancer, however, considerable interest has been placed into the use of vaccines with conventional therapies such as local radiation of tumor, chemotherapeutic agents, hormones, and monoclonal antibodies.

A. Vaccine Therapy in Combination with Radiation Therapy

Radiation is the standard of care for many cancer types and has conventionally been exploited for its direct cytotoxic effect on tumors or palliative effects in patients. However, it has been reported that local radiation of tumor cells given at doses insufficient to kill tumor will modulate numerous classes of genes and consequently alter the phenotype of the tumor cell (Chakraborty *et al.*, 2004; Garnett *et al.*, 2004). Several genes that have been shown to be upregulated in both murine and human tumors postirradiation are Fas, MHC Class I, ICAM-1, and the tumor-associated antigens CEA, MUC-1, HER-2/*neu*, p53, and CA125. The upregulation of any one of these genes has the ability to render a tumor cell more susceptible to T-cell–mediated immune attack. Preclinical studies by Chakraborty *et al.* (2004) have shown that the regulation of Fas expression in tumor cells via sublethal local tumor irradiation significantly improved the therapeutic efficacy of a recombinant anticancer vaccine regimen in a preclinical model. In that study, tumor bearing CEA-transgenic (Tg) mice were vaccinated s.c. with recombinant vaccinia and recombinant fowlpox CEA/TRICOM vectors and GM-CSF in combination with local tumor irradiation. Mice were vaccinated on days 8, 15, 22, and 29; posttumor transplant and tumors received 2 Gy/day of radiation for 4 days (days 11–14). Subcutaneous vaccination alone and irradiation alone did not significantly inhibit tumor growth (Fig. 1). However, therapy of tumors with the combination of vaccine regimen and irradiation resulted in a marked and significant decrease in tumor growth rate and volume (Fig. 1D); 55% of the mice treated with the combination regimen resolved their tumor mass and remained tumor free for the duration of the experiment. These studies thus demonstrated a new paradigm for the use of local tumor irradiation in combination with active specific vaccine therapy to elicit durable antitumor responses of established tumors.

B. Vaccine Therapy in Combination with "Standard-of-Care" Chemotherapy

If cancer vaccines are to be used early in the disease process, they would need to be used in combination with certain chemotherapeutic agents used as standard-of-care. It has been shown that drugs commonly used in cancer chemotherapy can also upregulate tumor antigens and/or histocompatibility antigens. For example, 5-fluorouracil treatment of tumor cells has been shown to upregulate CEA, MHC Class I, ICAM-1, and Fas (Bergmann-Leitner and Abrams, 2000). Adriamycin has also been shown to increase effector T-cell


No treatment
Vaccine
Irradiation
Vaccine + irradiation
CEA-positive tumors
CEA-negative tumors
Tumor volume (mm³)
Days post tumor transplant
A
n = 12
12 (36)
B
n = 13
11 (41)
C
n = 12
66 (435)
D
n = 9
54 (415)
n = 0
n = 0
n = 0
n = 5
E
n = 10
7 (16)
F
n = 10
6 (21)
G
n = 9
64 (593)
H
n = 9
68 (654)
n = 0
n = 0
n = 0
n = 0
0 100 200 300 400 500
0 7 14 21 28 35 42 49

activity. In preclinical models, Michiels *et al.* (2001) have shown that cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of a whole-tumor cell vaccine. A comprehensive review on combining antineoplastic drugs with tumor vaccines has been published (Terando and Mule, 2003).

C. Vaccine Regimens and Monoclonal Antibody Therapy

There are several ways in which mAbs are being used in combinatorial regimens to enhance vaccine-induced antitumor responses. One of the examples is the use of anti-cytotoxic T lymphocyte antigen (CTLA) to block negative regulatory signals. In several preclinical animal models, the use of anti-CTLA-4 has been shown to enhance antitumor activity by whole-tumor or peptide-based vaccines (Davila *et al.*, 2003; Hurwitz *et al.*, 1998b). The use of antibodies directed against regulatory $CD4^{+}CD25^{+}$ T cells has also shown to augment the generation of specific immune responses in preclinical tumor models (Jones *et al.*, 2002; Koido *et al.*, 2002). The combination of antibodies directed against $CD4^{+}CD25^{+}$ T cells with live viral or DC-based vaccines is another strategy that is being explored for augmenting the efficacy of immune interventions against cancer (Kudo-Saito *et al.*, 2005; Prasad *et al.*, 2005; Shimizu *et al.*, 1999).

Fig. 1 Irradiation of tumor cells *in vivo* enhances efficacy of vaccine therapy. CEA-Tg mice were transplanted subcutaneously with murine colon carcinoma cells expressing CEA; MC38-CEA^{+} tumor cells. (A) Mice received no treatment. (B) Mice were vaccinated with rV-CEA/TRICOM on day 8 (closed triangle), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). (C) Tumors in mice were subjected to external-beam irradiation (2 Gy) *in situ* on days 11, 12, 13, and 14 (open inverted triangles). (D) Mice were vaccinated with rV-CEA/TRICOM on day 8 (closed triangle). Tumors were subjected to external-beam irradiation (2 Gy) *in situ* on days 11, 12, 13, and 14 (open inverted triangles), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). CEA-Tg mice were injected with 3×10^5 MC38 tumor cells (CEA-negative) s.c. (E) Mice received no treatment. (F) Mice were vaccinated with rV-CEA/TRICOM on day 8 (closed triangle), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). (G) Tumors in mice were subjected to external-beam irradiation (8 Gy) *in situ* on day 14 (open inverted triangle). (H) Mice were vaccinated with rV-CEA/TRICOM on day 8 (closed triangle). Tumors were subjected to external-beam irradiation (8 Gy) *in situ* on day 14 (open inverted triangle), followed by boosting with rF-CEA/TRICOM on days 15, 22, and 29 (gray triangles). In a subset of mice from each treatment group, tumors were surgically removed at day 21 posttumor transplant and costained with CEA and Fas antibodies. Inset figure parts: % Fas positive cells (mean fluorescent intensity). All vaccines were coadministered with rF-GM-CSF. Tumor volume was monitored. Adapted from Chakraborty *et al.* (2004).

VI. NEXT-GENERATION VACCINE DESIGN AND DELIVERY

A. Manipulating the Mechanism of T-Cell Activation

Optimization of the mechanism of T-cell activation is crucial to achieve a successful immune response to an antigen. As described previously, activation of T cells has been shown to require at least two signals. For weak antigens, such as TAAs, the requirement for accessory molecules is greater for optimal T-cell activation. The most studied of the costimulatory molecules are B7-1, B7-2, ICAM-1, LFA-3, and CD40, each with its own ligand on the T cells (Allison *et al.*, 1995; Schultze *et al.*, 1996; Schwartz, 1996; Swain *et al.*, 1996; Wingren *et al.*, 1995).

While costimulatory molecules are found on professional APCs, such as DCs, activated B cells, and activated macrophages, the vast majority of solid tumors do not express costimulatory molecules on the cell surface. This may constitute one of the reasons for why the immune system may fail to recognize tumors. An approach to enhance the activation of T cells in response to a tumor has been the introduction of costimulatory molecules into the tumor. Experimental models have used retroviral, adenovirus, and poxvirus vectors to deliver expression of various costimulatory molecules into nonimmunogenic tumor cells to make them more immunogenic (Abrams *et al.*, 1996a; Baskar *et al.*, 1994; Gilboa, 1996; Gilligan *et al.*, 1998; Hodge and Schlom, 1999; Hodge *et al.*, 1994; Hurwitz *et al.*, 1998a; Melero *et al.*, 1997; Townsend and Allison, 1993).

Preclinical studies have also shown that mice immunized with an admixture of a poxvirus encoding for a TAA (CEA) and the costimulatory molecule B7-1 were able to generate CEA-specific T-cell responses that delivered antitumor immunity to the immunized mice (Akagi *et al.*, 1997; Hodge *et al.*, 1995). Furthermore, it was demonstrated that combinations of various costimulatory molecules act synergistically to further enhance T-cell activation. Using recombinant vaccinia or avipox vectors, each containing three different costimulatory molecules (B7-1, ICAM-1, and LFA-3), T-cell responses and antitumor immunity were further enhanced over the levels acquired when only one costimulatory molecule was used (Hodge *et al.*, 1999).

Studies have shown that the functional avidity of antigen-specific CTLs constitutes a major determinant in the outcome of T-cell–mediated immunotherapy of tumors (Hodge *et al.*, 2005; Oh *et al.*, 2003; Rubio *et al.*, 2003). Since high-avidity CTLs are functionally defined as being responsive to very low levels of antigen, they are able to recognize low amounts of peptide presented in the context of the MHC molecules on the surface of a

tumor cell and, therefore, able to kill tumor targets. It has been shown that the use of vaccines with T-cell costimulation and certain cytokines can enhance T-cell avidity (Hodge *et al.*, 2005). Employing CEA as an immunogen in CEA-Tg mice, Hodge *et al.* (2005) have evaluated the effect of costimulation and the use of cytokines on the avidity of CEA-specific T cells. Mice were vaccinated with a viral vector expressing (1) CEA alone; (2) CEA and B7-1; and (3) CEA and three costimulatory molecules (B7-1, ICAM-1, and LFA-3). Additionally, mice also received anti-CTLA-4 mAb and/or GM-CSF. $CD8^+$ T-cell quantity was measured by tetramer binding precursor frequency; avidity was measured by quantitative cytolytic function (Table III). The experiments demonstrated that the combined use of multiple costimulatory molecules, the cytokine GM-CSF, and the administration of anti-CTLA-4 mAb, each contributed to the avidity enhancement of the antigen-specific T cells. In another study, immature DCs and CD40L-matured DCs generated from PBMCs of healthy individuals were modified via infection with a recombinant fowlpox vector encoding for the human B7-1, ICAM-1, and LFA-3 molecules (TRICOM). *In vitro* stimulation of

Table III Effect of Multiple Costimulatory Modalities to Enhance CTL Avidity

Vaccine modality	T-cell quantity (Precursor frequency/10^5 CD8 T cells)		T-cell quality (Peptide concentration for CTL; nM)	
Buffer (HBSS)	133		NA	
rV-CEA	321	1×	510	1×
rV-CEA/B7-1	584	1.8×	110	4.6×
rV-CEA/TRICOM	769	2.4×	5	102×
rV-CEA	455	1×	950	1×
rV-CEA + CTLA-4 mAb	784	1.7×	237	4×
rV-CEA/B7-1	674	1.5×	135	7×
rV-CEA/TRICOM + CTLA-4 mAb	1303	4×	0.4	1275×
rV-CEA/TRICOM + GM-CSF	1289	4×	0.6	850×
rV-CEA/TRICOM + GM-CSF + CTLA-4 mAb	1690	5.3×	0.02	25,500×

The quantity of CEA-specific CTL in CEA-Tg mice after vaccination was measured by tetramer binding directly from fresh mouse splenocytes and expressed as T-cell precursor frequency. The avidity of the CEA-specific CTL was determined by peptide dilution CTL assay after a short *in vitro* restimulation. These data were expressed as percentage of maximum lysis versus peptide concentration, and the natural logarithm of the normalized data was plotted against peptide concentration. The avidity of each T-cell population was defined as the negative log of the peptide concentration that resulted in 50% maximal target lysis and was expressed in nanomolars. Adapted from Hodge *et al.*, 2005.

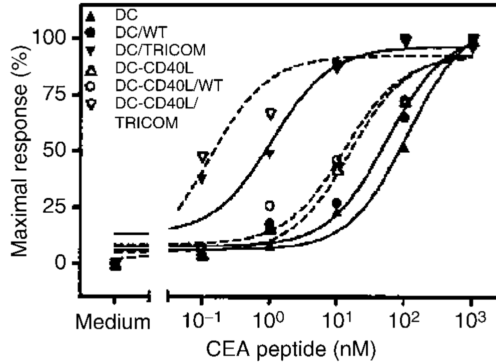


Fig. 2 Effect of TRICOM on avidity of CTLs generated by peptide-pulsed immature or CD40L-matured DCs. Immature DCs (closed symbols) and CD40L-matured DCs (open symbols) were used for *in vitro* generation of human CEA-specific T cells. Both types of DCs were left uninfected or were infected with a control wild-type fowlpox vector or the recombinant rF-TRICOM vector. After three rounds of stimulation in the presence of CEA peptide-pulsed DCs, CTLs from each group were tested for their ability to produce IFN-gamma in response to T2 cells pulsed with various concentrations of CEA peptide. Results were normalized as percentage of maximal response, as described. Adapted from Yang *et al.* (2005).

autologous T cells used DCs as APCs that were CEA peptide-pulsed and TRICOM-modified. This resulted in the generation of higher numbers of CEA-specific T cells and, more importantly, higher-avidity CTLs as compared with T cells stimulated in the presence of unmodified, peptide-pulsed DCs (Fig. 2) (Yang *et al.*, 2005).

B. Regulatory T Cells

Since many of the targets for cancer immunotherapy are self-antigens, a successful intervention would require breaking tolerance mechanisms present in the host against those antigens. One of the tolerance mechanisms for self-antigens is the existence of immunoregulatory T cells, which normally function by suppressing autoreactive T cells. Therefore, it is expected that depletion of these regulatory T cells would result in induction of antitumor immune responses. There are several types of regulatory T cells: (1) $CD4^{+}CD25^{+}$ T cells; (2) myeloid cells; and (3) $CD4^{+}NKT^{+}$ cells (Kusmartsev and Gabrilovich, 2002; Serafini *et al.*, 2004).

In preclinical studies, reagents that can potentially reduce the number or the function of these regulatory T cells are being investigated, which include cyclophosphamide (Karanikas *et al.*, 2001; Lutsiak *et al.*, 2005; MacLean *et al.*, 1996), the fusion protein Ontak (Foss, 2001), and anti-CD25 antibodies (Kudo-Saito *et al.*, 2005; Shimizu *et al.*, 1999).

C. Role of Antigen Cascade

Antigen cascade refers to the situation in which vaccination with a given antigen results in induction of an immune response not only directed against the antigen in the vaccine but also against other antigens in the tumor (Chakraborty *et al.*, 2004; Kudo-Saito *et al.*, 2005; Ribas *et al.*, 2003). Antigen cascade could be the consequence of some degree of tumor cell destruction induced by the vaccine; APCs will engulf tumor fragments and then "cross-present" those tumor fragments to the T cells, thus initiating the antigen cascade phenomenon. This has now been observed in several preclinical models and in clinical trials, and may eventually be correlated with antitumor activity.

D. Diversified Prime and Boost Immunization Strategies and Route of Immunization

Most of the various methods of immunization described in Table I have been studied as single agents in preclinical models. However, the most effective immunization protocol may involve priming with one type of immunogen and boosting with another. This method may be advantageous because (1) one methodology may be more effective in priming naïve cells, while another modality may be more effective in enhancing memory cell function; (2) two different arms of the immune system may be enhanced by using two different modalities (i.e., $CD4^+$ and then $CD8^+$ T cells); and (3) as noted earlier, some of the most effective methods of immunization, like the use of recombinant vaccinia virus or adenoviruses, can be used only a limited number of times because of host antivector responses. These vectors may be most effective when used as priming agents, followed by boosting with other agents. Numerous preclinical studies demonstrate the advantages of diversified prime and boost protocols (Abrams *et al.*, 1997; Bei *et al.*, 1994; Chappell *et al.*, 1999; Irvine *et al.*, 1997; Murata *et al.*, 1996).

Conventional vaccinations involve subcutaneous or intradermal inoculations. It has been shown in several preclinical models and some clinical studies that intratumoral and/or intranodal vaccination may be more efficacious in some cases. In a study it was shown that the sequential use of primary vaccination s.c. followed by booster vaccination intratumorally is more efficacious in terms of antitumor effects than the use of either route alone (Kudo-Saito *et al.*, 2004).

VII. CHALLENGES IN CANCER VACCINE DESIGN

Information obtained from preclinical studies conducted in animal tumor models have contributed enormously to the development of cancer vaccines for treatment of human cancers. A major issue, however, is related to the relevance of the utilized models. To date, most preclinical work has been conducted with transplanted murine tumors that grow rapidly and are usually noninvasive and fail to metastasize. Most human tumors do not grow as quickly and do not represent the percent of body mass that murine tumors do. The short time span of mouse models does not permit multiple booster vaccinations and, as a result, very few cycles of vaccine immunotherapy can be given. This is in contrast to the vaccine therapy in a patient with minimal residual disease, whose tumor burden represents a relatively small fraction of body mass and who can receive many cycles of immunotherapy over the course of several years. Tg mouse models that form spontaneous cancers were developed, such us those for HER-2/*neu* (Esserman *et al.*, 1999), CEA (Kass *et al.*, 1999), MUC-1 (Soares *et al.*, 2001), and TRAMP (in prostate cancer) (Hurwitz *et al.*, 2000). Tg mice with spontaneously arising tumors offer a greater opportunity over transplantable tumors to evaluate novel and diversified immunotherapies.

A major challenge in the development of cancer vaccines is related to the fact that many defined tumor antigens are self-proteins and therefore generally fail to initiate strong antitumor T-cell responses. Thus, a key for developing successful cancer vaccines is to overcome potential mechanisms of immune suppression against antigenic but weakly immunogenic tumors (Pardoll, 2003; Van Parijs and Abbas, 1998). Tg mice expressing self-TAA (Greiner *et al.*, 2002; Hodge *et al.*, 2003; Hurwitz *et al.*, 2000; Sakai *et al.*, 2004; Wolpoe *et al.*, 2003) have shown that, most likely, tumor-specific T-cell precursors exist in the periphery but in a tolerant state. Cancer vaccines are constructed to enhance the immunogenicity of the tumor and to promote proinflammatory reactions necessary to sustain T-cell responses against the tumor (Emens and Jaffee, 2003; Finn, 2003; Hodge *et al.*, 2000,

2003; Merad *et al.*, 2002; Nair *et al.*, 2002; Pardoll, 2003; Tsang *et al.*, 2001).

In the case that a therapeutic T-cell response is initiated, the development of undesirable immune reactions could occur if the specific antigen is not only expressed in the tumor but also in normal tissues. Little if any evidence of autoimmunity has been observed in clinical trials. Immune responses to some melanoma-associated antigens were able to induce vitiligo in experimental and clinical studies (Dudley *et al.*, 2002; Overwijk *et al.*, 2003).

However, in preclinical studies using Tg mice expressing TAAs, induction of immune responses against the TAA has not led to the induction of autoimmunity in normal tissues expressing those same TAAs (Chakraborty *et al.*, 2003; Esserman *et al.*, 1999; Gendler and Mukherjee, 2001; Gong *et al.*, 1998; Greiner *et al.*, 2002; Shibata *et al.*, 1998).

VIII. CONCLUSIONS

Progress in the understanding of the molecular mechanisms that govern immune activation as well as the advent of recombinant DNA technologies have led to great progress in the development of immune therapies against cancer. Identification of multiple TSAs and TAAs, as well as a better understanding of the mechanisms of T-cell activation, tumor escape, and tumor-induced immune suppression have helped in the design of novel and more efficient vaccine strategies. Moreover, combination therapies, involving vaccination and other treatment modalities, such as radiation, chemotherapy, and cytokines, among others, may prove beneficial to facilitate long-term survival with a lower risk of generating aggressive tumor escape variants.

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Clinical Results of Vaccine Therapy for Cancer: Learning from History for Improving the Future

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Active, specific immunotherapy for cancer holds the potential of providing an approach for treating cancers, which have not been controlled by conventional therapy, with very little or no associated toxicity. Despite advances in the understanding of the immunological basis of cancer vaccine therapy as well as technological progress, clinical effectiveness of this therapy has often been frustratingly unpredictable. Hundreds of preclinical and clinical studies have been performed addressing issues related to the generation of a therapeutic immune response against tumors and exploring a diverse array of antigens, immunological adjuvants, and delivery systems for vaccinating patients against cancer. In this chapter, we have summarized a number of clinical trials performed in various cancers with focus on the clinical outcome of vaccination therapy. We have also attempted to draw objective inferences from the published data that may influence the clinical effectiveness of vaccination approaches against cancer. Collectively the data indicate that vaccine therapy is safe, and no significant autoimmune reactions are observed even on long term follow-up. The design of clinical trials have not yet been optimized, but meaningful clinical effects have been seen in

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B-cell malignancies, lung, prostate, colorectal cancer, and melanoma. It is also obvious that patients with limited disease or in the adjuvant settings have benefited most from this targeted therapy approach. It is imperative that future studies focus on exploring the relationship between immune and clinical responses to establish whether immune monitoring could be a reliable surrogate marker for evaluating the clinical efficacy of cancer vaccines. © 2006 Elsevier Inc.

I. INTRODUCTION

There exists a paradox in the field of cancer immunotherapy. Monoclonal antibodies (mAbs) like trastuzumab, rituximab, and alemtuzumab, targeting various cancer-associated molecules, are increasingly becoming established treatment modalities for various cancers. In contrast, harnessing the cellular arm of the immune system for therapy of cancer through vaccination and adoptive transfer of tumoricidal lymphocytes remain largely within the realms of experimental therapy. There are no simple and clear explanations for this discrepancy. One of the reasons may be that generation of a cellular antitumor immunity of sufficient magnitude and duration to mediate a therapeutic effect is complex and involves multifactorial processes whose individual components have not yet been clearly defined. Yet, the generation of therapeutic cellular responses necessitates that each of these individual components be perfectly harmonized. In contrast, tumor cell death induced by mAbs is more straightforward. On binding of the antibody molecules to the relevant tumor antigen, death is induced by antibody dependent cellular cytotoxicity (ADCC), complement dependent lysis, or induction of apoptosis or growth arrest by inhibition of various signaling pathways associated with the tumor antigen. A commentary suggested that vaccination approaches against cancer have failed to be effective and it is time to move beyond to other approaches with greater promise of therapeutic efficacy (Rosenberg *et al.*, 2004). The commentary initiated intense debate (Mocellin *et al.*, 2004; Timmerman and Levy, 2004) that not only focused on the success, or lack thereof, of vaccine therapy for cancer but also on the need for evaluating success or failure on the basis of accepted criteria for clinical responses in oncology.

In this chapter, we have attempted to review vaccination trials in several malignancies that have been conducted over the years and that have reported clinical results. We have for the most part concentrated on clinical trials with patient numbers large enough to draw clinically relevant conclusions. In addition, we have attempted to summarize any general conclusions that can be drawn on the basis of the collective data.

II. VACCINE TRIALS IN B-CELL MALIGNANCIES

Few large clinical trials have been conducted in B-chronic lymphocytic leukemia (B-CLL) and B-cell lymphoma and are summarized in Table I.

Each B-cell expresses an immunoglobulin molecule that contains unique variable region sequences within the heavy and light chains. During tumorigenesis, these unique sequences are preserved by the malignant clone and the proteins they encode (idiotype, Id) represent tumor-specific antigens. The Id protein can be isolated from the malignant B-cells and utilized as a vaccine target.

A correlation between the development of an immune response and a better clinical outcome has been shown in vaccination studies of lymphoma patients with the Id protein (Hsu *et al.*, 1997). However, it is not yet known which component of the immune response (humoral or cellular) is associated with the better outcome. Weng *et al.* (2004) determined the predictive value of humoral anti-Id immune responses on the clinical outcome of a large patient population who received Id vaccination either in the form of Id-pulsed dendritic cells (DCs) or Id-protein administered with either a chemical adjuvant or granulocyte-macrophage colony-stimulating factor (GM-CSF) after gaining complete remission with chemotherapy. In this study, 48 patients (35%) developed specific humoral anti-Id responses and 27 patients (20%) developed specific cellular anti-Id responses. It was also shown that utilizing DCs or GM-CSF as adjuvant induced higher cellular response rates (RRs) than combining the vaccine with a chemical adjuvant ($p = 0.0002$). The patients who developed anti-Id antibodies (Abs) had longer progression-free survival (PFS) than those who did not. The estimated PFS at 5 years was 61% for patients with anti-Id Abs and 38% for patients without anti-Id Abs, with median time-to-progression (TTP) estimates of 8.21 and 3.38 years for the two groups, respectively ($p = 0.018$). In contrast, the development of a cellular immune response had no correlation with PFS; TTP in patients with and without cellular immune response was 2.47 and 4.92 years respectively. Because FcγR allotypes have been shown to affect the ability of macrophages and natural killer (NK) cells to mediate ADCC, the aim of this study was also to evaluate the predictive value of FcγR polymorphisms on the clinical outcome of the patients. It was shown that patients with certain FCγR genotypes have better clinical outcome after Id vaccination. For the FCγRIIIa 20 patients were homozygous for valine/valine (158V/V), 60 were heterozygous for valine/phenylalanine (158V/F), and 56 were homozygous for phenylalanine/phenylalanine (158F/F). The estimated PFS at 5 years was 77%, 38% and 48% respectively. The estimated PFS of the patients with 158 V/V was significantly longer than that of 158V/F or 158F/F carriers (= 0.009). In contrast to FCγRIIIa,

Table I Vaccine Trials in B-Cell Malignancies

Diagnosis	Trial stage	Number of pts	Study design	Vaccine	Adjuvant	Number of immunizations	Immune response	Clinical outcome	References
Indolent phase (Rai stage 0–IV) B-CLL	Phase I–II	18	3 vaccination schedules	Oxidized, autologous leukemic cells	None	12 or 4	7 cellular	5 PR; 6 SD	Spaner *et al.* (2005)
Stage III or IV follicular B-cell NHL	Pilot followed by phase I	35	10 pts with measurable disease (pilot); 25 pts in adjuvant setting	DC pulsed with ld-KLH	Syntex adjuvant formulation	4 DC-ld-KLH booster with ld-KLH + SAF	17/35 (48%) cellular; 9/35 (25%) anti-ld humoral (mainly IgM)	Of first 10 pts: 2 CR, 2 PR; of 25 adjuvant pts: 5 CR, 11 Cru[a], 7 PR	Timmerman *et al.* (2002)
Stage III–IV follicular lymphoma	Phase I	20	Adjuvant vaccine therapy after clinical remission post chemotherapy	FL-lg-KLH	GM-CSF	4	19/20 cellular; 15/20 anti-FL-lg humoral	18/20 pts maintain CR (28–53 mo, median 42 mo); 8/11 pts were t(14;18) PCR positive before vaccination and turned negative after vaccination	Bendandi *et al.* (1999)
Stage III–IV follicular lymphoma	Phase I–II Retrospective analysis	136	Adjuvant vaccine therapy after clinical remission post chemotherapy	ID-KLH	86 chemical[b]; 32 DC; 18 GM-CSF	Not shown	27/136 (20%) cellular; 48/136 (35%) humoral; 12/136 pts had both	PFS was greater in pts with humoral response (8.21 v 3.38 yrs; $p = .018$). PFS correlation to cellular response was not significant ($p = .312$)	Weng *et al.* (2004)

[a]Cru: complete remission unconfirmed.

[b]Chemical adjuvants used: syntex adjuvant formulation (SAF), incomplete SAF (ISAF), QS-21 (Antigenics, New York, NY), and SBAS-2 (GlaxoSmithKline Biologicals, Rixensart, Belgium). Cytokine: granulocyte-macrophage colony-stimulating factor.

CR, complete response; GM-CSF, granulocyte/macrophage-colony stimulating factor; KLH, keyhole limpet hemocyanin; PD, progressive disease; PR, partial response; pts, patients; SD, stable disease; mo, months; yrs, years.

the FCγRIIa 131 histidine/arginine (H/R) polymorphism had no correlation with PFS. These results indicate that FcγR-bearing cells mediate an antitumor effect by killing antibody-coated tumor cells and that ADCC is one of the possible mechanisms of action for anti-Id Abs.

In another clinical trial, Bendandi *et al.* (1999) have utilized idiotype protein vaccine after chemotherapy-induced first clinical complete remission. Minimal residual disease (MRD) was assessed by PCR blinded to clinical information about the samples. TNF (tumor necrosis factor)-α, IFN-γ, and GM-CSF release to autologous follicular lymphoma (FL) cells was detected in the peripheral blood mononuclear cells (PBMCs) of 19 out of 20 vaccinated patients. Vaccine-induced cell-mediated immune response was also shown in 6 out of 6 patients. In the same 6 patients autologous FL target cells were lysed by positively selected T-cells from PBMC (23–29% specific lysis at E:T ratio, 20). Immune PBMC restimulated by CD40L for a single period of 5 days lysed unmodified autologous tumor cells (27–44% E:T ratio, 100). Furthermore, a postvaccination-specific antibody response (mainly IgG1) against autologous Id was detected in 15 out of 20 patients. Analysis of molecular RR showed clear evidence for an antitumor effect of lymphoma-specific vaccination, indicating that after cytoreduction by chemotherapy, vaccination can induce a tumor-specific T-cell response capable of clearing residual tumor cells from the blood.

Timmerman *et al.* (2002) used Id-pulsed DCs for vaccinating 35 patients with B-cell lymphoma. The study was conducted in two steps. In the pilot phase of the study, 10 patients with measurable disease were treated with DC pulsed with tumor-derived Id protein and complete response (CR) was achieved in 20%, partial response (PR) in 10% and molecular response in 10% of them. Cellular immune response was found in 80% of the patients taken as a whole and in 75% (3 out of 4) of the patients who achieved a clinical response.

Subsequently, 25 additional patients with advanced stage FL were treated after an attempt at remission induction by chemotherapy. Of these, 18 still had measurable disease at the start of vaccinations, and in this subgroup a 22% RR was achieved. Putting the RRs from the pilot and the second study together, a 36% RR was achieved by this vaccination approach.

At a median of 43 months after chemotherapy withdrawal, 70% of the patients had still not experienced disease progression. Immune response evaluation was possible in 23/25 patients. Globally, 65% of these patients achieved an immune response (T-cell or humoral). The development of humoral anti-Id response could not be significantly correlated to the clinical outcome (86% of patients demonstrating humoral responses had long-lasting stabilization of disease (SD) versus 63% of patients without antibody response, $p = 0.4$) and, similarly, even considering T-cell and humoral responses together, no correlation was found with PFS (67% of patients

with immune response remained progression free compared to 75% of those without immune response).

Cumulatively, 6 patients (5 from the pilot study and 1 from the second study) progressed after primary DC vaccination and were treated with booster injections of Id-keyhole limpet hemocyanin (KLH) protein. Clinical response was obtained in 3 of them and correlated with the development of an immune response (2 patients had T-cell response and 1 had a humoral response). This can be regarded as an interesting finding, showing that subsequent boosting with Id-KLH can lead to tumor regression after progression during primary DC vaccine.

A phase III randomized clinical trial is recruiting patients in first complete remission to be treated with a patient-specific vaccination made of follicular lymphoma-derived idiotype (FNHLIdl). Another Phase III ongoing clinical trial has started in February 2000 to assess the clinical efficacy of BiovaxId, an autologous tumor-derived immunoglobulin idiotype vaccine, in patients with indolent follicular non-Hodgkin's lymphoma (NHL) in the first complete remission.

III. B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

Spaner *et al.* (2005) have published the results of a Phase I/II clinical trial of oxidized autologous tumor cell vaccine for CLL. They exposed 10 ml of whole blood of B-CLL patients with advanced stage disease to heat ($42.5 \pm 1^{\circ}$C), medical grade oxygen and UV-C light (at a wavelength of the 253.7 nm), and then injected the cells intramuscularly either 12 times over 6 weeks (group 1), or 12 times over 16 days (group 2), or 4 times over 6 weeks (group 3). Of the total 18 patients, 6 (33%) achieved a SD, 7 had progressive disease (PD) and 5 had PR. Patients who achieved PR had lower starting WBC count ($37.4 \pm 5.2 \times 10^9$/l; mean $\pm$ SE) than patients with SD ($61.8 \pm 11.3 \times 10^9$/l, $p < 0.05$), indicating that vaccination is more effective when the tumor burden is low.

The proliferative T-cell response against autologous tumor cells after vaccination was higher than that at baseline in patients who achieved PR compared to patients who had SD or PD ($p < 0.02$). No significant toxicity and adverse side effect was reported.

IV. MULTIPLE MYELOMA

Clinical trials in multiple myeloma (MM) are summarized in Table II. In MM vaccination trials have been performed so far mainly with the Id-protien as a vaccine. One of the first vaccination trials in MM using the Id

was published 1996 (Bergenbrant *et al*., 1996). The Id alone (precipitated in alum) was given to 5 MM patients with stage I–IIA disease. A transient, low level anti-Id T-cell response was observed in 2/5 patients. No clinical response was noted. In a following study, the Id was used together with GM-CSF in 5 MM patients with stage II disease. All of the patients developed Id-specific T-cell immunity and in 1 patient a partial remission (>50% reduction of M-component level) was observed (Osterborg *et al*., 1998). Coscia *et al*. (2004) vaccinated patients, following high-dose chemotherapy and stem cell support with the Id-protein while they were in MRD in the first remission. Fifteen patients received Id conjugated to KLH with the addition of GM-CSF as an adjuvant. Eighty-five percent of the patients developed Id-specific positive skin test, but no clinical effects were seen.

In an attempt to augment the induction of an Id-specific T-cell response, Id-pulsed DCs have been used. The first clinical study on DCs was published in 1998. An MM patient was vaccinated with autologous Id-protein-pulsed DCs generated from blood adherent cells. Enhanced Id-specific cellular and humoral responses were observed as well as a transient minor fall in the M-component levels (Wen *et al*., 1998). In a study by Lim and coworkers, 6 MM patients were treated with autologous Id-protein-pulsed DCs. A minor response was observed in one patient (Lim and Bailey-Wood, 1999). Liso *et al*. (2000) reported a study in which 4/26 MM patients developed an Id-specific T-cell response. These patients were in PR or CR following high-dose therapy and were vaccinated with Id-pulsed DCs without concomitant cytokine administration. Titzer *et al*. (2000) used Id-pulsed DCs together with GM-CSF and showed that in patients with advanced disease, 3 and 2 out of 10 patients developed a humoral and cellular specific response respectively. In one patient a decreased plasma cell infiltration in the bone marrow was observed, but this patient was not evaluated for increase in Id-specific T-cells. Lacy *et al*. (2000) vaccinated 17 patients with Id-pulsed DCs after high-dose chemotherapy treatment. Three patients entered CR and 2 PR. Yi *et al*. (2002) vaccinated 5 patients in PR following high-dose chemotherapy using DCs and IL-2 and administered the DCs s.c. to improve the efficacy. An Id-specific T-cell response was elicited in 4 patients. Reichardt *et al*. (1999) at the Stanford University reported their experience of Id-pulsed DC vaccination in 12 MM patients after autologous peripheral blood stem cell transplantation (PBSCT). Two of 12 patients developed cellular Id-specific proliferation. Reichardt *et al*. (2003) also vaccinated 12 patients in remission after high-dose chemotherapy following peripheral blood stem cell transplant, using serum-free generated DCs and GM-CSF as additional adjuvant and could show that 2 developed Id-specific proliferation and out of those, 1 patient also developed a low level of cytotoxic T lymphocyte (CTL). Curti *et al*. (2003) reported that among 6 patients in PR after autologous stem cell transplantation, vaccinated with

Table II Vaccine Trials in Myeloma

Diagnosis	Trial stage	Number of pts	Vaccine	Adjuvant	Number of immunizations	Immune responses	Clinical outcome	References
Stage I–II	Phase II	28	Id	Alum + IL-12 ± GM-CSF	12	IL-12 group: 11/13 cellular, 3/11 DTH. IL-12/GM-CSF group: 5/15 cellular, 0/10 DTH	IL-12 group: 1/28 PR, 1/28 minor response. No significant difference in clinical outcome	Hansson *et al.* (2004)
1st remission after HDCT and PBSCT		15	Id	KLH + GM-CSF	7	3/15 Id-specific Ab; 8/8 Recovery of T cell diversity; 11/13 DTH	0/15 clinical response. No significant difference in mOS compared to control group	Coscia *et al.* (2004)
Remission after HDCT and PBSCT		26	Id-pulsed DC → boost with Id	± KLH-pulsed DC → Kl, H	7 (2 Id pulsed DC + 5 Id)	0/10 ld-specific Ab; 4/26 cellular	2/21 PR→CR, 6/21 decrease M component	Liso *et al.* (2000)
Advanced disease		11	Id-pulsed DC → boost with Id	GM-CSF	4 (1 Id-pulsed DC + 3 Id)	3/10 Id-specific Ab; 2/10 cellular	1/10 minor response	Titzer *et al.* (2000)
Remission after HDCT and PBSCT		17	Id-pulsed DC			5/17 cellular	3/17 CR, 2/17 PR	Lacy *et al.* (2000)

1st remission after HDCT and PBSCT	Phase I	12	Id-pulsed DC → boost with Id	KLH	7 (2 Id-pulsed DC + 5 Id)	2/12 cellular	1/12 decrease M component	Reichardt *et al.* (1999)
Remission after HDCT and PBSCT	Phase I	12	Id-pulsed DC → Id	KLH + GM-CSF	7 (2 Id-pulsed DC+ 5 Id)	2/10 cellular	None	Reichardt *et al.* (2003)
Remission after HDCT and PBSCT × 2	Phase I– II	10	Id/peptide pulsed DC	KLH-pulsed DC	5	0/6 Id-specific Ab; 6/6 cellular; 2/8 DTH	1/6 CR	Curti *et al.* (2003)
Remission after receiving HDCT and ASCT		16	Irradiated tumor cells Vaccinations pre- and post ASCT + reinfusion of primed lymphocytes	GM-CSF-secreting K562 cells	9 (1 pre-transplantation and 8 post-transplantation)	Humoral and cellular responses seen. 4/10 DTH 1 yr post-transplantation	3/16 with decrease in M component, after vaccination	Borrello *et al.* (2004)

[a]Abnormal metaphase cytogenetics.
[b]Autologous transplantation.
HDCT, high dose chemotherapy; ASCT, autologous stem cell transplantation; PBSCT, peripheral blood stem cell transplantation; PC, plasma cell; Ab, Antibody; CR, complete response; ctr, control; KLH, keyhole limpet hemocyanin; MR, minor or mixed response; PR, partial response; SD, stable disease.

Id or Id (VDJ)-derived HLA class I-restricted peptides coupled to KLH pulsed DCs, all 6 patients developed an Id-specific T-cell proliferative response and 4/6 showed circulating IFN-γ secreting T-cells by ELISPOT. One patient developed CR. Advanced patients have also been vaccinated with Id-pulsed DCs. Two patients with advanced refractory myeloma, received Id-pulsed DCs combined with GM-CSF (Cull *et al.*, 1999). An anti-Id T-cell proliferative response as well as T-cell cytokine release was obtained in both patients.

Among novel strategies to improve the results from vaccination, Szmania *et al.* (2004) have tried to combine immunotherapy with high-dose chemotherapy and autologous transplantation (AT) in high-risk myeloma patients. Antimyeloma T-cells have been primed and protected from high-dose chemotherapy by apheresis before AT. After high-dose chemotherapy the primed T-cells were reinfused early after AT and expanded by a series of booster vaccinations. The vaccines were DCs loaded with myeloma cell lysate and KLH combined with IL-2. Cellular responses were found in 3 out of 3 evaluable patients and in one of these patients a near CR was seen 719 days post-transplantation. This model has also been tried by Borrello *et al.* (2004). Untreated myeloma patients underwent a bone marrow harvest to obtain tumor cells for vaccine processing. Primed lymphocytes, obtained by pretransplant vaccination, were collected by leukapheresis and reinfused with the stem cell graft in patients eligible to receive autologous stem cell transplantation. The patients also received vaccinations post-transplant. The vaccine consisted of irradiated autologous tumor cells admixed with GM-CSF–secreting K562 cells. Both humoral and cellular responses were seen and in 3 out of 16 evaluable patients, with rising paraprotein levels early posttransplant, a decrease in paraprotein levels were observed following initiation of posttransplant vaccines.

To get an effective presentation of a broad array of tumor-associated antigens (TAAs) in the context of DC derived costimulation, Avigan *et al.* (2004b) have fused myeloma cells with DCs. Four patients have so far been vaccinated with autologous mature DCs administered with GM-CSF. Cellular responses have been seen. Clinical responses are being monitored.

Trudel *et al.* (2001) have demonstrated the possibility to vaccinate myeloma patients post-transplant with autologous plasma cells infected with an IL-2 expressing adenovirus. Eight patients have been enrolled and one of them was evaluable. In this patient no cellular response or clinical response was seen.

DNA vaccination in MM has been investigated by Stevenson *et al.* (2004). DNA vaccines offer a simplified strategy for delivering Id antigens encoded by the variable region genes, *VH* and *VL*. The *V* genes were assembled in a single chain Fv (scFv) format and fused in to the Fragment C of tetanus toxin to make DNA scFv-FrC vaccines. One patient has been vaccinated so

far and a strong and durable anti-Id T-cell response was seen combined with a slow fall of the paraprotein.

The collective data from these trials in myeloma illustrate that several factors should be considered while developing effective vaccination strategies. These include the nature of the elicited immune response and the best approach to generate such a response. The nature of the antigen(s) as well as the immunological adjuvants coadministered are important factors. Furthermore, delineation of the patients group that is most likely to respond to vaccination therapy is a critical issue. Based on the experiences it appears that the best means of achieving a broad immune response is through the use of DCs as it ensures NK cell activation as well as MHC class I and class II presentation (Lizee *et al.*, 2003). Another approach is the "prime-boost" concept. DNA vaccination is most likely to induce a potent $CD8^{+}$ T-cell response, while proteins preferentially generate $CD4^{+}$ T-cell response and antibodies.

Which antigen should be chosen? The Id is the most specific tumor antigen but may not be optimal. Cancer–testis (CT) antigens (MAGE and sperm protein-17) and MUC-1 are other potential target structures also expressed by myeloma cells but not in all patients (Pellat-Deceunynck, 2003). The use of whole-tumor cells has the advantage of utilizing the whole repertoire of rejection antigens.

Regarding adjuvants, GM-CSF seems to be a crucial adjuvant cytokine that augments the functional activity of antigen-presenting cell (APC) (Fischer *et al.*, 1988) and facilitate MHC class I as well as class II antigen presentation. IL-12 directs the immune response toward Th1 and is important for the induction of a $CD8^{+}$ T cytotoxic response (Trinchieri, 1998). In an attempt to expand the tumor-specific T-cells generated by the cancer vaccine, IL-2 or other immune stimulatory agents might be added.

Selecting the appropriate patient group seems to be essential to obtain clinical effect. Patients with pre-existing type I immunity against the antigen seem to be the candidates most likely to respond to vaccine therapy (Speiser *et al.*, 2003).

V. VACCINE TRIALS IN SOLID TUMORS

A. Renal Cell Carcinoma

Treatment with interferon-α both as monotherapy or in combination with other cytokines as well as chemotherapeutic agents has been extensively studied and has shown to provide a significant, though modest, survival benefit in patients with advanced renal cell carcinoma. The efficacy of a

specific immunotherapeutic approach, however, still remains to be assessed. Available data is derived from a very limited number of clinical trials. The ones discussed here used autologous or allogenic tumor cells (Table III). The largest trial published to date is a Phase III study (Jocham *et al.*, 2004) in which patients who had undergone radical nephrectomy were randomized either to receive six administrations of an autologous renal tumor cell vaccine as adjuvant treatment or to receive no adjuvant treatment. The assessment of the immune response was not an endpoint of the trial, which aimed at evaluating PFS as the primary endpoint. Analysis was performed on 379 evaluable patients and showed a 5-year PFS of 77% in the vaccine group versus 68% ($p = 0.0204$) in the control group. Seventy-months PFS was 72% versus 59%. The treatment was well tolerated.

In the advanced setting, autologous or allogenic tumor cells-loaded DC together with KLH were administered to 27 evaluable patients, and 7% CR, 3% PR and 26% SD were achieved (Holtl *et al.*, 2002). Cellular immune response against KLH was evaluated in only 11 patients and proved to be positive in all of them (40% of the total number of patients). As antigen for the assessment of a renal cell carcinoma-specific immune response oncofetal antigen/immature laminin receptor (OFA/LRP) was used, but testing was done only in 6 patients. Five patients developed an at least twofold increase in T-cell proliferation compared to pretreatment.

Ongoing clinical trials will hopefully provide further information. At present at least seven clinical trials (Phase I/II or II) are ongoing in the United States, investigating different vaccine approaches, either with fibroblast growth factors 5 (FGF-5)-derived peptides, or heat shock protein (HSP) PC-96 derived from autologous tumor cells as well as autologous tumor cells transfected with adenovirus B7–1. Finally, vaccines composed of DC treated *in vitro* with autologous tumor cells and vaccines made of tumor-antigen gene–expressing viruses, such as the Trovax study, are ongoing.

B. Prostate Cancer

A number of clinical trials have been conducted in prostate cancer and are summarized in Table IV. The fact that prostate cancer cells express unique proteins, such as prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) and prostatic acid phosphatase (PAP), which can be specifically targeted, makes this disease suitable for an active vaccine-based approach aimed at breaking the immunological tolerance to the tumor. The disease setting of the trials performed to date is generally that of PD, defined from study to study either as rising PSA value (biochemical progression) or as metastatic or non-metastatic, hormone-refractory disease.

Table III Vaccine Trials in Renal Cell Carcinoma

Diagnosis	Trial stage	Number of pts	Study design	Vaccine	Adjuvant	Number of immunizations	Immune response	Clinical outcome	References
Adjuvant	Phase III	379 (177 vaccine; 202 control)	Multicenter, randomized	Autologous renal tumor cell	None	6	Not evaluated	5-yr PFS 77% vs 68% ($p = 0.0204$); 70-mo PFS 72% vs 59%	Jocham *et al.* (2004)
Advanced		27		Autologous or allogenic tumor cells-loaded DC	KLH	3	40% cellular response to KLH[a]; 18% to OFA[b]	7% CR, 3% PR, 26% SD	Holtl *et al.* (2002)

[a]T-cell proliferation (only 11/27 pts evaluated).
[b]T-cell proliferation (only 6/27 pts evaluated).
KLH, keyhole limpet hemocyanin; PFS, progression-free survival; OFA, oncofetal antigen; PR, partial response; pts, patients; SD, stable disease; mo, months; yrs, years.

Table IV Vaccine Trials in Prostate Cancer

Diagnosis	Trial stage	Number of pts	Study design	Vaccine	Adjuvant	Number of immunizations	Immune response	Clinical outcome	References
Local recurrence	Phase II	37		HLA-A2-specific PMSA peptide-loaded DC	None	6	DTH+[a] in 73% responders vs 57% non responders	RR 30%	Murphy *et al.* (1999b)
Hormone-refractory metastatic disease	Phase II	25		HLA-A2-specific PMSA peptide-loaded DC	None	6	DTH+[b] in 75% responders vs 30% non responders	RR 32%	Murphy *et al.* (1999a)
Hormone-refractory metastatic disease	Phase III	127 (82 vaccine; 45 control)	Double-blinded, placebo-controlled	PAP/GM-CSF (PA2024) fusion protein-pulsed DC (Provenge®)	None	3	Median cellular response: 17 in treated vs 2 in controls ($p = 0.0004$)[c]	PFS 11.1 wks vaccine arm vs 10 wks control arm ($p = 0.061$). OS 30.7 mo vaccine arm vs 22.3 control arm ($p = 0.047$) if Gleason ≤ 7	Schellhammer and Hershberg (2005), Small *et al.* (2005)
Hormone-refractory disease	Phase I–II	31		PAP/GM-CSF (PA2024) fusion protein-pulsed DC (Provenge®)	None	3	100% cellular[d] response to PA2024, 32% to PAP; 52% humoral response to PAP, 81% to GM-CSF	RR 10% mPFS phase I: 12 wks; mPFS phase II; 29 wks; mPFS 34 wks responders vs 13 wks non-responders ($p < 0.027$)	Small *et al.* (2000)
Hormone-refractory disease	Phase II	19		PAP/GM-CSF (PA2024) fusion protein-pulsed DC (Provenge®)	None	5	79% cellular response to PA2024[d]. 68% humoral response to PA2024	PFS: 3.9 mo	Burch *et al.* (2004)

Biochemical PD (+/– metastases)	Phase I	21		Recombinant murine PAP-pulsed DC	None	2	100% cellular[d] response to mouse PAP; 52% to human PAP (100% SD pts vs 33% PD pts)	SD 29%; PD 71%	Fong *et al.* (2001)
Biochemical PD	Phase II	64	Randomized (3 arms)	PSA-expressing poxvirus +/– PSA-expressing-vaccinia virus	None	4	46% cellular[e] response, 0% humoral response	RR: 0%. mPFS all arms: 9.7 mo	Kaufman *et al.* (2004)
Biochemical PD	Phase II	24	Multicenter, open-label	PSA-pulsed DC	None	9	46% cellular response[f], 0% humoral response	No PSA response; circulating cancer cells disappeared in 6/6 pts	Barrou *et al.* (2004)

[a]No change or increase vs pre-vaccine.

[b]No change or increase vs pre-vaccine (only 6/17 responders evaluated).

[c]Specific T-cell response (median T-cell stimulation (TCSI) ratio in proliferation assay. TCSI-ratio defined as median TCSI at 8 wks/median TCSI at pre-treatment).

[d]T-cell proliferation.

[e]IFN-γ–secreting cells after a 7-day *in vitro* stimulation with an HLA-A2–restricted PSA peptide and autologous PBMC as antigen-presenting cells (APC). Reported data refer to the HLA-A2–positive pts only (54% of total).

[f]IFN-γ–secreting T cells.

KLH, keyhole limpet hemocyanin; mPFS, median progression-free survival; PR, partial response; pts, patients; SD, stable disease; PD, progressive disease; mo, months; yrs, years; RR, response rate.

Three of these trials (Burch *et al.*, 2004; Schellhammer and Hershberg, 2005; Small *et al.*, 2000) used APC8015 (Provenge®), an immunotherapeutic product made of DC loaded *ex vivo* with the recombinant fusion protein PA2024, consisting of PAP linked to GM-CSF. In all three studies, the vaccine proved to be safe and well tolerated and a specific immune response to the vaccine could be seen, which could be correlated to a better clinical outcome in two of them. In a sequential Phase I/II trial (Small *et al.*, 2000), a correlation was seen between the development of an immune response to PAP and the time to disease progression. Median PFS was in fact significantly longer in responders compared to nonresponders (34 weeks vs 13 weeks, $p < 0.027$).

In the only double-blinded placebo-controlled Phase III clinical trial, named D9901 (Schellhammer and Hershberg, 2005; Small *et al.*, 2005), a total of 127 patients with asymptomatic, metastatic, androgen-independent prostate cancer (AIPC) were randomized in a 2:1 ratio to receive three doses of APC8015 or control. To be eligible for the study, the patients had to have $\geq$25% of cancer cells staining positive for PAP. The improvement in clinical outcome in terms of PFS, though, was seen only in patients with Gleason score $\leq$7 (median PFS 16.1 weeks vs 9.1 weeks, $p = 0.001$) and not in patients with Gleason $\geq$8. In the intent-to-treat population, no benefit in PFS was seen in the vaccine arm compared to the control arm (11.1 weeks vs 10 weeks, $p = 0.061$). With regard to overall survival (OS) in the intent-to-treat population, a 3.9 months OS advantage was seen in the treated group (25.9 months vs 22 months, $p = 0.020$). The advantage was again more striking in patients with Gleason score $\leq$7 (median OS 30.7 months vs 22.3 months, $p = 0.047$). The importance of Gleason score as a variable predicting the response to APC8015 was also confirmed by the immune monitoring. An eightfold higher PA2024-specific T-cell response (T-cell stimulation index 17 vs 2, $p = 0.0004$) could be seen in vaccine-treated patients compared to controls. What was remarkable was that this T-cell response was sevenfold higher in the group with Gleason scores $\leq$7 than in the group with Gleason $\geq$8 (T-cell stimulation index 49.6 vs 7.26, $p = 0.0065$). Based on the results of this study, a confirmatory Phase III study (named D9902B) enrolling patients with asymptomatic metastatic AIPC with Gleason score $\leq$7 is ongoing. Encouraging data was obtained from an open-labeled Phase II study (trial D9905) suggesting that Provenge® as single agent could delay PSA-doubling time in men with rising PSA values after definitive local therapy (androgen-depedent prostate cancer). Based on these results a Phase III study, named P-11, is ongoing in this disease setting. Finally, the combination of Provenge and bevacizumab (Avastin®) is also being investigated in the same disease setting in an ongoing Phase II trial (P-16).

In two stage II trials by Murphy *et al.*, DCs loaded with two HLA-A2-specific PSMA peptides (PSM-P1 and -P2) were administered to patients

with either metastatic (Murphy *et al.*, 1999a) or locally recurring (Murphy *et al.*, 1999b) hormone-refractory prostate cancer. Overall immunological responsiveness of the patients was measured at the beginning and after conclusion of the study with delayed-type hypersensitivity (DTH) test against a number of recall antigens (streptococcus, tuberculin, glycerin, candida, trychophyton and proteus). In the first study, 32% of the patients had a clinical response (24% PR and 8% CR) and DTH test remained stable in 75% of the responders vs 30% of the nonresponders. In the second study, an overall RR of 30% (27% PR + 3% CR) was achieved and at DTH skin testing 73% of patients in the responder group maintained or improved response compared to 57% in the nonresponder group.

The possibility of inducing a PSA-specific immunity was evaluated in a Phase II clinical trial (Kaufman *et al.*, 2004) testing a prime/boost vaccine strategy using Vaccinia virus and fowlpox virus expressing human PSA. Eligible patients had biochemical progression after local therapy for prostate cancer and were randomly assigned to three different treatment arms, either fowlpox-PSA for four admnistrations or three fowlpox-PSA vaccine doses followed by one Vaccinia-PSA vaccine dose or one Vaccinia-PSA vaccine dose followed by three fowlpox-PSA vaccine doses. Treatment was well tolerated with no evidence of autoimmunity. No PSA response was seen in any of the treatment arms. Median time to biochemical or clinical progression in the three arms altogether was 9.7 months. At 19.1 months of follow up, 45.3% of patients were free of biochemical progression and 78.1% had not progressed clinically. Although the vaccines used in this trial were designed to express the full-length *PSA* gene, the cellular immune monitoring was performed only in HLA-A2-positive patients based on the choice to use two well-characterized HLA-A2-restricted PSA epitopes for the ELISPOT assay. PSA-reactive T-cells were found in 46% of HLA-A2-positive patients. No increase in anti-PSA antibody titres was detected.

In another Phase II trial (Barrou *et al.*, 2004), DCs pulsed with human PSA were used to vaccinate 24 patients in biochemical relapse. The vaccine was administered by combined intravenous, subcutaneous and intradermal routes. No antibody response to PSA could be seen while PSA-specific T-cells were detected in 46% of patients after vaccination versus 30% before vaccination. No PSA response was seen. However, 6 patients who tested positive by PCR-based methods for the presence of circulating cancer cells before vaccination became PCR-negative post-vaccination. No statistically significant relationship could be found between the specific immune response and the disappearance of circulating cancer cells.

Finally, a Phase I clinical trial (Fong *et al.*, 2001) investigated the efficacy of DC pulsed with recombinant mouse PAP (mPAP) in the metastatic disease setting. A stabilization of disease was seen in 29% of the patients, while all the others progressed. A cellular response to human PAP (hPAP) was seen,

which could be correlated with the clinical response. All patients who achieved disease stabilization, developed a cellular response to hPAP versus 33% of those with PD. Moreover, in all 21 vaccinated patients a cellular response to mPAP could be induced. Interestingly, PBMCs from 16 patients were also assessed for TNF-α, IFN-γ, IL-4, and IL-10 secretion in response to mPAP and hPAP. IFN-γ–secreting cells were found in 43% and 62% of the patients in response to mPAP and hPAP respectively, while TNF-α secreting cells were found in 6% and 31% of the patients. No production of either IL-4 or IL-10 could be seen, consistent with the induction of Th1 immunity. The data provided by this study provide evidence that immunological tolerance to human self-antigens can be broken by xenoantigen immunization.

Data is awaited from studies presented at ASCO 2005 in a preliminary form. Here we report the ones with enough mature data and investigating a correlation between clinical and immunological response. In a pilot trial (Hallmeyer *et al.*, 2005), two different vaccination strategies using a PSA peptide either injected i.d. in combination with GM-CSF or pulsed on DC and intraveneously infused were compared in two groups of patients (high risk, local disease or metastatic, hormone sensitive). Specific DTH responses to the PSA peptide could be seen in 50% of the patients and could be correlated to clinical response (78% DTH responders had stabilization or reduction of PSA levels, while 64% of the nonresponders had biochemical or clinical progression).

Moreover, data from a confirmatory Phase II study of GVAX® (Simons *et al.*, 2005), a vaccine made of allogenic prostate carcinoma cell lines genetically modified to secrete GM-CSF, were presented. Eighty patients with metastatic hormone-refractory prostate cancer have been accrued in the study. The occurrence of tumor-reactive antibodies was measured by Western blot of vaccine lysates against the patient's serum. Stabilization or reduction of bone metastases were seen in 43% of patients. Although median survival has not yet been reached, a trend toward prolonged survival was seen in patients generating a specific 280-kD band in Western blot indicating that immune reactivity of the patients to this antigen of the allogeneic cell line vaccine may be critical for therapeutic efficacy. Based on this study, a Phase III study named VITAL-1 is underway, randomizing 600 patients to GVAX® versus docetaxel/prednisone. A number of other studies are also presently ongoing in the United States with different vaccine preparations, for example, hyperacute-prostate cancer vaccine made of alpha-(1,3) galactosyltransferase expressing allogenic tumor cells, recombinant Vaccinia-PSA/TRICOM (PROSTVAC®-V/TRICOM™) or recombinant fowlpox-PSA/TRICOM (PROSTVAC®-F/TRICOM™) viruses, and TG40101, a modified Vaccinia virus carrying the *MUC1* and the *IL-2* genes.

C. Melanoma

The large number of clinical trials on vaccine therapy for melanoma make it unfeasible to have a listing and discussion of each trial in this chapter. We have summarized some of the larger trials in Table V. Vaccine preparations for melanoma can basically be categorized as: (1) modified tumor cells or tumor cell-derived polyvalent vaccine, (2) synthetic peptides or recombinant proteins, (3) glycolipid and anti-Id vaccines, and (4) DNA/RNA-based vaccines. These have been delivered along with a variety of adjuvants including incomplete Freund's adjuvant (IFA), alum, Bacillus Calmette-Guérin (BCG), cytokines such as GM-CSF, IL-2, and IL-12 as well as DC as cellular adjuvant.

There are a few observations that form a synopsis of the clinical trials. First, melanoma is one of the few malignancies that have an exceptional degree of responsiveness to immunotherapy. In contrast to other solid tumors like breast, ovarian and pancreas, vaccination therapy for metastatic melanomas, utilizing widely different antigen preparations, patient characteristics and immunological adjuvants, have reported varying degrees of clinical efficacy. On close scrutiny, very few of the clinical trials in metastatic melanoma patients have had clinical responses in $\geq$20% of accrued patients. These results emphasize the need to improve the clinical outcome following vaccination therapy in melanoma.

A second observation is that polyclonal vaccination with modified tumor cells or tumor lysate tend to favor clinical responsiveness compared to monoepitopic vaccine strategies (Belardelli *et al.*, 2004). This observation may be biased partly due to the promising results of the large Phase II studies performed with Canvaxin (Morton *et al.*, 2002) and Melacine (Sondak and Sosman, 2003). However, when Canvaxin was compared to an observational group, no significant effect was noted with Canvaxin in stage IV melanoma in the adjuvant setting. Based on these interim results the study was closed. The randomized trial in stage III adjuvant setting is, however, continuing based on the same interim analysis (Athos Gianella-Borradori, personal communication). The spontaneous occurrence of escape variants that have downregulated the targeted melanoma-associated antigen is a well-established phenomenon (Khong *et al.*, 2004; Slingluff *et al.*, 2000) and polyclonal vaccine preparations may minimize the generation of antigen-loss variants.

A third observation was the infrequent relationship between measured immune reactivity to the immunizing antigen and the clinical response. Patients with robust immune reactivity to the antigen as measured with DTH tests or *in vitro* assays for antigen-specific responses often demonstrated little or no clinical benefit. Conversely, clinical responders in many

Table V Vaccine Trials in Metastatic Melanoma

Diagnosis	Trial stage	Number of pts	Study design	Vaccine	Adjuvant	Number of immunizations	Immune responses	Clinical outcome	References
Stage III–IV		17	Single arm	DC-tumor fusion hybrid	None	3–25 (average 8)	11/14 cell mediated	1 CR, 1 PR, 6 SD	Trefzer *et al.* (2005)
State III–IV non resected	Phase I	54	Single arm analysis	Polyclonal melanoma cell vaccine (PMCV; Canvaxin)	BCG +: 1) Cytoxan 2) Dinitrochlorobenzene (DNCB) 3) Indomethacin 4) Cimetidine 5) Indomethacin plus cimetidine 6) Ranitidine	24 over 5 yrs	35/51 positive DTH to PMCV; 16/51 negative DTH to PMCV	7/54 CR, 2/54 PR, 11/54 MR, 13/54 SD, 32/54 PD	Hsueh *et al.* (1999)
Stage III adjuvant	Phase II	739	Non-randomized retrospective Matched-pair analysis	Polyclonal melanoma cell vaccine (PMCV; Canvaxin)	BCG +: 1) Cytoxan 2) GMCSF 3) Indomethacin 4) Cimetidine 5) Ranitidine	>10 variable	Not reported	OS 55.3 vs 31.6 mo. 5-yr OS 48.8 vs 36.8%, 10-yr OS 42 vs 31% ($p < 0.001$)	Morton *et al.* (2002)
Stage IV adjuvant	Phase II	107	Non-randomized retrospective Matched-pair analysis	Polyclonal melanoma cell vaccine (PMCV; Canvaxin)	BCG +: 1) Cytoxan 2) GMCSF 3) Indomethacin 4) Cimetidine 5) Ranitidine	>10 variable	OS correlated to DTH ($p = .0001$)	OS 38 vs 19 mo. ($p = .0009$). 5-yr OS 39 vs 20%	Hsueh *et al.* (2002)
Clark stage IV adjuvant	Phase III	553 total, 294 vaccinated	Randomized, observation controlled	Allogeneic melanoma cell lysate (Melacine™)	DETOX	4 × 10	Not reported	5-yr RFS, 83% vs 59%; $p = .0002$ in pts expressing ≥M5 antigens. 5-yr RFS 77% vs 64% ($p = .004$) for pts expressing HLA-A2 and/or HLA-C3	Sosman *et al.* (2002)

Stage III–IV adjuvant	Phase I–II	42	Double-blind placebo controlled	NY-ESO-1 protein	ISCOMATRIX	3	20/20 with Ag + ISX vs 4/16 with Ag developed Ab response; 10/16 with Ag + ISX vs 1/16 with Ag alone developed DTH	5/7 placebo, 9/16 Ag alone and 2/19 Ag + ISX relapsed over median follow up of 748 days	Davis *et al.* (2004)
Stage III adjuvant	Phase I–II	214	Non-randomized, uncontrolled	DNP haptenized autologous tumor cells	BCG + cytoxan pretreatment	6, 8, or 12	47% of pts developed DTH to unmodified tumor. All pts developed DTH to hapten modified tumor	5-yr OS for DTH positive vs DTH negative pts[b] (59.3% v 29.3%; $p < .001$). 5-yr OS after relapse for DTH positive vs DTH negative pts[b] (25.2% v 12.3%; $p < .001$)	Berd *et al.* (2004)
Stage III–IV	Phase II	29	Randomized	Gp 100 + tyrosinase peptide	Immature DC (arm 1) or IFA + GM-CSF (arm 2). IL-2 both arms	6	T-cell responses in 42% PBLs and 80% of SINs[c] of GM-CSF arm vs 11% and 13% in DC arm ($p < .02$)	Objective clinical response noted in 2 pts in GM-CSF arm and 1 patient in DC arm	Slingluff *et al.* (2003)
Stage IV	Phase II	45 vaccinated, 39 evaluable	Randomized	HSPPC-96	None	4 or 8	11/23 had increased T-cell response ($p <.05$ or $< .01$)	2 CR, 3 SD	Belli *et al.* (2002)
High-risk resected stages IIB, III, and IV	Phase I	25		MART-1 (27–35) peptide	IFA	4	13/25 DTH, 10/22 y-IFN ELISA	RFS correlates with y-IFN ELISA ($p < 0.003$)	Wang *et al.* (1999)
III and IV	Phase I	25		MAGE-3.A1 peptide	None	3	No detectable CTL activity	3 CR, 4 PR	

(*continues*)

Table V (*continued*)

Diagnosis	Trial stage	Number of pts	Study design	Vaccine	Adjuvant	Number of immunizations	Immune responses	Clinical outcome	References
Not reported	Phase I–II	46	3 sequential	Fowlpox viruses encoding (a) the full-length gp 100 molecule; (b) the gp 100 molecule with two amino acids modified to increase HLA-A2 binding; and (c) a "minigene" encoding a single, modified epitope gp 100;209–217	II-2 was administered when pts progressed with vaccination alone	Variable	1/7, 10/14 and 12/16 pts with (a), (b) and (c) respectively showed cellular response (y-IFN)	1 PR with virus alone. 6/12 pts in group c had objective responses after receiving IL-2 including 3 CR	Rosenberg *et al.* (2003)

[a] M5 antigens were IILA-A2, IILA-A28, IILA-B44, IILA-B45, and IILA-C3.
[b] DTH response to unmodified tumor (correlation of DTH response against haptenized tumor to OS was not significant).
[c] Sentinel immunized lymph node.

Ab, antibody; BCG, Bacillus Calmette-Guérin; CR, complete response; ctr, control; DT, diphtheria toxoid; GM-CSF, granulocyte/macrophage-colony stimulating factor; PD, progressive disease; PR, partial response; pts, patients; SD, stable disease; mo, months; yrs, years; MR, minor response; RFS, relapse-free survival.

vaccine trials did not universally demonstrate a potent induction of immune response to the tumor antigen. The discrepant relationship between immune reactivity and clinical response emphasizes the critical need for improved immunomonitoring strategies and raises the question whether sampling of peripheral blood lymphocytes can serve as a putative indicator of the therapeutic immune response responsible for tumor regression at the site of the disease.

D. Gastrointestinal Malignancies

1. PHASE II/III RANDOMIZED CONTROLLED TRIALS

Randomized controlled therapeutic vaccine trials in patients with gastrointestinal (GI) malignancies are summarized in Table VI. A total of 704 stage II–III colorectal carcinoma (CRC) patients were enrolled in three separate Phase III trials and were randomized to undergo surgery alone or to receive vaccination with OncoVAX after tumor resection (Harris *et al.*, 2000; Hoover *et al.*, 1993; Vermorken *et al.*, 1999). OncoVAX is an autologous tumor cell vaccine mixed with BCG. Hoover *et al.* (1993) reported a statistically significant prolongation of overall and disease-free survival (DFS) in patients with colon cancer, whereas no benefit of OncoVAX was seen in rectal cancer. The subsequent Phase III studies using OncoVAX failed to show a statistically significant improvement in overall survival of colon cancer patients in the intent-to-treat population. Subgroup analysis by Harris *et al.* (2000) revealed, however, a significantly longer overall survival in stage II colon cancer patients mounting a DTH response as compared to patients lacking DTH response. Vermorken *et al.* (1999) reported a significantly longer DFS in vaccinated patients with stage II but not stage III disease. Meta-analysis of the three trials with OncoVAX did not show a statistical difference in OS between the vaccine and control groups. However, the disease-specific survival in the intent-to-treat patient population was significantly improved ($p = 0.047$), and a significantly longer OS was noted in patients with stage II colon cancer receiving an additional booster vaccine ($p = 0.018$) (Hanna *et al.*, 2001).

Encouraging results were also observed in a large randomized study using autologous tumor cells infected with Newcastle-disease virus (NDV) as an adjuvant (Liang *et al.*, 2003). Postsurgical stages I–IV CRC patients ($n = 567$) were enrolled into the study. The overall survival duration was significantly improved in vaccinated patients as compared to those treated with surgery alone. Patients mounting a DTH response survived significantly longer. Twenty-five patients with metastatic disease were also vaccinated, of which 6 (24%) showed a complete or partial response.

Table VI Vaccine Trials in Gastrointestinal Malignancies

Diagnosis	Clinical stage[a]	Trial stage[b]	Number of pts	Study design	Vaccine	Adjuvant	Number of immuni-zation	Immune responses	Clinical outcome	References
Colon	II, III adjuvant	Phase II–II	182/188	Resection + ASI vs resection	Autologous TC	BCG	3	88% DTH	OS↑ in stage II in DTH+ vs DTH− ($p = 0.03$) (7.6 yrs)	Harris *et al.* (2000)
Colon	II, III adjuvant	Phase II–III	128/126	Resection + ASI vs resection	Autologous TC	BCG	4	98% DTH	DFS↑ ($p = 0.03$) in stage II (5.3 yrs)	Vermorken *et al.* (1999)
Colon, rectum	II, III adjuvant	Phase II–III	41/39	Resection + ASI vs resection	Autologous TC	BCG	3	80% DTH	OS↑ ($p = 0.02$), DFS↑ ($p = 0.04$) in colon ca (6.5 yrs)	Hoover *et al.* (1993)
Colon, rectum	I–IV adjuvant	Phase II–III	310/257	Resection + ASI vs resection	Autologous TC	NDV	4 + booster biweekly or monthly	>90% DTH	OS↑ ($p < 0.01$) (7 yrs). OS↑ in DTH+ vs DTH− ($p < 0.01$) (5 yrs)	Liang *et al.* (2003)
Colon, rectum	Advanced disease	Phase II–III	18/21	SCV106 vs ctr anti-ld	Anti-ld mimicking Ep-CAM (SCV106)	Alum	8	67% humoral	PD↓ ($p = 0.001$), OS↑ ($p = 0.01$) in Ab+ vs Ab− (9.7 mo)	Samonigg *et al.* (1999)

Colon, rectum	Advanced disease	Phase II–III	85/77	105AD7 vs placebo	Anti-ld mimicking CD55 (105AD7)	Alum	3	ND	No significant difference in OS	Maxwell-Armstrong *et al*. (2001)
Colon, rectum, other	III–IV	Phase II–III	120/119	IGN101 vs placebo	mAb17-1A (IGN101)	Alum	9	Almost 100% humoral	OS↑ ($p = 0.037$) in stage IV rectal ca ($n = 53$) (1 yr)	Himmler *et al*. (2005)
Pancreas	Advanced disease	Phase II–III	79/75	G17DT vs placebo	Gastrin-17-linked to DT (G17DT, Gastrimmune)	–	8	54% humoral	OS↑ ($p = 0.03$) (151 vs 82 days)	Gilliam *et al*. (2004a)
Liver	I–III	Phase II–III	18/21	Resection+ ASI vs resection	Autologous TC fragments	GM-CSF, IL-2 microparticles, BCG	3	67% DTH	OS↑ ($p = 0.01$), DFS↑ ($p = 0.003$) 15 mo). Recurrence-free pts; 92% in DTH+ vs 33% in DTH–	Kuang *et al*. (2004)
Colon, rectum	II, III adjuvant	Phase I/II	57		Autologous TC	NDV or BCG	3	68% DTH	OS↑ 98% in NDV vs 67% in BCG vs 74% in historical ctr (2 yrs)	Ockert *et al*. (1996)
Colon, other	IV	Phase I/II	37		CEA mRNA – transfected DC	IL-2+/–	4	31% DTH	2 MR, 3 SD (16 mo), 4% tumor market↓	Morse *et al*. (2003)
Colon, rectum, other	Advanced disease	Phase I/II	18		CEA peptide-pulsed DC	–	5x booster up to 39	18% DTH, 17% celluar[c]	11% tumor marker↓	Ueda *et al*. (2004)

(*continues*)

Table VI (*continued*)

Diagnosis	Clinical stage[a]	Trial stage[b]	Number of pts	Study design	Vaccine	Adjuvant	Number of immuniza-tion	Immune responses	Clinical outcome	References
Colon, rectum, other	Advanced disease	Phase I/II	113		Peptides to which pts had pre-existing cellular response	IFA	3, booster up to 15	37% DTH, 66% humoral, 47% cellular	5 PR, 2 MR	Mine *et al.* (2004)
Colon, rectum, other	Advanced disease	Phase I/II	41		MUC-1 peptide linked to mannan	–	3, booster up to 6	60% humoral, 28% cellular	5 SD	Karanikas *et al.* (2001)
Colon, rectum, other	ND	Phase I/II	63		MUC-1 peptide	BCG	4	5% DTH, 80% cellular	No clinical response	Goydos *et al.* (1996)
Colon, rectum	Advanced disease	Phase I/II	17		Survivin peptide	–	6	40% DTH 7% cellular	1 MR, 40% tumor market↓	Tsuruma *et al.* (2004)
Colon, rectum	II–IV resected	Phase I/II	32		Anti-ld mimicking CEA (CeaVac)	Alum or QS-21	4, booster monthly	100% humoral, 100% cellular	78% SD (16 mo)	Foon *et al.* (1997, 1999)
Colon, rectum	I–IV resected	Phase I/II	35		Anti-ld mimicking CD55 (105AD7)	Alum	3–4	73% cellular, 61% NK activity	65% NED (4 yrs)	Amin *et al.* (2000), Durrant *et al.* (2000a,b)

Colon, rectum	Advanced disease	Phase I/II	17	Plasmid CEA co-expressing HbsAg	–	1–3	23% cellular[c]	5 SD (2 mo)	Conry *et al.* (2002)
Colon, rectum, other	III–IV resected or metastatic	Phase I/II	32	Vaccinia-CEA (full-length or truncated CEA)	–	2	22% humoral	No clinical response	Conry *et al.* (1999, 2000), Tsang *et al.* (1995)
Colon, rectum	Advanced disease	Phase I/II	16	ALVAC-p53	–	3	19% humoral, 12% cellular	1 SD (7 wks)	Menon *et al.* (2003), van der Burg *et al.* (2002)
Colon, rectum, other	Advanced disease	Phase I/II	60	ALVAC-CEA B7.1	+/– GM-CSF	4		44% SD in GM-CSF+ (up to 13 mo) vs 27% in GM-CSF– (up to 6 mo)	von Mehren *et al.* (2001)
Colon, rectum, other	Advanced	Phase I/II	58	Fowlpox-CEA-TRICOM; Vaccinia-CEA-TRICOM	+/– GM-CSF	6, booster every 3 mo	18% humoral; 77% cellular	1 CR, 40% SD (4–>6 mo), 19% tumor marker response	Marshall *et al.* (2005)
Stomach	I–IV	Phase I/IV	52	Gastrin-17-linked to DT (G17DT, Gastrimmune)	–	5	92% humoral	ND	Gilliam *et al.* (2004b)

(*continues*)

Table VI (*continued*)

Diagnosis	Clinical stage[a]	Trial stage[b]	Number of pts	Study design	Vaccine	Adjuvant	Number of immunization	Immune responses	Clinical outcome	References
Pancreas	I–III adjuvant	Phase I/II	14		Allogeneic GM-CSF secreting TC	GM-CSF	4	21% DTH, 36% cellular	DFS↑ in DTH+ (25 mo)	Jaffee *et al.* (2001), Thomas *et al.* (2004)
Pancreas, liver, bile ducts, thyroid	Advanced disease	Phase I/II	20		TC lysate-pulsed DC	IL-2	4–10	90% DTH, 75% cellular	2PR, 2SD (22, 24 mo), 40% tumor marker↓	Stift *et al.* (2003)
Pancreas, colon, rectum, breast	Advanced disease	Phase I/II	63		MUC-1 peptide	BCG	4	5% DTH, 80% cellular	No clinical response	Goydos *et al.* (1996)

[a]American Joint Committee on Cancer staging.
[b]Number of eligible pts in treatment vs control group.
[c]Cellular responses are collective data obtained from any of the following assays or observations: Lymphoproliferation, CTL, IFN-γ secretion, TNF-α secretion, tetramer positivity, NK activity, lymphocyte infiltration at vaccine site, increase in tumour infiltrating lymphocytes and tumor cell apoptosis.

Ab, Antibody; ADCC, Antibody-dependent cellular cytotoxicity; ASI, active specific immunotherapy; BCG, Bacillus Calmette-Guerin; β-hCG, human chorionic gonadotropin β; ca, carcinoma; CR, complete response; ctr, control; DFS, disease free survival; DT, diphtheria toxoid; GM-CSF, granulocyte/macrophage-colony stimulating factor; HSP, heat-shock protein; KLH, keyhole limpet hemocyanin; MPL, monophosphoryl lipid A; MR, minor or mixed response; NDV, Newcastle-disease-virus; NED, no evidence of disease; ND, not determined; OFA, oncofetal antigens; OS, overall survival; PD, progressive disease; PR, partial response; pts, patients; SD, stable disease; TC, tumor cell; TIL, tumor infiltrating lymphocytes; trt, treatment.

Three randomized controlled trials were carried out in metastatic CRC making use of anti-idiotypic network responses. Anti-idiotypic antibodies representing the internal image of the three-dimensional structure of the bona fide antigen can be used as surrogate TAAs for vaccination. Immunization with an anti-idiotypic antibody mimicking the TAA, CD55, did not have an impact on patient survival, but it should be noted that only 50% of patients received the planned vaccine dose (Maxwell-Armstrong *et al.*, 2001). In another randomized study, vaccination with an anti-idiotypic antibody mimicking Ep-CAM (SCV106) was compared to an unspecific isotype matched antibody (Samonigg *et al.*, 1999). No tumor response and no difference in OS for the intent-to-treat population were observed. However, the frequency of PD, measured by the development of new metastasis, was significantly reduced among patients receiving SCV106 as compared to the control group. Although the number of patients analyzed was relatively limited, it seemed that immune responders vaccinated with SCV106 survived significantly longer than patients in the control group.

Treatment with an mAb, particularly if delivered as an active immunotherapy with adjuvants, can induce anti-idiotypic antibodies. In a Phase II study, the anti-Ep-CAM murine mAb 17–1A conjugated to alum (IGN101) was used to vaccinate patients with stage III–IV epithelial cancers (mainly CRC) and compared to placebo. Almost all patients in the IGN101 group mounted antibodies against Ep-CAM. For the whole intent-to-treat population, no difference in OS was seen. Subgroup analysis showed, however, a statistically significant survival prolongation for patients with metastatic rectal cancer ($n = 53$; $p < 0.05$). The 1-year survival was doubled in the IGN101 group as compared to placebo (Himmler *et al.*, 2005).

G17DT (a synthetic gastrin-like peptide linked to diphtheria toxoid, DT) induces neutralizing antibodies against gastrin, which is believed to be a growth factor for GI malignancies. Vaccination with G17DT significantly prolonged the OS of patients with advanced pancreatic cancer as compared to placebo (Gilliam *et al.*, 2004b). When G17DT vaccination was combined with gemcitabine in advanced pancreatic carcinoma and compared to gemcitabine alone, no survival difference was noted between the two groups. However, a subgroup analysis showed that patients with the highest anti-G17DT IgG titres survived significantly longer—75% of the patients mounted a specific antibody response (Shapiro *et al.*, 2005).

In postresection hepatocellular carcinoma (HCC), a tumor cell-based vaccine in conjunction with adjuvant cytokines significantly improved the overall and disease-free survival compared to surgery alone ($p = 0.01$). Recurrences were less frequent in patients mounting a DTH response (Kuang *et al.*, 2004).

2. PHASE I/II TRIALS

Phase I/II therapeutic vaccine trials in GI malignancies are shown in Table VI. Whole-tumor cell-based vaccines have been used in conjunction with BCG or NDV (Habal *et al.*, 2001; Ockert *et al.*, 1996). As compared to historical controls, an increased 2-year survival rate was noted in CRC patients vaccinated with autologous tumor cells infected with NDV but not BCG (Ockert *et al.*, 1996). CancerVax, an allogeneic tumor cell vaccine preparation expressing the TA90 tumor antigen, was coadministered with BCG in advanced CRC patients and induced antibody responses. Peak IgM titres against TA90 were shown to be a significant predictor of OS (Habal *et al.*, 2001). Vaccination with tumor-cell lysate–pulsed DCs induced tumor-specific cellular immune responses, tumor marker and occasional clinical responses in patients with various adenocarcinomas, including pancreatic cancer (Stift *et al.*, 2003). After vaccination with GM-CSF–transfected tumor cells, the DFS seemed to increase in pancreatic carcinoma patients mounting a DTH response. IFN-γ producing CD8^{+} T-cells against an MHC class I restricted tumor antigen, mesothelin, were only detected in DTH positive patients (Jaffee *et al.*, 2001; Thomas *et al.*, 2004).

Vaccination with tumor-derived heat-shock protein gp96 also induced *in vivo* expansion of MHC class I restricted IFN-γ producing T-cells against CRC cells. Immune responses correlated with prolonged disease-free and overall survival (Mazzaferro *et al.*, 2003). Vaccination with HSP70–peptide complexes induced CTL responses in CRC and pancreatic carcinoma patients (Dong and Wei, 2005).

With regard to antigen-specific vaccination, the most commonly targeted antigens in GI malignancies are CEA, Ep-CAM, and MUC-1. Vaccination with DC pulsed with a CEA-derived peptide induced DTH, CTL, and tumor marker responses in a relatively small proportion of patients (Ueda *et al.*, 2004). Despite advanced disease status, vaccination with mutant *p53* peptide–pulsed DCs induced IFN-γ production in 45% of patients, and patients survived longer than expected (Behrens *et al.*, 2003; Ibrahim *et al.*, 2004b).

Vaccination with peptides derived from MUC-1, SART3 or other tumor antigens, linked to a carrier protein or delivered with adjuvants, have also been able to induce both humoral and cellular responses to a varying degree. DTH and CTL responses, IFN-γ and TNF-α production were detected (Goydos *et al.*, 1996; Karanikas *et al.*, 2001; Mine *et al.*, 2004). Vaccination with a human chorionic gonadotrophin (β-hCG) peptide linked to DT showed a significant survival benefit for antibody responders (Moulton *et al.*, 2002). Vaccination with a peptide derived from the antiapoptosis protein survivin induced DTH and tumor-marker responses in CRC patients. One minor clinical response was noted (Tsuruma *et al.*, 2004).

Mutant *ras* peptide combined with GM-CSF and/or IL-2 induced ras peptide-specific cellular immune responses in patients with CRC and pancreatic cancer. One CR and disease stabilization in several patients were reported (Achtar *et al.*, 2003). Immune responses were associated with a positive clinical outcome (Gjertsen *et al.*, 2001).

An anti-idiotypic antibody mimicking CEA (CeaVac) induced humoral and cellular CEA-specific responses in all CRC patients vaccinated in the adjuvant setting (Foon *et al.*, 1997, 1999). A Phase III study evaluating CeaVac in patients with metastatic CRC is ongoing. Although the anti-idiotypic antibody mimicking CD55 (105AD7) failed to influence patient survival in advanced CRC (possibly due to poor compliance) (Maxwell-Armstrong *et al.*, 2001), 105AD7 was shown to have immunological activity in the adjuvant setting. NK cell, $CD4^+$ as well as $CD8^+$ T-cell activation was detected. Enhanced lymphocytic infiltration in tumor tissues as well as tumor-cell apoptosis were observed (Amin *et al.*, 2000; Durrant *et al.*, 2000a,b; Maxwell-Armstrong *et al.*, 2001).

GM-CSF potentiated CEA-specific immune responses in patients vaccinated with a recombinant CEA protein. The magnitude of humoral and cellular immune responses was significantly higher in patients receiving the vaccine in conjunction with GM-CSF than without the adjuvant cytokine. A positive correlation between the anti-CEA IgG titre and OS was suggested (Samanci *et al.*, 1998; Ullenhag *et al.*, 2004).

A large number of patients with various adenocarcinomas, including GI malignancies have been vaccinated with Theratope (the sialyl-Tn antigen conjugated to KLH). Patients generating high IgG titres against mucin sialyl-Tn (STn) epitopes survived longer, and anti-STn IgM titre was shown to be an independent positive prognostic factor for colon cancer (MacLean *et al.*, 1996b; Reddish *et al.*, 1996). Vaccination with G17DT targeting the growth factor gastrin-17 showed a significant survival benefit for antibody responders in pancreatic carcinoma (Brett *et al.*, 2002). In a Phase II study, G17DT induced gastrin-specific antibody responses in a high proportion of gastric carcinoma patients (Gilliam *et al.*, 2004b). G17DT has also induced antigastrin antibodies when combined with irinotecan in metastatic CRC patients and the immune responses were shown to be a predictor of survival (Rocha Lima *et al.*, 2004).

DNA and viral vector-based vaccine trials have also been carried out in GI malignancies. An ALVAC-p53 vaccine induced humoral and cellular immune responses (Menon *et al.*, 2003; van der Burg *et al.*, 2002). Vaccination with plasmid DNA or recombinant Vaccinia virus encoding CEA induced limited CEA-specific immune and no clinical responses (Conry *et al.*, 1999, 2000, 2002; Tsang *et al.*, 1995). A prime-boost approach utilizing Vaccinia-CEA and ALVAC-CEA frequently induced CEA-specific CTL responses in patients with advanced CRC, and disease stabilization for up to 21 months

was observed. The CEA-specific IFN-γ response was associated with improved OS. GM-CSF significantly augmented the cellular response in contrast to IL-2 (Marshall, 2003; Marshall *et al.*, 2000; Slack *et al.*, 2001). An ALVAC-CEA vector containing the costimulatory molecule B7.1 was shown to induce CTL responses in patients with metastatic adenocarcinomas (Horig *et al.*, 2000; von Mehren *et al.*, 2000). Fewer patients receiving the cytokine adjuvant GM-CSF mounted a cellular response as compared to ALVAC-CEA alone but more patients had stable disease in the GM-CSF group (von Mehren *et al.*, 2001). The number of prior chemotherapy regimens correlated negatively, whereas the number of months from the last chemotherapy regimen correlated positively to the generation of immune response (von Mehren *et al.*, 2001). Furthermore, patients vaccinated with ALVAC-CEA containing B7.1 mounted a CTL response of a higher magnitude than patients vaccinated with ALVAC-CEA alone (Marshall *et al.*, 1999). Immunization with a dual pox virus-based vaccine (PANVAC-VF) containing the transgene for CEA, MUC-1 and a triad of costimulatory molecules (B7.1, ICAM-1, and LFA-3) was safe, and survival data in patients with metastatic pancreatic cancer is encouraging. A randomized controlled Phase III trial is in progress (Schuetz *et al.*, 2005). One patient had a CR after prime-boost vaccination with fowlpox-CEA-TRICOM and Vaccinia-CEA-TRICOM containing the transgene for CEA, B7.1, ICAM-1 and LFA-3. Several patients with PD had disease stabilizations for more than 6 months after vaccination. Tumor marker responses were observed (Marshall *et al.*, 2005). Vaccination with DC modified with fowlpox-CEA-TRICOM induced a high frequency of CEA-specific CTL precursors in patients with a minor clinical response or stable disease as compared to those who progressed. An increased frequency of $CD4^{+}CD25^{+}$ regulatory T-cells correlated inversely with the magnitude of the $CD4^{+}$-anti-CEA immune response (Morse *et al.*, 2004).

Several Phase II and a few Phase III (see the preceding paragraph) vaccine trials are ongoing in GI malignancies. Some of them are listed in the National Cancer Institute, NIH Physician's data query, and the Office of Biotechnology Activities' Human Gene Transfer Clinical Trial Database. In a Phase II randomized study using autologous tumor cell vaccine, the addition of IFN-γ and GM-CSF is compared. Another Phase II randomized trial compares vaccination with a CEA peptide in IFA versus GM-CSF. CEA-MUC-1-TRICOM plus GM-CSF is being tested in a Phase II pilot study using Vaccinia- and fowlpox-based prime-boost approach. A Phase II randomized trial is planned to compare this vaccine regime using GM-CSF or DCs as adjuvants after complete resection of hepatic metastasis. A Phase I/II study combines the 105AD7 anti-Id vaccine plus alum with allogeneic tumor-cell–based vaccines in metastatic CRC. A Phase II study with autologous tumor cell vaccine plus GM-CSF, followed by adoptive

transfer of activated T-lymphocytes and IL-2 administration in postresection stages III–IV CRC is ongoing.

Combination of immuno- and chemotherapy regimes suggest that immune responses can be induced and maintained during chemotherapy (Harrop *et al.*, 2005). Chemotherapy might also enhance cell-mediated immune responses and reduce the number of regulatory T-cells (Correale *et al.*, 2005). The ALVAC-CEA-B7.1 vaccine, with or without tetanus toxoid, is being tested in a Phase II randomized study in combination with chemotherapy versus chemotherapy alone. A randomized Phase II study is ongoing using sequential administration of Vaccinia-CEA-TRICOM and fowlpox-CEA-TRICOM plus GM-CSF with or without chemotherapy.

3. SAFETY

No short-term serious adverse events or long-term autoimmune side effects have been observed using therapeutic vaccines in over 2000 patients with GI malignancies. Six vaccine trials in GI malignancies showed no evidence of autoimmune side effects after an extended observation time (minimum 4 years) in a total of 720 patients (Durrant *et al.*, 2000b; Harris *et al.*, 2000; Hoover *et al.*, 1993; Liang *et al.*, 2003; Ullenhag *et al.*, 2004; Vermorken *et al.*, 1999).

E. Gynecologic Malignancies

The MUC-1, STn, Her-2/neu, TAG-72, and CEA antigens have been targeted in a relatively small number of patients with ovarian carcinoma (Holmberg and Sandmaier, 2004).

The cancer antigen CA125 is a clinical marker for ovarian carcinoma. An anti-idiotypic antibody mimicking CA125 (ACA125) conjugated to alum was used to vaccinate patients with advanced ovarian carcinoma. More than 100 patients were enrolled in a multicenter Phase Ib/II uncontrolled study. Patients underwent debulking surgery and chemotherapy before vaccination. Half of the patients developed an antibody response against CA125. In 27% of the patients, ADCC was detected. A statistically significant improvement of OS was noted in immune responders as compared to nonresponders (Reinartz *et al.*, 2004). Delivering the ACA125 vaccine by varying routes and doses is currently under investigation. A Phase I/II pilot study in ovarian carcinoma patients without macroscopic disease is ongoing using a p53 peptide vaccine in combination with different vaccine adjuvants (http://www.controlled-trials.com/mrct/).

The majority of cervical cancers are associated with human papilloma virus (HPV). The protein products of the early genes *E6* and *E7* of the most common high-risk HPV types 16 and 18 have been implicated in the malignant transformation and disease maintenance. These proteins provide a potential target for vaccine therapy. Patients with advanced cervical cancer were vaccinated in two Phase II pilot studies using preimmature DCs pulsed with E6 and E7 peptides respectively (Achtar *et al.*, 2005; Ibrahim *et al.*, 2005). The vaccines were well tolerated. CTL responses were induced in 65% and 43% of patients respectively. Epitope spreading against the E7 peptide occurred after vaccination with E6 peptide. The OS of patients mounting an immune response was doubled as compared to those without immune responses. A Phase II study in early cervical cancer is ongoing using recombinant Vaccinia vector expressing E6 and E7 proteins (http://www.controlled-trials.com/mrct/).

F. Lung Cancer

Several human carcinomas express aberrantly glycosylated form of the antigen MUC-1. As a tumor associated antigen MUC-1 functions as an oncogene and confers resistance to genotoxic agents. The levels of expression correlate directly with stage and inversely with prognosis (Baldus *et al.*, 2002; Gu *et al.*, 2004). MUC-1 is a mucinous glycoprotein that has truncated carbohydrate side chains increasing the exposure of the protein backbone. A synthetic lipopeptide (L-BLP25) has been synthesized in which the peptide portion is identical in sequence to a portion of the MUC-1 protein backbone. The sialated peptide (STAPPAHGVTSAPDTRPAPG-STAPP-lus (PAL) G) has been fused with monophosphoryl lipid A (adjuvant) derived of a *Salmonella* species, which binds to the Toll receptor 9 on NK cells. The lipopeptide is encapsulated into a liposomal component.

Patients with non-small cell lung carcinoma (NSCLC) stage III B locoregional (LR) disease ($n = 65$) as well as patients with stage III B with pleural effusion (PE) and stage IV disease ($n = 106$) with stable or responding disease following any first line chemotherapy were enrolled in a clinical trial. Patients were randomized to MUC-1 vaccination (L-BLP25) preceded by depletion of regulatory T-cells with a single low dose of cyclophosphamide (300 mg/m^2) (Lutsiak *et al.*, 2005) or best supportive care (BSC). In the overall analysis of all patients no difference in survival between the two groups was noted. However, in stage III B LR (limited disease burden) the survival median for vaccinated patients has not yet been reached (54% alive at 24 months) while the median survival for the BSC-group was 13.3 months (Cox analyses $p = 0.09$, HR 0.57 (95% CI (0.29–1.10)) (Fig. 1) (Murray *et al.*, 2005). Furthermore, quality-of-life (QoL) assessment indicated a

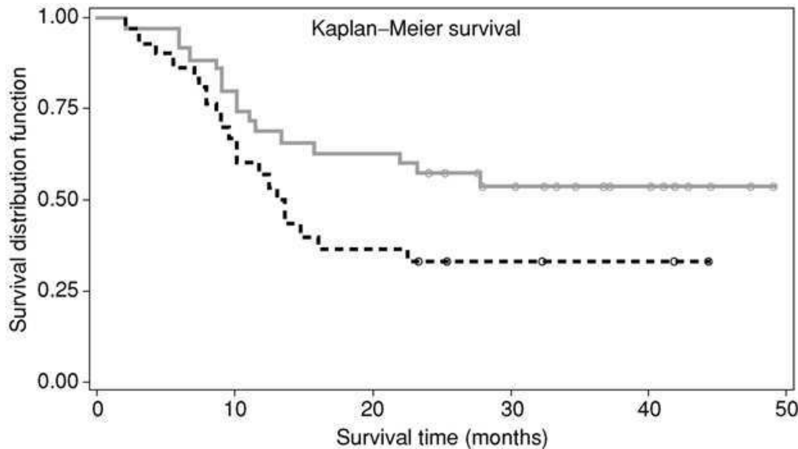

Fig. 1 Kaplan–Meir survival curve of NSCLC patients stage IIIB LR ($n = 35$) vaccinated with MUC-1 (—) compared to the control group receiving BSC (- - -) ($n = 30$) (significance levels: adjusted Cox analyses $p = 0.0924$; unadjusted log-rank $p = 0.0731$) (Murray *et al.*, 2005).

better QoL evaluated to the baseline for patients with stage III B LR treated with the vaccine as compared to BSC both at 19 weeks and at 31 weeks of follow up (p <0.0.5 and <0.01, respectively) (Soulieres *et al.*, 2005). This very encouraging vaccine preparation is now in a Phase III study of NSCLC patients and will also be explored in other diseases as myeloma.

G. Breast Cancer

There are very few vaccine trials in breast cancer that have demonstrated significant clinical benefit. This is a surprising revelation in view of the fact that many potential TAAs have been defined in breast cancer. These include MUC-1, HER-2/neu, CEA, p53, STn, the CT antigens MAGE, BAGE, GAGE, and XAGE, and the putative universal tumor antigens survivin and telomerase (hTERT) (Emens and Jaffee, 2003). Several clinical trials on vaccination in breast cancer patients have demonstrated the induction of immune responses to the TAAs (Adluri *et al.*, 1999; Reece *et al.*, 2003; Svane *et al.*, 2004) in the absence of any major clinical responses. A study by Murray *et al.* (2002) demonstrated a trend toward prolongation of TTP ($p = 0.06$) in a small number of patients vaccinated with a Her-2/neu peptide and GM-CSF. A study utilizing DC–tumor cell hybrids demonstrated significant regression of tumor in 2/16 breast cancer patients and disease stabilization in 1 patient (Avigan *et al.*, 2004a).

Arguably, the largest vaccine trial in breast cancer was performed using the Theratope vaccine (THERATOPE, Biomira Inc., Edmonton, Alberta, Canada). The vaccine consisted of the MUC-1 STn carbohydrate epitope conjugated to KLH together with the adjuvant DETOX-B (Enhanzyn, Corixa Corp., Seattle, WA, USA). Phase II trials of Theratope in breast cancer patients showed demonstrable immunologic activity and clinical benefit (Ibrahim and Murray, 2003; Miles *et al.*, 1996). In the study by Miles *et al.* (1996), 18 patients received 4 injections of the vaccines. Two patients demonstrated minor responses and 5 patients demonstrated stable disease. Some of the patients receiving the vaccine had received cyclophosphamide (300 mg/m^2) for 3 days prior to vaccination. Based on these results a multicenter, randomized, double-blind Phase III study of 1028 patients with metastatic breast cancer was performed. The patients were entered into the trial following primary therapy and had no evidence of disease or had no PD. All patients received a single infusion of cyclophosphamide before vaccine (or control). Five hundred twenty-three patients were randomized to the THERATOPE (STn-KLH) vaccine arm and 505 patients to the control arm (KLH alone). Comparable numbers of patients in both arms received concurrent hormone therapy ($n = 180$; T/$n = 170$ C) (Ibrahim *et al.*, 2004a; Mayordomo *et al.*, 2004). No differences in TTP or OS emerged in an intent-to-treat analysis. However, a subgroup analysis revealed that there was a trend (Cox $p = 0.22$) toward improved TTP in the subgroup of the patients who concurrently received hormone therapy. Median OS of patients receiving hormone therapy who generated a high-titer antibody response was 41.1 months versus 25.4 months in patients on hormone therapy who did not develop high titre of antibodies against the STn antigen ($p = 0.01$).

VI. INCORPORATION OF VACCINE TREATMENT INTO CHEMOTHERAPY REGIMENS

The clinical trials discussed in the previous section provide evidence for the premise that vaccine-based therapies can provide clinical benefit in several malignancies. It is also clear that this approach to therapy may have greater efficacy when utilized in settings of low-tumor burden. Combining anticancer vaccines with chemotherapy can potentially produce a synergistic effect by achieving a favorable tumor reduction, providing the optimum immunological milieu by depletion of regulatory T-cells (discussed in a subsequent section) and enhance the magnitude and duration of the immune response generated by the vaccine. The chemotherapeutic agent most extensively studied in conjunction with immunotherapy is cyclophosphamide. As early as 1986, Berd *et al.* (1986) demonstrated that metastatic melanoma

patients, who received 300 g/m^2 of cyclophosphamide 3 days prior to receiving vaccination therapy, tended to have DTH responses of greater magnitude and a higher frequency of clinical responses. Tumor-infiltrating lymphocytes (TILs) expanded *ex vivo* were reinfused into patients with metastatic melanoma following partial lymphodepletion using fludarabine and cyclophosphamide (Dudley *et al.*, 2002). Six of 13 patients demonstrated objective clinical responses and 5/13 had accompanying immunopathologic manifestation such as vitiligo and, in 1 case, anterior uveitis.

In addition to cyclophosphamide, doxorubicin and taxanes have been tested in animal experiments for "conditioning" prior to administration of therapeutic vaccines (Eralp *et al.*, 2004; Machiels *et al.*, 2001; Nigam *et al.*, 1998). In these animal models, the drugs enhanced T-cell responses postvaccination, enhanced the efficacy of antitumor responses and facilitated the reversal of tolerance to TAAs. However, none of the drugs had any significant potentiating effect when administered after the administration of the vaccine, underlining the importance of the schedule of administration when combining vaccines with chemotherapy.

It can be envisioned that combinations of chemotherapy and vaccine will increasingly replace the use of vaccines as a stand-alone single agent for adjuvant therapy of patients who have been debulked of their tumor by conventional therapeutic approaches. It may be speculated that restricted radiation therapy to sentinel draining lymph nodes prior to administration of vaccines may also be one approach to achieve localized lymphodepletion without the associated adverse effects of systemic chemotherapy.

VII. RELEVANCE OF REGULATORY T-CELLS FOR VACCINE THERAPY OF CANCER

Tumor cells have evolved different mechanism to evade immune surveillance. Counteracting tumor escape mechanisms is a key issue and a great challenge for successful immunotherapy. Both systemic immunological factors as well as the local tumor microenvironment play important roles in immune evasion of cancer.

Much attention has been paid to CD4$^+$CD25$^+$ regulatory T (Treg) cells that are known to be essential to maintain peripheral tolerance, that is, suppress self-reactive T-cells (Sakaguchi, 2005). As most tumor antigens targeted by cancer immunotherapy are nonmutated self-antigens, Treg cells may play a role in regulating vaccine-induced immune responses against tumor antigens. Vaccination strategies against cancer may induce the expansion of Treg cells (Chakraborty *et al.*, 2004).

Besides naturally occurring and induced Treg cells, regulatory T-cells of the immune system include T regulatory 1 (Tr1) and T helper 3 (Th3) cells. These cells do not possess the $CD4^{+}CD25^{+}$ phenotype, but are characterized by their cytokine profile (Bluestone, 2005; Wang and Wang, 2005).

$CD4^{+}$ Treg cells constitutively express the cell surface molecules CD25 (IL-2Rα), glucocorticoid-induced tumor-necrosis factor-receptor related protein (GITR), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and CD62L. They also express an intracellular protein, forkhead box P3 (FOXP3), which controls the development and the function of Treg cells (Antony and Restifo, 2005; Bluestone, 2005). Hitherto, no Treg specific cell surface receptor has been identified. These molecules can also be expressed, although transiently, by activated responder T-cells. Treg cells are best defined by their immunosuppressive function (IL-10, TGF-β production, inhibition of immune effector cells and so on). It has also been shown that the CD4 homolog lymphocyte activation gene-3 (LAG-3) was selectively upregulated by Treg cells (Huang *et al.*, 2004). Signaling through LAG-3 may exert suppressive functions through Treg cells (Macon-Lemaitre and Triebel, 2005; Triebel, 2003).

Tumor-specific Treg cells suppressing DC maturation as well as $CD4^{+}$ and $CD8^{+}$ effector functions has been shown to accumulate in the peripheral blood and in the tumor microenvironment of patients with various tumors (Wang and Wang, 2005). Trafficking of Treg cells to the tumor site is probably mediated by the production of the chemokine CCL22, produced by tumor cells and macrophages in the tumor microenvironment (Curiel *et al.*, 2004). Increased frequency of Treg cells has been reported in gastric, esophageal, colorectal, pancreatic, breast, hepatocellular, ovarian and lung carcinoma as well as CLL (Beyer *et al.*, 2005; Bueter *et al.*, 2005; Curiel *et al.*, 2004; Ichihara *et al.*, 2003; Liyanage *et al.*, 2002; Ormandy *et al.*, 2005; Unitt *et al.*, 2005; Woo *et al.*, 2001, 2002). The frequency of Treg cells seem to increase in advanced disease stage or in untreated/progressing patients (Beyer *et al.*, 2005; Bueter *et al.*, 2005). These cells were shown to inhibit effector T-cell functions. Failure of imatinib mesylate to exert antitumor activity in patients with gastrointestinal stromal tumors (GIST) was attributed to the presence of high number of Treg cells inhibiting NK cell activity (Zitvogel *et al.*, 2005). Accumulation of Treg cells at the tumor site is associated with poor survival (Curiel *et al.*, 2004).

Pretreatment of cancer patients with Treg inhibitors may enhance the efficacy of immunotherapy. This has been demonstrated in several preclinical studies (Antony and Restifo, 2005). Cyclophosphamide has been shown to inhibit immune suppressor functions and to deplete Treg cells as well as augment antitumor immune effector functions (Loeffler *et al.*, 2005; Mitchell, 2003). The molecular mechanism behind this effect has been elucidated (Loeffler *et al.*, 2005).

Patients with advanced breast cancer receiving low-dose intravenous cyclophosphamide prior to vaccination with Theratope STn-KLH had a statistically significantly improved survival as compared to patients receiving oral cyclophosphamide alone or vaccine alone. Vaccine-induced immune responses were augmented in patients pretreated with intravenous cyclophosphamide (MacLean *et al.*, 1996a; Miles and Papazisis, 2003). A Phase II study demonstrated that pretreatment of advanced pancreatic carcinoma patients with immunopotentiating dosages of cyclophosphamide prior to vaccination with a whole-tumor cell vaccine had a significant effect on survival. More than twice as many patients who received cyclophosphamide plus vaccine reached PFS at 16 weeks compared to patients receiving the vaccine alone (Laheru and Jaffee, 2005). In line with the observation that immunopotentiating dosages of cyclophosphamide reduce Treg functions, the inhibitory function of Treg cells was also decreased after fludarabine treatment of patients with B-CLL (Beyer *et al.*, 2005).

Besides combining immunotherapy with low-dose chemotherapy, or using other lymphodepleting regimens, attempts have been made to selectively deplete Treg cells. In animal models, it has been demonstrated that depletion of CD25-expressing cells by anti-CD25 mAb promotes tumor rejection (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999). A humanized anti-CD25 mAb has been approved for human use. Another FDA approved Treg depleting drug is DAB(389)IL-2 (ONTAK) (IL-2 conjugated to active DT), which induces apoptosis in $CD25^{+}$ cells (Foss, 2000). Although these drugs may have some potential to augment anti-tumor immunity in conjunction with cancer vaccines, their effects on Treg cells and on effector T-cell populations have not been elucidated warranting further preclinical and clinical studies.

CTLA-4 is a negative regulator of T-cell activation. Blockade of the regulatory effects of CTLA-4 may potentiate antitumor responses. The first study showing that administration of antibodies to CTLA-4 results in tumor rejection was published by Leach *et al.* (1996). In vaccinated cancer patients, treatment with inhibitory mAbs against CTLA-4 not only augmented antitumor immunity but also resulted in significant autoimmune side effects (Hodi *et al.*, 2003; Phan *et al.*, 2003).

IL-6 may play a role in blocking the suppressive activity of Treg cells by rendering effector cells refractory to Treg suppressive activity (Pasare and Medzhitov, 2003). Another potential way of blocking Treg function may be blockage of suppressive cytokine signaling pathways or administration of anti-CCL22 antibodies to reduce Treg trafficking to the tumor site (Curiel *et al.*, 2004). Manipulating LAG-3 signaling may also be an interesting alternative to inhibit Treg suppressor activity (Macon-Lemaitre and Triebel, 2005; Triebel, 2003).

Taken together, combining cancer vaccines with direct or indirect inhibitors of Treg suppressive function may be vital for successful cancer vaccine therapy.

VIII. IMMUNOLOGICAL ADJUVANTS FOR CANCER VACCINES

It is considered to be an established fact at present that immunological adjuvants are indispensable for the induction of immune responses of sufficient magnitude and duration against TAAs to have a clinical benefit. The most commonly used, noncytokine, adjuvants in the vaccine trials include alum, BCG and IFA (Belardelli *et al.*, 2004; Dredge *et al.*, 2002). While these adjuvants have long standing history of use and excellent safety profiles, repeated use of BCG can cause severe local ulceration in a significant number of patients (Vermorken *et al.*, 1999). Furthermore, alum favors the cellular response toward a Th_2 type (Brewer *et al.*, 1999), which may not be the optimal immune response type for rejection of tumors. Other adjuvants that have been used in clinical trials include QS-21, a highly purified saponin derivative from the *Quillaja saponaria* Molina tree and DETOX-B™ (Enhanzyn, Corixa Corp., Seattle, WA, USA) that consists of an oil droplet emulsion of monophosphoryl lipid A, lecithin and mycobacterial cell wall skeleton. Among the recombinant cytokines, GM-CSF, IL-2 and IL-12 have been used as immunological adjuvants (Belardelli *et al.*, 2004; Dredge *et al.*, 2002). GM-CSF has probably been the cytokine most frequently used as an adjuvant. GM-CSF is known to be a pleiotropic cytokine that supports the differentiation of myelomonocytic cells from hematopoietic precursors, promotes differentiation and functional activation of DCs, increases MHC class I expression and antigen-presentation function (Fischer *et al.*, 1988; Hamilton and Anderson, 2004). However, the universal applicability of GM-CSF as an adjuvant for cancer vaccines has been questioned and may be related to dose, time and frequency of administration (Belardelli *et al.*, 2004). It is known that several tumors produce GM-CSF in an autocrine manner and the high dose of GM-CSF may result in immune dysfunction (Bronte *et al.*, 1999; Rokhlin *et al.*, 1996; Tsuchiya *et al.*, 1988). Another cytokine that holds promise as a potential adjuvant for vaccine therapy is IFN-α (Belardelli *et al.*, 2002). IFN-α has been used for the treatment of several malignancies including hairy cell leukemia, chronic myelogeneous leukemia (CML), melanoma and renal cell carcinoma, and its immunopotentiating and antitumor effects in these diseases are documented. Studies in animal models and vaccination with tumor cells that have been genetically modified to express IFN-α have demonstrated its adjuvant-like properties for the induction of antitumor responses (Belardelli *et al.*, 2002). IFN-α is also known to facilitate the maturation of DC (Radvanyi *et al.*, 1999).

Among a large number of chemical adjuvants that have been subjects of preclinical and clinical testing, two that are worthy of mention are deoxycytidyl-deoxyguanosin oligodeoxynucleotides (CpG ODNs) and thalidomide.

CpG oligonucleotides are known to trigger Toll-like receptor 9, resulting in DC maturation that can enhance immunogenicity of peptide-based vaccines in mice. Speiser *et al.* (2005) have performed a clinical trial using CpG ODNs as adjuvant for immunizing melanoma patients with a Melan-A peptide. Eight patients who had the vaccine and CpG adjuvant had immune responses that were an order of magnitude higher than 8 patients who received the vaccine alone. Thalidomide is a drug that has been shown to be clinically useful in several diseases including cancer. However, the teratogenic effects of the drug have greatly limited its use. Clinical activity of this drug is attributed to a wide range of immunological and nonimmunological properties including the stimulation of T-cells to produce IL-2. Newer analogs of thalidomide are being tested for maximizing the immunostimulatory properties while decreasing the side effects. Preclinical studies in animal models have demonstrated that such analogs can enhance immune responses to autologous tumor and promote the secretion of Th-1 cytokines (Dredge *et al.*, 2002).

The use of DC as cellular adjuvants for cancer vaccination has been clearly established in a variety of *in vitro* experiments, animal studies and clinical trials. Tables I–VI summarize the clinical trials in various malignancies in which DCs have been used both as a delivery vehicle as well as a cellular adjuvant. The use of DC for cancer vaccine therapy has been greatly facilitated by the delineation of methods for generating large numbers of DC from $CD14^+$ monocyte precursors. However, the generation of adequate number of DC loaded with the relevant antigen(s) under good manufacturing practice (GMP) conditions is not without complications. An array of preclinical and clinical data also suggest that terminal maturation of DC using agents like TNF-α, LPS or recombinant CD40 ligand is essential for optimum *in vivo* responses following DC-based vaccination (Jeras *et al.*, 2005).

The collective data on immunological adjuvants for cancer vaccines does not provide any distinct indication as to which adjuvant may be the most suited for a particular form of vaccine therapy. Logistical limitations restrict the parallel direct comparison of the various adjuvants in randomized clinical trials with substantial numbers of patients in each arm. In the absence of this clinical information, extrapolation of data from animal models is the only pointer currently available for selecting a particular adjuvant for a vaccination study.

IX. CONCLUSIONS

There are a few significant observations that can be made from the cumulative data of all the trials summarized previously. First, vaccine therapy against cancer, in general, has very low-associated toxicity. Adverse effects

are normally limited to local reactions, transient mild fever, erythema, and systemic reactions are generally rare. Second, the widely speculated concerns with regard to induction of crippling autoimmune reactions appear to be largely unfounded. Several of the melanoma trials have reported vitiligo as an autoimmune consequence of vaccination with melanoma antigens. Immunopathologic reactions of any greater severity have been anecdotal at best. A clinical follow up of colorectal cancer patients over 76 months after vaccination with CEA did not reveal any autoimmune manifestations despite the generation of significant immune responses (Ullenhag *et al.*, 2004). Data obtained from this and other studies strongly suggest that vaccination against a cancer-associated molecule can be safely performed with minimal associated risk of acute or chronic autoimmune reactions. However, it must be borne in mind that few trials have reported major clinical responses. Discovery of vaccination strategies that yield a higher frequency and magnitude of immune responses may be associated with greater therapeutic effectiveness but also more frequent autoimmune reactions.

The third observation and perhaps the most essential to address is the question of clinical efficacy. Several of the clinical trials listed have reported clinical responses often in patient populations whose disease have progressed on standard treatment or have a large tumor burden. Vaccine therapy has a high-QoL index as well as low toxicity which extend vaccine therapy to aged or debilitated patients, otherwise ineligible for more intensive chemoradiotherapy regimens. These positive attributes are counterpoised against the unambiguous fact that clinical responses with vaccine therapy have been documented only in a minor fraction of vaccinated patients. To improve the clinical efficacy of vaccine therapy there is a need for a two-pronged strategy, that is, eliciting immune responses of greater therapeutic efficacy and selection of patients who have better odds of benefiting from vaccine therapy. With regard to the former, the trend noted from clinical trials is that polyclonal vaccines, such as modified autologous or allogeneic tumor cells and multiple (at least four or more) vaccinations, tend to produce superior clinical responses. The other observation is that immune responses and clinical responses are often discrepant. This has raised questions, albeit intensely controversial, on the value of clinical studies reporting immune responses as "surrogate end points" without addressing the issue of clinical effectiveness (Rosenberg *et al.*, 2004). However, there are other clinical studies that do show a correlation between immune and clinical responses (Mosolits *et al.*, 2005). Confirmation of these results in extended prospective studies would represent a significant advancement in vaccine therapy since immune responses could be used as a surrogate endpoint especially for evaluating vaccine therapy in the adjuvant settings.

Patient selection may be a critical factor in ultimately improving the clinical effectiveness of vaccine therapy. The poor clinical outcome in many vaccine trials can undoubtedly be attributed to the fact that the accrued patients had advanced disease and had exhausted practically every other therapy option. It is an unjustified expectation that vaccine therapy can produce significant clinical responses in this group of patients. It is also clear that patients with a large tumor burden have much less clinical benefit from vaccine therapy compared to patients who receive vaccines as adjuvant therapy or in the MRD settings. It is not inaccurate to say that reports of complete remissions following vaccination in patients with bulky disease are at best anecdotal. In contrast, several clinical trials in which vaccine therapy has been administered as adjuvant therapy or under conditions of MRD have reported statistically significant improvement in DFS, PFS and overall RR.

A significant issue relating to the design of clinical trials relates to the number of patients treated with any given schedule. In our survey of the literature, we have often found vaccine trials in which patients have been divided into several groups that have received different doses, number of vaccinations or adjuvants. The consequence of such a design is that the numbers of patients within each group have been greatly reduced. Clinical responses within such small groups of patients are often difficult to assess or evaluate for statistical significance. Trial designs with larger number of patients and fewer arms may yield information of greater impact and utility.

It is our opinion that over the next 10 years the results of ongoing Phase III trials as well as novel immunological adjuvants and criteria for patient selection will improve the therapeutic outcome of vaccine therapy. It is likely that this therapeutic modality will be an established form of targeted therapy and integrated into the standard therapeutic arsenal for the care of patients with cancer.

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Immunodominance and Immunodomination: Critical Factors in Developing Effective CD8^{+} T-Cell–Based Cancer Vaccines

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The focusing of cellular immunity toward one, or just a few, antigenic determinant, even during immune responses to complex microorganisms or antigens, is known as immunodominance. Although described in many systems, the mechanisms of determinant immunodominance are only just beginning to be appreciated, especially in relation to the interplay between T cells of differing specificities and the interactions between

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T cells and the antigen-presenting cells (APCs). The outcome of these cellular interactions can lead to a form of immune suppression of one specificity by another—described as "immunodomination". The specific and detailed mechanisms involved in this process are now partly defined. A full understanding of all the factors that control immunodominance and influence immunodomination will help us to develop better viral and cancer vaccines. © 2006 Elsevier Inc.

I. INTRODUCTION

The terms "immunodominance" and "immunodominant" were originally used to describe prominent humoral responses to various antigens in the late 1960s and early 1970s (Benacerraf and McDevitt, 1972; Curtiss and Krueger, 1975; Johnston and Simmons, 1968). This was not long before the discovery of major histocompatibility complex (MHC) restriction (Zinkernagel and Doherty, 1974) but a decade prior to the discovery that antigenic peptides are presented by MHC molecules (Allen *et al.*, 1984; Bixler and Atassi, 1983; Townsend *et al.*, 1986). It was originally noticed that the cytotoxic T-lymphocyte (CTL) responses to antigens, such as the minor histocompatibility antigen (MiHA) H-Y and lymphocytic choriomeningitis virus (LCMV), were restricted by a single or just a few H-2 haplotypes (Bevan, 1975; Zinkernagel and Doherty, 1975). More detailed studies of CTL responses to mouse sarcoma virus (MSV) (Gomard *et al.*, 1977) and influenza A viruses (IAV) (Doherty *et al.*, 1978) revealed that responses were strongly linked to mouse *H-2* alleles. Using recombinant inbred mouse strains expressing different *H-2* genes and then transplanting lymphomas as targets, Gomard *et al.* (1977) identified $H\text{-}2K^d$ as the major restriction allele for anti-MSV (mouse sarcoma virus) CTL responses whereas the $H\text{-}2D^d$ allele was not involved in these responses. Doherty *et al.* (1978), using similar mouse strains and fibroblast cell lines as CTL targets, defined H-2K^b as a "nonresponder" *MHC* gene locus in anti-IAV responses despite this being a responder in antirecombinant vaccinia virus (rVV) responses. These observations were made as part of the demonstration of MHC-restriction of CTL responses and constituted the first indication of the *H-2* allele-associated immunodominance. It is now well established that antigen-specific immunodominant responses are linked to particular *MHC* alleles (Belz *et al.*, 2000a; Schirmbeck *et al.*, 2002; Tourdot and Gould, 2002; Tussey *et al.*, 1995), known initially as "immune response (*Ir*)" genes (Solinger *et al.*, 1979; Zinkernagel *et al.*, 1978).

From such beginnings, it was soon found that even within the strains that responded to a given virus, the CTL responses were highly focused on just a few proteins and often on a single polypeptide (Bennink and Yewdell, 1988; Bennink *et al.*, 1987). These "immunodominant responses" were traced to

a subregion of the protein antigen (Lamb and Green, 1983) around the time when the peptide nature of antigenic determinants was being elucidated (Allen *et al.*, 1984; Bixler and Atassi, 1983; Townsend *et al.*, 1986).

Nearly simultaneously, Sercarz and colleagues studying $CD4^{+}$ T-cell responses to an artificial antigen, hen egg lysozyme (HEL), observed that the $CD4^{+}$ T-cell responses to some determinants were easily detectable whereas responses to other determinants were much smaller and consequently harder to demonstrate. There were yet other determinants that were not detected under normal circumstances unless very high levels of antigen were used for priming. Determinants involved in detectable responses were defined as either immunodominant determinants (IDDs) or subdominant determinants (SDDs) depending upon their reproducibility and magnitude; the third category of undetectable determinants were called cryptic determinants (Sercarz *et al.*, 1993), a term derived from their earlier studies on anti-HEL B-cell responses and autoimmune responses (Furman and Sercarz, 1981; Wicker *et al.*, 1984a,b).

Similar phenomena were observed in mouse models of IAV, LCMV, herpes simplex virus (HSV), and *Listeria monocytogenes* (*LM*) infection. In a given mouse strain, the major responses to these pathogens were often directed toward a single IDD (Table I). However, progress in unraveling the factors that controlled immunodominance was limited by the lack of tools for immune monitoring. The field accelerated when novel methods for enumerating $T_{CD8^{+}}$ arrived in the late 1990s. These technologies included MHC-peptide tetramers (Altman *et al.*, 1996) and intracellular cytokine staining (ICS) of antigen-specific T cells (Jung *et al.*, 1993). These new techniques allowed accurate enumeration of specific T cells in combination with their surface and functional markers in the absence of *in vitro* T-cell expansion (Butz and Bevan, 1998; Flynn *et al.*, 1998; Murali-Krishna *et al.*, 1998). Moreover, the newer methods were up to 100-fold more sensitive in detecting Ag-specific $T_{CD8^{+}}$ than the established limiting dilution analysis (LDA) that measured CTL precursor frequencies indirectly through target killing (Lalvani *et al.*, 1997; McMichael and O'Callaghan, 1998; Murali-Krishna *et al.*, 1998). This observation suggested that historical estimates of Ag-specific $T_{CD8^{+}}$ numbers might have been drastically underestimated. Since then, many experiments have reassessed these systems and revised the estimates of Ag-specific $T_{CD8^{+}}$ numbers (Flynn *et al.*, 1998; Murali-Krishna *et al.*, 1998). The newer technologies were not only more sensitive in detecting the immunodominant $T_{CD8^{+}}$ but in particularly the subdominant $T_{CD8^{+}}$ were more readily appreciated. It was then possible to quantitate $T_{CD8^{+}}$ responses reproducibly and define "immunodominance hierarchies" (Belz *et al.*, 2000a,b; Chen *et al.*, 2000).

While tetramers, or multimers of MHC/peptide complexes, allowed assessment of specific T cells at various stages of their development and

differentiation following antigen-specific activation, another key technological advance was the development of T-cell receptor (TCR) transgenic (Tg) mice expressing a "monoclonal" T-cell repertoire (Berg *et al.*, 1988; Bluthmann *et al.*, 1988). Transfer experiments involving TCR Tg-T cells enabled these T cells to be tracked directly *ex vivo* at early stages of the immune response and also permitted the *in vivo* study of T cell–T cell (T-T) and/or T cell–antigen-presenting cell (T-APC) interactions.

A large body of work has since shown how immunodominance might be controlled or influenced by the steps involved in the creation of antigenic peptides (Ag-processing) and their presentation by the MHC molecules on the APC (Ag-presentation). Therefore, in this chapter, we begin by addressing the potential contribution of antigen processing and presentation to the establishment of immunodominance hierarchies; we then focus on T-T and T-APC interactions; we next discuss the positive and negative roles that immunodominant T_{CD8^+} play during viral and tumor escape of immune surveillance. Finally, we explore the possibility of better vaccine development, utilizing the knowledge accumulated from studying immunodominance. We confine our discussions to MHC class I (MHC-I)–restricted T_{CD8^+} responses as MHC class II-restricted immunodominance has been reviewed elsewhere (Latek and Unanue, 1999; Sercarz and Maverakis, 2003).

II. THE PHENOMENON: IMMUNODOMINANCE

Immunodominance has been mostly observed in antiviral and antibacterial immune responses, both in mouse models and in human diseases. Table I shows a list of the best-studied antigen systems in mouse models illustrating the generality of well-focused T_{CD8^+} immune responses in antiviral and antibacterial immunity. Compared to laboratory mouse models, human populations express many different human leukocyte antigen (HLA) haplotypes and are repeatedly exposed to various pathogens (some simultaneously). Therefore, immunodominance hierarchies are generally not as easily dissected in humans, and quite often the immunodominant T_{CD8^+} responses cannot be simply predicted based on HLA genotype (Betts *et al.*, 2000; Day *et al.*, 2001) because the impact of HLA allotypes (alleles) on immunodominance hierarchy is context dependent.

Immune responses specific to tumor antigens are relatively poorly understood. Tumor-specific immunodominance has not been well characterized except for tumor antigen systems in which viral antigens become "neo" antigens such as the simian virus 40 (SV40) antigen system in mouse models (Jennings *et al.*, 1988; Rawle *et al.*, 1988) and Epstein–Barr Virus (EBV)

Table I Summary of Major Studied Immunodominance Systems in Mice

Pathogen	Gene number	Genome length	Number of proteins	IDD/SDD in C57BL/6 mice	IDD/SDD in BALB/c mice	References
SV40	1	5243 bp	7	1 $K^b/\geq 3$	1 $L^d/\geq 2$	Deckhut *et al.*, 1992; Newmaster *et al.*, 1998; Schirmbeck *et al.*, 1996
IAV	8	13,585 bp	11	2 $D^b/\geq 16$	1 $K^d/\geq 5$	Deng *et al.*, 1997; Zhong *et al.*, 2003
LCMV	2	10,056 bp	4	~(2–3) $D^b/\geq 3$	1 $L^d/\geq 2$	van der Most *et al.*, 1997, 1998
Pichinde virus (PV)	2	10,416 bp	4	2 D^b, 1 $K^b/\geq 1$	Not known	Brehm *et al.*, 2002
rVV	1	191,636 bp	~250	1 $K^b/\geq 4$	1 $L^d/\geq 2$	Tscharke *et al.*, 2005; Tscharke, personal communication
MHV	1	31,526 bp	11	1 $D^b/\geq 1$	1/not known	Bergmann *et al.*, 1993, 1999; Pewe *et al.*, 1996
HSV	1	152 Kb	~(30–35)	1 K^b/not known	Not known	Bonneau *et al.*, 1993; Wallace *et al.*, 1999
Sendai virus	1	15,384	6	1 K^b/not known	Not known	Kast *et al.*, 1991
VSV	1	11,161	5	1 K^b/not known	Not known	Van Bleek and Nathenson, 1990
LM	Single chromosome	2.94 Mb	~2600	Not known	1 $L^d/\geq 3$	Pamer, 1994

Due to limited space in this table, the IDD and SDD sequences and some of the original references are not listed. It is highly likely that in each of these systems other SDD exist, so the symbol "≥" is used to indicate that likelihood. Novel IDD(s) may also remain unidentified for some systems. The immunodominance hierarchy is ultimately determined as a ranking of the number of detected antigen-specific T cells and is functionally based.

(Khanna and Burrows, 2000; Rickinson and Moss, 1997) and human papilloma virus (HPV) (Frazer, 2004) infections in humans. Most nonviral tumor antigens are self-antigens differentially expressed by tumors and normal tissues (Levitsky, 2000; Romero *et al.*, 2002). Accordingly, the T_{CD8^+} response hierarchy to tumor-associated self-Ags may manifest differently to that of antiviral immune responses due to the shaping of the T_{CD8^+} repertoire by self-tolerance mechanisms.

III. THEORETICAL CONTRIBUTIONS OF ANTIGEN PROCESSING AND PRESENTATION TO IMMUNODOMINANCE

The demonstration that antigen presentation occurs in the form of short peptides bound to MHC-I molecules (Allen *et al.*, 1984; Bixler and Atassi, 1983; Bjorkman *et al.*, 1987; Townsend *et al.*, 1986) led to the elucidation of the detailed biochemistry and pathway of this process, which has been reviewed extensively (Kloetzel, 2004b; Trombetta and Mellman, 2005; Van Kaer, 2002; Yewdell and Bennink, 1999; Yewdell *et al.*, 1999) and will only be briefly addressed here.

The steps involved in antigen processing and presentation predict a number of factors that might lead to immunodominant T_{CD8^+} responses. First, it is essential that a determinant is generated efficiently by the immunoproteasomes, which are constitutively expressed by professional APCs such as dendritic cells (DCs) (Macagno *et al.*, 2001). DCs are believed to be the main priming APC that activates naïve T_{CD8^+} (Belz *et al.*, 2004b; Carbone and Heath, 2003; Jung *et al.*, 2002; Norbury *et al.*, 2002; Probst and van den Broek, 2005; Smith *et al.*, 2003), although there is recent evidence that macrophages may also prime naïve T_{CD8^+} (Pozzi *et al.*, 2005). The immunoproteasome differs from the constitutive proteasome by the presence of three γ-IFN–inducible subunits, named LMP2, LMP7, and MECL-1, that replace the constitutive δ, X, and Z subunits, respectively (Kloetzel and Ossendorp, 2004). The constitutive, or housekeeping, proteasome and the immunoproteasome are believed to degrade protein or polypeptide antigens with different preferences (Gaczynska *et al.*, 1994) to generate short peptides with carboxyl termini most suitable for direct MHC-I loading. Second, precursor peptides containing T_{CD8^+} determinants must be transported into the endoplasmic reticulum (ER) by the membrane-bound TAP heterodimer (transporter associated with antigen processing) prior to their association with the nascent MHC-I molecules in the peptide-loading complex (McCluskey *et al.*, 2004). Third, the loaded peptide has to possess a suitable binding affinity for the MHC-I heavy chain that is over a

certain threshold to enable the MHC/peptide/β-2 microglobulin tricomplex to be released by the peptide-loading complex for surface expression (Howarth *et al.*, 2004; Momburg and Tan, 2002). Finally, these complexes need to be recognized by T_{CD8^+} bearing the correct antigen-specific TCR. The T_{CD8^+} responses to IDDs and SDDs are not only dictated by the sum of the above-mentioned factors but also the T-T and T-APC interplay (see later).

The potential discrepancy between *in vitro* assessment and the *in vivo* reality of antigen processing and presentation has limited our understanding of immunodominance. Our current understanding of immunodominance is potentially biased given that much of it is based on the results of *in vitro* assays. For example, the information so far derived from *in vitro* studies has largely relied upon direct antigen presentation. It appears likely that cross-priming/presentation plays an essential role in guiding immunodominance *in vivo*—a point that is probably underrecognized (Chen *et al.*, 2004a; Nevala *et al.*, 1998). The other critical block in the better understanding of immunodominance is the inability to directly measure naïve T_{CD8^+} precursor frequencies—even for the most abundant immunodominant T_{CD8^+}—simply because it is beyond the detection limit of current technologies. Notwithstanding these issues, there is good evidence indicating that the immunodominance hierarchy in various systems is not imprinted in the precursor or naïve T_{CD8^+} repertoire. Rather, immunodominance is determined during the course of the immune response and this can be demonstrated experimentally. For example, Sandberg *et al.* (1998) engineered five known IDDs together as a linear artificial antigen and showed that a novel immunodominance hierarchy emerged following immunization of mice with the construct. In another system involving IAV-infected C57BL/6 (B6) mice, T_{CD8^+} responses to SDD $PB1_{703-710}$ were dependent on the route of infection. For instance, when mice were infected intranasally the $PB1_{703-710}$ response was quite prominent (Belz *et al.*, 2001) but when an intraperitoneal route was used the response was negligible (Chen *et al.*, 2001). These findings highlight the complex basis of immunodominance *in vivo*.

A. IDDs Are Normally Efficiently Processed

Many predicted peptides are perfectly immunogenic in mice when administered as synthetic peptides, yet are never observed as part of the natural immune response toward the parent antigen, either as part of an antiviral or antitumor response. This suggests that such determinants are not naturally generated by the antigen-processing machinery (Chen *et al.*, 2000; Deng *et al.*, 1997; van der Most *et al.*, 1997; Zhong *et al.*, 2003) and demonstrates an inherent selectivity of Ag-presentation pathways. There are now emerging rules for predicting antigen-processing or proteasome-mediated

cleavage patterns for any given protein sequence (Kuttler *et al.*, 2000). The antigen "cleavage motifs" used by the proteasomes still require further refining and similar prediction motifs may be needed in the future to cover other cellular proteases such as tripeptidyl-peptidase II (Kloetzel, 2004a). However, the benefit of combining both peptide-binding motifs and cleavage motifs should allow more accurate prediction of the potential antigen-processing outcome for a particular antigen (Tenzer *et al.*, 2005).

The failure to generate a determinant or a given peptide naturally could have two main potential explanations, assuming the precursor protein or polypeptide is produced at a sufficiently abundant level. One possibility is that unsuitable flanking sequences prevent some determinants from being generated by the proteolytic apparatus (Eisenlohr *et al.*, 1992). Another may involve overprocessing of determinants due to internal proteolytic cleavage sites, which destroys suitable peptide determinants (Basler *et al.*, 2004).

The antigen processing of IDDs is not always necessarily more efficient than for SDDs. In IAV-infected BALB/c mice, for instance, where the regular immunodominance hierarchy is composed of five T_{CD8^+} determinants all restricted by the same H-2K^d molecules, the immunodominant $NP_{147-155}$ determinant is processed at only intermediate efficiency (Chen *et al.*, 2000). In IAV-infected B6 mice, the IDD $PA_{224-233}$ is processed and presented relatively slowly (Chen *et al.*, 2004b). Similarly, in the H-2^d restricted anti-*LM* response, there are three IDDs and a single SDD, and yet the SDD $p60_{449-457}$ is processed most efficiently (Sijts *et al.*, 1996).

In the same *LM* system, Vijh *et al.* (1998) studied a series of mutants predicted to disrupt the antigen-processing efficiency of the second IDD $P60_{217-225}$. Their study showed that antigen-processing efficiency correlated only partially with immunity. Thus, a mutant with a poor efficiency for generating an IDD (efficiency of equivalent to one IDD generated for every 350 translated copies degraded (1/350)) produced no detectable T_{CD8^+} activation. However, when the processing efficiency increased from 1/350 to 1/70, the T_{CD8^+} response was restored to a normal level; further enhancement of the Ag-processing efficiency to 1/50 or 1/17 did not further enhance the T_{CD8^+} responses.

It seems likely that once a certain antigen-processing/presentation threshold is achieved for a given determinant, its pattern of T_{CD8^+} activation and ranking in an immunodominance hierarchy will be determined by other factors such as competition from other T_{CD8^+} recognizing alternate specificities (see later). However, we should bear in mind that most of the above knowledge has been acquired from *in vitro* analysis using established cell lines or cultured DCs as APC (Chen *et al.*, 2004b; Jenne *et al.*, 2000; Schnurr *et al.*, 2005). With *in vitro* models of viral infection and immunodominance, infection might involve the wrong cell types or lead to inappropriate infection

doses that might not be applicable *in vivo*. Another significant drawback of *in vitro* analysis is the common use of a 1:1 ratio of APCs and T_{CD8^+}, a situation that is unlikely to reflect what occurs *in vivo* where multiple T_{CD8^+} of various specificities may simultaneously access a single APC and cause T-T competition and subsequent immunodomination (see later). Moreover, as already noted, *in vitro* derived data largely represent direct antigen presentation, which may not be the major antigen-presenting mode *in vivo* either because some viruses do not directly infect DC or because tumor cells that cannot directly prime T_{CD8^+} rely upon cross-presentation as the major mechanism of Ag-presentation. So, it remains essential to assess antigen presentation by professional APC directly *ex vivo* (Belz *et al.*, 2004a; Crowe *et al.*, 2003) or *in vivo* although this may pose a substantial challenge (Yewdell and Haeryfar, 2005).

B. IDDs Generally Bind to their MHC Molecules Efficiently

The antigen-binding motifs for many MHC-I molecules have been elucidated following the pioneering peptide elution work of the Rammensee group (Falk *et al.*, 1990). These motifs are of practical use for predicting and identifying major T_{CD8^+} determinants derived from various antigens (DiBrino *et al.*, 1993; Nijman *et al.*, 1993; Pamer *et al.*, 1991). It is now appreciated that beyond a certain point additional binding affinity does not necessarily enhance or guarantee a peptide's ranking in the immunodominance hierarchy. The MHC-binding threshold for immunogenicity correlates with a K_D of <50 nM originally studied for common HLA molecules (Sette *et al.*, 1994a,b) and subsequently validated in mouse systems (van der Most *et al.*, 1996, 1997; Vitiello *et al.*, 1996). However, this may vary depending on the particular *MHC-I* alleles. The MHC-binding ability of synthetic peptides is normally tested either using cells deficient for TAP in a direct binding assay or using a radiolabeled reference peptide in an indirect binding competition assay (Sette *et al.*, 1994b). In the latter, the binding ability of the peptide to be screened is expressed as the concentration needed to compete off half the reporter peptide.

There is good evidence that most IDDs are generally of higher MHC-binding affinity than SDDs for both class I and class II molecules (Chen *et al.*, 1994; Lazarski *et al.*, 2005; Sette *et al.*, 1994b; van der Most *et al.*, 1997, 1998). Nonetheless, there are also clear exceptions in which higher affinity MHC-binding peptides are not more immunogenic (Chen *et al.*, 2000; Mullbacher *et al.*, 1999; Regner *et al.*, 2001) and lower affinity MHC-binding peptides become IDDs (Chen *et al.*, 2000). However, the binding conditions in these

assays may not necessarily reflect the biological conditions in the ER or other related compartments where the peptide loading events are heavily facilitated by chaperone proteins and other proteins of enzymatic potential (Peh *et al.*, 2000; Van Kaer, 2001). Taken together, the most important parameter in controlling immunodominance hierarchy, though still not a guarantee of relative immunodominant status, is that a peptide is processed efficiently and achieves sufficient binding to a given MHC molecule. In the absence of these criteria, determinants will not be any different from the multitude of non-immunogenic peptides randomly generated as part of the normal protein degradation process and amino acid recycling within the APC (Yewdell, 2001).

C. Contribution of T_{CD8^+} Repertoire to the Immunodominance Hierarchy

Although the potential contribution of the T_{CD8^+} repertoire to immunodominance has always been appreciated theoretically, there have been few demonstrations of this point experimentally. There are examples demonstrating the importance of having abundant responding T_{CD8^+} in the repertoire for a given T_{CD8^+} response to become immunodominant (Choi *et al.*, 2002) but most examples rely upon indirect evidence. For instance, by demonstrating efficient antigen processing of a particular SDD *in vitro*, despite no detectable T_{CD8^+} response *in vivo*, many studies have concluded that there is a lack of responding T_{CD8^+} in the T - cell repertoire (Chen *et al.*, 2000; DiPaolo and Unanue, 2002; Regner *et al.*, 2001). This concept has been explored more directly in transgenic systems. Thus, Daly *et al.* (1995) utilized single TCR β-chain Tg mice to deliberately constrain the T-cell repertoire. They then demonstrated that the subsequent T-cell repertoire for an IDD was significantly impaired which in turn lead to a different immunodominance hierarchy. However, Otahal *et al.* (2005) obtained a slightly different result in their experiments. They transferred large number of TCR Tg-T_{CD8^+} specific to a SDD from SV40 large T Ag (489–497) into B6 mice. The mice were then primed with either wild-type SV40 large T Ag, which contained four well-characterized T_{CD8^+} determinants, or a mutant SV40 large T Ag with three determinants silenced by anchor residue mutations leaving only the SDD specifically recognized by the Tg-T_{CD8^+}. The transferred Tg-T_{CD8^+} were all activated when the mice were cross-primed with an antigen-expressing cell line that only presented the SDD. Surprisingly, when the mice were immunized with a cell line that expressed the wild-type large T Ag, the T_{CD8^+} response to the IDD proliferated extensively at the expense of activation of the transferred Tg-T_{CD8^+} specific for the SDD (Otahal *et al.*, 2005). These experiments strongly argue against a simple relationship between precursor frequency and immunodominance.

Because the precursor T_{CD8^+} repertoire for either IDDs or SDDs has never been properly assessed in naïve animals, it might be possible to exploit TCR Tg-T_{CD8^+} to reconstitute defined numbers of T_{CD8^+} precursors for both IDDs and SDDs. This might permit the relationship between precursor frequency and immunodominance to be more directly tested. However, even this approach would fail to take into account TCR heterogeneity.

In another approach, not involving TCR Tg mice, the lack of the immunoproteasome in the thymus due to LMP-2 deficiency dramatically changed the T_{CD8^+} repertoire and the immunodominance hierarchy toward IAV, suggesting altered selection of thymic precursors (Chen *et al.*, 2001). However, none of these experimental conclusions was based on the direct assessment of the naïve immunodominant and/or subdominant T_{CD8^+} repertoire because of the rarity of these antigen-specific T_{CD8^+} precursors. The naïve precursor frequency for the immunodominant T_{CD8^+} specific to LCMV $GP_{33–41}$ was estimated to be only 100–200 per mouse. Thus, 30 million T_{CD8^+} cells per mouse would give a frequency equivalent to 7 precursors per million T_{CD8^+} (assuming 200 precursors per mouse) (Blattman *et al.*, 2002). The most powerful enumeration methods, such as tetramer staining combined with multiplestep T_{CD8^+} enrichment, only detect antigen-specific T_{CD8^+} around 0.01% frequency (100 per million T_{CD8^+}) (Pittet *et al.*, 1999; Valmori *et al.*, 2000). This detection threshold falls well short of detecting naïve immunodominant T_{CD8^+} precursors, let alone the frequencies of T_{CD8^+} specific to SDDs (assuming they are rarer in some cases).

The antimelanoma T_{CD8^+} response restricted by the HLA-A2 molecule in humans provides a relatively convincing example of immunodominance due to TCR repertoire. Many healthy HLA-A2 expressing individuals have elevated T_{CD8^+} precursors specific to Melan-$A_{27–36}$, which can be directly identified using specific tetramers *ex vivo*. These cells are part of the normal thymic output (Zippelius *et al.*, 2002) and possess naïve phenotypes. They can subsequently be activated *in vitro* using peptide-loaded autologous DCs (Pittet *et al.*, 1999; Salio *et al.*, 2001).

A less convincing example linking precursor frequency and immunodominance involves the T_{CD8^+} response against the H60 MiHA. Specific T_{CD8^+} recognizing the H60-derived octamer peptide LTFNYRNL presented by the H-2K^b (Choi *et al.*, 2002) are easily detected with tetramers after mixed lymphocyte culture, but T_{CD8^+} specific to other MiHA SDDs are barely detectable under similar conditions. The H60 specific T_{CD8^+} precursors were estimated to represent ~15,000 precursors per spleen (~22,500/mouse), which is significantly higher than the estimated T_{CD8^+} frequency specific for LCMV-derived IDD $GP_{33–41}$ (~100–200/mouse) (Blattman *et al.*, 2002) and only ~10–20-fold lower than allospecific T_{CD8^+} recognizing H-2K^d alloantigen (Choi *et al.*, 2002). However, under similar culture conditions, T_{CD8^+} precursor frequency specific to a SDD

from IAV ($PB1F2_{62-70}$) was similar to that of H60-specific T_{CD8^+} (Choi *et al.*, 2002).

Taken together, most data suggest that the T_{CD8^+} precursor frequency has little relationship to immunodominance. This makes sense when one considers that antigen-specific T_{CD8^+} proliferate every 6 hours in the immediate period after antigen priming (Mueller *et al.*, 2002). Therefore, a single T_{CD8^+} precursor could divide 8 times in 48–72 hours, producing 256 daughter cells. Assuming such daughter cells are still capable of proliferating at that point, minor starting differences in naïve precursor frequency could easily be overcome by other factors in a short time period. In other words, a lower precursor frequency could be irrelevant in the face of stronger stimulation by the relevant antigen (Oh *et al.*, 2003). Accordingly, it is problematic to ascribe a relatively small difference in the starting frequencies as the basis for immunodominant and subdominant responses.

IV. IMMUNODOMINATION AND ITS POSSIBLE MECHANISMS

Immunodomination, also described as T-T competition (Kedl *et al.*, 2003), refers to circumstances in which the T-cell response to a given antigenic determinant is inhibited or suppressed either directly or indirectly by T cells specific to other antigenic determinant(s). Immunodomination usually involves T cells specific for determinants from the same antigen or same pathogen. The suppressing T_{CD8^+} are usually specific to IDD, although this might not be necessarily true in the context of vaccination. Following vaccination, if T_{CD8^+} specific to SDD(s) are first primed, these T_{CD8^+} might then dominate naïve responses to other IDDs and/or SDDs. Immunodomination can also influence the pattern of priming of T_{CD8^+} responses to complex antigens or pathogens containing multiple immunogenic determinants. The best evidence for this is provided by the MiHA system (detailed later) and by the various model antigen systems with transferred DCs and TCR Tg-T_{CD8^+}.

A. Immunodomination in Primary Responses

Immunodomination in primary T_{CD8^+} responses is highlighted in studies on anti-MiHA responses, which cause graft-versus-host (GVH) responses. This system is particularly relevant since there are data showing that the IDDs, but not the SDDs, drive GVH (Perreault *et al.*, 1996) and graft-versus-tumor responses (Pion *et al.*, 1995). In the MiHA systems, there

are well-characterized congenic mouse strains in which multiple MiHAs have been defined as MHC-I–restricted antigenic peptides (Perreault *et al.*, 1998). For example, the C3H.SW ($H\text{-}2^b$) female anti-C3H.SW male response forms the anti-H-Y male response coded by the *Uty* gene on the Y chromosome and is the most dominant response in the C3H.SW syngeneic system. However, in the C3H.SW anti-B6 male responses the anti-H-Y response becomes barely detectable due to immunodomination by the more vigorous responses toward B6-derived MiHAs (at least 10). These B6-derived MiHAs are collectively called $B6^{dom}$ determinants (Pion *et al.*, 1999). Amongst these, the best IDD is called $B6^{dom1}$. Both the H-Y and $B6^{dom1}$ determinants are 9-mer peptides and presented by the $H\text{-}2D^b$ (Greenfield *et al.*, 1996; Perreault *et al.*, 1996). In naïve C3H.SW mice there are equivalent number of T_{CD8^+} precursors to both H-Y and $B6^{dom1}$ determinants (Pion *et al.*, 1997). The proposed mechanism for the immunodominance hierarchy in this system involves differences in antigen-processing efficiency for the two MiHA determinants. The B6 APC surface expresses more than 1000 $B6^{dom1}/D^b$ complexes compared with only $\sim$10 $H\text{-}Y/D^b$ complexes (Pion *et al.*, 1997, 1999).

However, the above observation does not apply to MiHA responses in the B6 anti-BALB.B direction. Here, there are at least 40 MiHAs with known gene loci, although many of these T_{CD8^+} determinants are yet to be defined for their exact peptide sequences. The BALB.B splenic cells present all the IDDs and SDDs restricted by K^b and D^b; on the other hand, the congenic strains present either IDD ($H\text{-}28^c$ strain, K^b) or SDDs ($H\text{-}19^c$ strain, K^b; $H\text{-}8^c$ strain, D^b). Wolpert *et al.* (1998) acid eluted the H-2-bound peptides of *ex vivo* spleen cells either from BALB.B or its congenic strains. They then showed that individual T_{CD8^+} lines raised from B6 mice immunized with splenic cells from single-gene congenic strain only responded to a single HPLC fraction from the peptide eluate. However, when each fraction containing either IDD or SDD activity was titrated using the corresponding T_{CD8^+} line, there was no significant difference in dose responsiveness toward the fractions containing the IDD versus the SDDs. Assuming all peptides were recovered with similar efficiency, then the antigen-processing and presentation efficiency for these IDDs and SDDs is likely to be similar. Therefore, the immunodominance hierarchy in this MiHA system does not superficially correlate with the density of MHC–peptide complexes during Ag-presentation. However, if specialized APC are involved in the initial priming and expansion steps, there is still scope for differential presentation to explain the immunodominance hierarchy.

An *in vitro* immunodomination system was also created from the B6 anti-BALB.B MiHA model. Here T_{CD8^+} primed *in vivo* to the $H\text{-}19^c$derived SDD could be stimulated *in vitro* using spleen cells from either $H\text{-}19^c$ or BALB.B. However, if the responding B6 splenic cells (primed with either spleen

cell type) were first mixed at 1:1 ratio then stimulated *in vitro* with only BALB.B spleen cells, the subdominant T_{CD8^+} specific to H-19^c–derived peptide were totally dominated by the T_{CD8^+} specific to the H-28^c–derived peptide (Wolpert *et al.*, 1998). Nonspecific T_{CD8^+} and/or APC crowding as a potential mechanism was excluded because addition of large number of naïve B6 splenic cells in nonmixed cultures did not affect T_{CD8^+} stimulation by either the IDDs or the SDDs (Wolpert *et al.*, 1998). However, antigen-specific crowding between immunodominant and subdominant T_{CD8^+} (Kedl *et al.*, 2003) might still have existed if the T_{CD8^+} were required to share a rare population of APCs, such as splenic DCs, rather than all the MiHA-bearing spleen cells.

As a note of caution, the above experiments were carried out by priming with MiHA followed by an *in vitro* stimulation step and CTL killing readout. In other words, the immunodomination *in vivo* was indirectly demonstrated after *in vitro* T_{CD8^+} expansion, which might exaggerate the difference between the immunodominant and subdominant T_{CD8^+} responses (and therefore overestimate the extent of immunodomination).

To directly assess immunodomination *in vivo*, Roy-Proulx *et al.* (2001) stimulated B10 female mice with B10 male splenocytes mismatched for only the H-Y MiHA. H-Y specific T_{CD8^+} were detected by tetramers *ex vivo*. The tetramer-positive T_{CD8^+} numbers in the immunized spleens peaked on day 15 postimmunization. When the B10 female mice were immunized with splenocytes from male B10.H7^b (H-Y+B6^{dom1}) mice, the major detectable T_{CD8^+} population was B6^{dom1} specific, which also peaked on day 15 but remained at this level for a further 5 days. In comparison, the T_{CD8^+} responses specific to H-Y antigen became much smaller over this period. However, when B10 female mice were immunized with male spleen cells from C3H.SW mice, which introduced many other MiHAs, the T_{CD8^+} specific to B6^{dom1} still dominated the overall response and peaked on day 15, but returned to baseline much sooner, while the T_{CD8^+} specific to H-Y became barely detectable. This latter experiment eliminated the possibility of *in vitro* interference with immunodomination and supported the general findings.

Immunodomination has also been addressed *in vivo* using the model OVA (ovalbumin) system. Kedl *et al.* (2000) transferred various numbers of OT-I Tg-T_{CD8^+} specific for the well-known IDD $OVA_{257–264}$ (SIINFEKL) into naïve B6 mice. The animals were then challenged with rVV encoding OVA. In this system, the large number of transferred OT-I Tg-T_{CD8^+} totally suppressed the response of host-derived $OVA_{257–264}$-specific T_{CD8^+}. Moreover, host-derived subdominant $OVA_{55–62}$-specific T_{CD8^+} response was also reduced. In analogous experiments, Probst *et al.* (2002) transferred TCR Tg-T_{CD8^+} specific for LCMV $GP_{33–41}$ into normal B6 mice and observed the same antigen-specific immunodomination of host-derived, $GP_{33–41}$-specific

T_{CD8^+}. However, utilizing the elegant observation that there are two independent determinants within $GP_{33–41}$ ($GP_{33–41}/D^b$ and $GP_{34–41}/K^b$), and transferring similar Tg-T_{CD8^+} as in the OVA system (Kedl *et al.*, 2000) mentioned above, these authors did not observe detectable immunodomination of host-derived T_{CD8^+} specific to SDD $GP_{34–41}/K^b$.

Although the efficiency of antigen presentation was different in various systems, immunodomination was consistently attributable to T_{CD8^+} sharing the same APC. Thus, when IDD and SDD were presented by separate APC, there was no immunodomination (Kedl *et al.*, 2000; Pion *et al.*, 1997; Wolpert *et al.*, 1998). Furthermore, if the APC:$T_{CD8}{}^+$ ratio was dramatically changed such that APCs were relatively abundant, by transferring limited numbers of T_{CD8^+} (Roy-Proulx *et al.*, 2001) or supplying larger number of APC (Grufman *et al.*, 1999; Kedl *et al.*, 2000), the immunodomination was completely alleviated.

Immunodomination could theoretically involve killing of APC. It has been shown that antigen-specific T_{CD8^+} acquire cytotoxicity after a single division (Oehen and Brduscha-Riem, 1998). When bulk alloantigen- or MiHA-bearing splenic cells were transferred into B6 mice, these transferred cells were eliminated in a perforin-dependent process (Loyer *et al.*, 1999). Similar findings were also made for viral peptide-pulsed bone marrow–derived DCs following subcutaneous transfer into either B6 or TCR Tg-mice (Hermans *et al.*, 2000; Yang *et al.*, 2005). In contrast, perforin-mediated APC-depletion was not demonstrated as a mechanism of immunodomination in the MiHA system (Roy-Proulx *et al.*, 2001). Instead, the recovery of transferred GFP-expressing DCs, in the presence or absence of IDD presentation, was not affected by the transfer of specific Tg-T_{CD8^+}. This finding suggests that antigen-specific APC elimination is not significant in the early phase of the primary response (Kedl *et al.*, 2000).

A curious observation was made by Kedl *et al.* (2002) who cotransferred genetically marked OT-I (specific for $K^b/OVA_{257–264}$) and P14 (specific for $D^b/GP_{33–41}$ from LCMV) TCR Tg-T_{CD8^+} into B6 mice. GFP-tagged bone marrow-derived DCs were then pulsed with both peptides at 1:1 ratio and injected intradermally. DCs isolated from draining lymph nodes were stained with the K^b/SIINFEKL-specific antibody 25D1.16 (Porgador *et al.*, 1997), revealing that the DCs recovered from the mice that received OT-I Tg-T_{CD8^+} showed decreased K^b/SIINFEKL expression. The authors proposed that this was possibly due to specific MHC/peptide complex "extraction" from APCs (Fig. 2), a phenomenon that could impair antigen presentation to T_{CD8^+} of the same specificity leading to antigen-specific immunodomination. However, such a mechanism does not explain immunodomination of T_{CD8^+} with different specificities unless the extraction of MHC–peptide complexes is nonspecific. Nor did diminished expression of MHC or costimulatory molecules on APC explain the data

even though loss of those molecules has been demonstrated in *in vitro* assays previously (Huang *et al.*, 1999; Hwang *et al.*, 2000). It will be interesting to determine whether other APC surface molecules and cellular functions are affected *in vivo*, potentially influencing the immunodominance hierarchy.

Generally, immunodomination between T_{CD8^+} with different specificities in the primary response has not been commonly observed. In IAV-infected B6 mice and *LM*-infected BALB/c mice, when the IDDs were silenced by mutating peptide anchor residues there was no enhancement of T_{CD8^+} responses to any of the SDDs, indicating a lack of immunodomination in such systems (Andreansky *et al.*, 2005; Vijh *et al.*, 1999). The apparent discrepancies between different systems may reflect different extremes of *in vivo* immunodomination. For instance, the SDD $OVA_{55–62}$ is known to be presented poorly due to its weak MHC-binding affinity compared to that of the immunodominant $OVA_{257–264}$ (Chen *et al.*, 1994). Therefore, in this system immunodomination of primary SDD-specific response by IDD-specific T_{CD8^+} is understandable.

In natural viral infections, the APC numbers might be greatly in excess compared to the naïve T_{CD8^+} precursors leaving little scope for competition at the APC level and thus minimal immunodomination (Fig. 1, upper half). When TCR Tg-T_{CD8^+} are artificially introduced, the APC number may become relatively limiting allowing immunodomination to become evident. As we have seen, in the LCMV model, immunodomination was limited to T_{CD8^+} of the same specificity and this could well be due to "antigen extraction" resulting in the functional silencing of APC (Fig. 2). On the other hand, in the rVV-OVA system (Kedl *et al.*, 2000), APC numbers are unlikely to be limiting since rVV infection is efficient (Norbury *et al.*, 2002). Perhaps, the host immunodominant responses toward rVV-derived IDD and SDDs (Harrington *et al.*, 2002; Tscharke *et al.*, 2005) facilitate immunodomination.

It is intriguing that there is immunodomination in the primary response against MiHAs and yet this is not seen in the response toward viral antigens. The simplest explanation might be that the priming of MiHA-specific T_{CD8^+} is largely dependent on host-APC rather than involving direct priming by the transferred donor APC. Although donor APC are excellent targets of MiHA-specific T_{CD8^+}, the host DCs that mediate cross-presentation of these specific MiHAs may be limiting because of the complexity of the Ag load (cellular debris), the amount of available source MiHAs (Norbury *et al.*, 2004; Shen and Rock, 2004), and the discrete number of specialized DCs with this function. Thus, cross-presentation of cellular material may lend itself to immunodomination as T cells compete for APCs, whereas DCs are less likely to be limiting during the early phase of many primary viral infections.

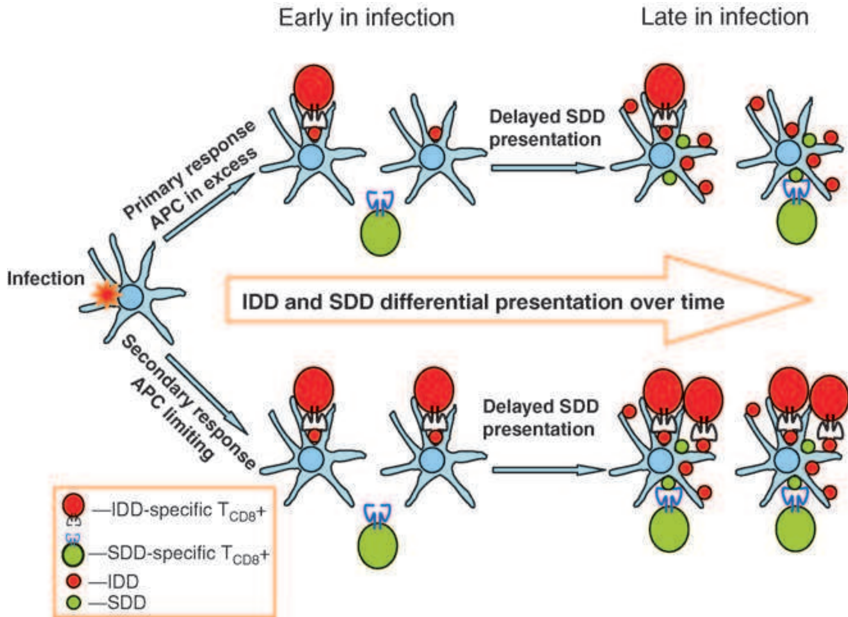

Fig. 1 Antigen-processing efficiency affects T_{CD8^+} stimulation in the primary and secondary responses. In the primary infection for most pathogens, the APC number is likely to be in excess because of very limited naïve Ag-specific T_{CD8^+} precursors. So there is generally no T-cell competition or immunodomination even if T_{CD8^+} determinants are presented with different kinetics (simplified as early and late infection, upper half). In a secondary response, antigen presentation becomes limiting because (1) the T_{CD8^+} frequencies, both for IDD and SDD, can be up to 1000-fold higher and (2) a more limited infection is established because of preexisting antiviral cellular immunity. Hence, the APC number and the extent of their infection in most cases may become relatively limiting (lower half). Also, memory T_{CD8^+} engagement will lead to APC elimination, the IDD-specific T_{CD8^+} are preferentially expanded and as a consequence, the SDD-specific T_{CD8^+} are poorly stimulated, viz., they are dominated. In IAV-infected B6 mice, DC might be the only APC presenting $PA_{224–233}$ and they do so with a slower kinetics, which could limit the activation of these memory T_{CD8^+} and render them subdominant.

B. Immunodomination on Secondary (Subsequent) Antigen Exposure

The ultimate goal of T_{CD8^+}-based vaccination strategies is to activate viral or tumor-specific T_{CD8^+} within an individual before an encounter with the authentic pathogen or tumor. Therefore, it is cogent to understand the subsequent immunodominance hierarchy in responding to the authentic antigen exposure in the presence of vaccine-primed memory T_{CD8^+} as well as the other antigen-specific naïve T_{CD8^+}. Transferring memory T_{CD8^+} into a naïve mouse mimics this situation and suppresses the priming of the naïve

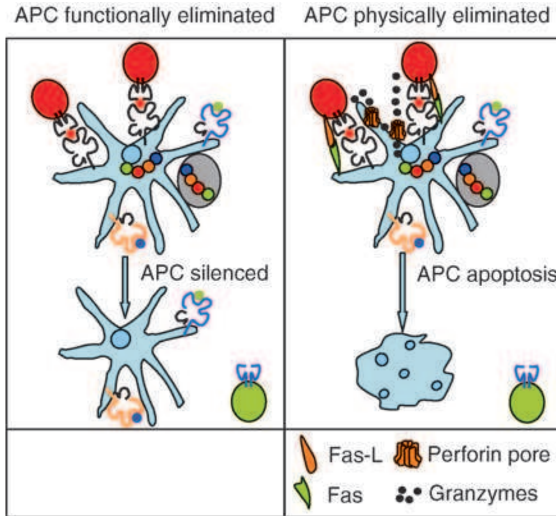

Fig. 2 APC elimination as a mechanism of immunodomination. In the primary response, if the APC number becomes relatively limiting, such as occurs experimentally with transfer of Tg-T_{CD8^+} or with Ag overload as in cell-associated MiHA taken up by specialized cross-presenting DCs, then the access to appropriate APC may depend on the avidity of T_{CD8^+}–APC interaction. The immunodominant and higher affinity T_{CD8^+} will interact with APC earlier and more avidly, which may lead to reduced expression of specific MHC/peptide complexes, or other APC surface molecules, rendering the APC functionally inactive. In the memory responses, established memory T_{CD8^+} will interact with APC according to antigen availability on their surface and the avidity of a given interaction. It is most likely that the immunodominant T cells will interact with APC first and eliminate the presenting cells through the combined impact of the Fas/Fas-L and perforin/granzyme killing pathways.

T_{CD8^+} (Jamieson and Ahmed, 1989). In an analogous approach, single specificity T_{CD8^+} lines against IAV were separately transferred into naïve BALB/c mice where they formed a detectable memory pool. The animals were then challenged with virus and their immune responses were compared to that of a normal primary response without T_{CD8^+} transfer. The memory cells derived from the transferred T_{CD8^+}, regardless of their specificity and rank in the immunodominance hierarchy, exerted profound immunodomination on the antigen-specific naïve T_{CD8^+}. However, the memory T_{CD8^+} specific to the IDD exerted more powerful immunodomination than the subdominant T_{CD8^+} (Chen *et al.*, 2000). Almost identical immunodomination patterns were observed when single-specificity T_{CD8^+} memory populations to IAV were primed using rVV-minigenes coding for the relevant IDD or SDD. This excluded the possibility that the findings were an artifact of using *in vitro*-cultured T_{CD8^+} lines (Chen *et al.*, 2000).

The contribution of specific, cross-reactive subsets of preprimed memory T_{CD8^+} to the immunodominance hierarchy in the secondary response has been examined in the mouse IVA system. Haanen *et al.* (1999) utilized the observation that D^b-tetramers complexed with either $NP_{366-374}$ (ASNENM*DA*M) from IAV strain A/NT/60/68 or $NP_{366-374}$ (ASNEN-M*ET*M) from A/PR/8/34 detect only a small subpopulation of cross-reactive T_{CD8^+} primed with the reciprocal heterologous virus. When the mice were primed with one virus and challenged with the heterologous viruses, the vast majority of the expanded T_{CD8^+} were stained by both tetramers. Given that there were relatively few cells positive for a single tetramer in the recall response, there is apparently little role for priming of naïve T_{CD8^+} in the secondary challenge indicating profound immunodomination from T_{CD8^+} that cross-react with the challenging virus (Haanen *et al.*, 1999).

Heterologous cellular immunity has also been studied in Pichinde virus (PV)-infected B6 mice. Here, a SDD $NP_{205-212}$ (K^b, YTVK*F*PN*M*) shared six of eight amino acids with a SDD from LCMV $NP_{205-212}$ (K^b, YTV-K*Y*PN*L*). The authors asked if the previously primed PV $NP_{205-212}$-specific subdominant T_{CD8^+} would affect the T_{CD8^+} immunodominance hierarchy induced by LCMV or vice versa. They demonstrated that memory $NP_{205-212}$-specific T_{CD8^+} primed with either infection with LCMV or PV became immunodominant when responding to a new challenge by the other virus. Hence, the previously established memory T_{CD8^+} are able to exert profound immunodomination upon naïve antigen-specific T_{CD8^+} even if their specificity was cross-reactive (Brehm *et al.*, 2002). The extent of cross-reaction between T_{CD8^+} specific to either $NP_{205-212}$ was not determined and so the fate of different T_{CD8^+} cells with varying levels of cross-reactivity could not be addressed (Brehm *et al.*, 2002).

Subtle changes in determinant composition can dramatically affect immunodominance hierarchy, especially during the secondary response. Using reverse genetic techniques, a series of recombinant IAV with either single or multiple IDD deletions was generated (Hoffmann *et al.*, 2000). Surprisingly, the T_{CD8^+} responses to the unmutated IDD and SDDs in the primary infection were not detectably different from that induced by the wild-type virus (Andreansky *et al.*, 2005). However, when these mutant viruses were used to challenge memory T_{CD8^+}, a dramatic increase in subdominant T_{CD8^+} responses was observed, indicating that under normal viral challenge conditions immunodominant T_{CD8^+} dominate the subdominant T_{CD8^+}. Similarly, Vijh *et al.* (1999) studied recall responses in *LM*-infected BALB/c mice to both IDDs and SDDs in the presence and absence of the best IDDs using *LM* mutants. Here, the T_{CD8^+} responses to the SDDs were not enhanced as a result of eliminating the IDDs. When the mice were primed with a mutant *LM* that lacked both IDDs and then challenged with wild-type *LM* a few

weeks later, the response was dominated by the memory T_{CD8^+} specific to the SDDs at the expense of naïve T_{CD8^+} specific to the IDDs (Vijh *et al.*, 1999). Importantly, the increased subdominant T_{CD8^+} populations were found to be qualitatively similar to those subdominant T_{CD8^+} obtained under a wild-type IAV challenge as reflected by their cytokine production after activation (Andreansky *et al.*, 2005; Webby *et al.*, 2003).

There are other studies that demonstrate similar immunodomination in the secondary T_{CD8^+} responses but these employ methods involving *in vitro* T_{CD8^+} expansion and less quantitative techniques, such as the ^{51}Cr-release assay (Schirmbeck *et al.*, 2002; Sherritt *et al.*, 2000), which makes their interpretation less accurate.

Taken together, the data discussed here allow some generalizations and speculations of a broad nature. In the secondary response, the antigen-specific memory T_{CD8^+} number increases up to 1000-fold compared to naïve precursors (Blattman *et al.*, 2002), rendering APC numbers relatively limiting for efficient Ag-presentation. As a result, only selected T_{CD8^+} are expanded either because of (1) their higher starting numbers, assuming comparable Ag-processing; (2) due to their greater avidity of TCR-MHC/peptide interactions stemming from selection of higher avidity T_{CD8^+} as a result of limiting antigen presentation during priming (Kedl *et al.*, 2000); or (3) because their cognate peptide antigen is processed and presented with faster kinetics (Chen *et al.*, 2004b) (Fig. 1, lower half).

C. Immunodominant T_{CD8^+} Can Become Subdominant in Secondary Responses

For adaptive immunity, it is widely thought that the memory cell pool is determined by the original clonal burst size (Hou *et al.*, 1994) and that the original immunodominant T_{CD8^+} response remains stable upon subsequent antigen encounter. This dogma has been challenged. There is evidence of nonproportional contraction of the primary clonal burst when forming the memory pools of immunodominant and subdominant T_{CD8^+} (Chen *et al.*, 2000). However, in this example, the discordance was relatively small and did not change the overall immunodominance hierarchy, so the point was not emphasized. On the other hand, more papers have highlighted that the immunodominance hierarchy in the primary response might not be reproduced upon secondary antigen encounter and that immunodominant T_{CD8^+}, as defined by their gross number, might even be functionally inferior and not protective against infection (Crowe *et al.*, 2003). In the following section we review the best examples in this category.

In the *LM* model, it was originally demonstrated that vigorous T_{CD8^+} responses were targeted to *N*-formyl methionine peptides presented by the

oligomorphic MHC class Ib molecules—H2-M3. Lack of polymorphism makes these molecules a promising target for peptide-based vaccines (Pamer *et al.*, 1992), although the relative importance of MHC class Ib-restricted T_{CD8^+} in antimicrobial immunity is still poorly understood. When the sizeable H2-M3–restricted T_{CD8^+} response was studied side by side with another immunodominant T_{CD8^+} response restricted by the normal class Ia molecule, H-2K^d, it was found that the H2-M3–restricted T_{CD8^+} had faster induction kinetics in the primary response. T_{CD8^+} with either specificity differentiated into the memory pool efficiently and were maintained to a similar extent. However, during secondary *LM* challenge, H2-M3–restricted memory T_{CD8^+}, although activated, expanded poorly and were rendered subdominant to the much larger H-2K^d–restricted responses (Kerksiek *et al.*, 1999). Moreover, peptide-primed memory H2-M3–restricted T_{CD8^+} expanded well in response to *LM* challenge indicating these memory T_{CD8^+} could be stimulated to proliferate when other immune cells were absent. The possibility that the two determinants might be presented differently in the secondary *LM* infection was not exhaustively tested (Kerksiek *et al.*, 1999).

Belz *et al.* (2000b) showed that the immunodominant T_{CD8^+} specific to $PA_{224–233}$ in the primary response to IAV infection became subdominant upon viral challenge. By assessing APCs directly enriched *ex vivo*, Crowe *et al.* (2003) found that different APC differentially presented $NP_{366–374}$ and $PA_{224–233}$. They put forward the hypothesis that $NP_{366–374}$ is presented universally by all infected APCs (it is still not clear that how many cell types *in vivo* can be infected by IAV), including DCs and macrophages, but $PA_{224–233}$ was only presented by DCs. Since in the primary response to IAV DCs are likely to be the only priming APC, as shown for quite a few viruses (Belz *et al.*, 2004a; Probst and van den Broek, 2005; Smith *et al.*, 2003), the differential antigen presentation of these two IDDs does not matter and the $NP_{366–374}$- and $PA_{224–233}$-specific T_{CD8^+} codominate the primary immune response. In the secondary infection, however, antigen-specific memory T_{CD8^+} might be able to engage any APC (provided they present sufficient peptide/MHC complexes) and thus expand their numbers. So, under these circumstances, $NP_{366–374}$-specific T_{CD8^+} will have more opportunity to engage appropriate APC, while $PA_{224–233}$-specific memory T_{CD8^+} can only engage DCs and are, therefore, constrained in their restimulation. If this is the case *in vivo*, the reversal of immunodominance hierarchy in the secondary infection would entirely be a consequence of differential antigen processing/presentation and the stimulation to $PA_{224–233}$-specific T_{CD8^+} should be totally independent from that of $NP_{366–374}$-specific T_{CD8^+}.

However, using the same system and a brefeldin A-based antigen-presentation kinetics assay, presentation of $PA_{224–233}$ occurred after a

4–6-hour delay and never reached saturating levels of presentation when compared to $NP_{366–374}$ (Chen *et al.*, 2004b). When antigen stimulation to $NP_{366–374}$-specific T_{CD8^+} was reduced, or when the frequency of memory $NP_{366–374}$-specific T_{CD8^+} was reduced, the T_{CD8^+} response to $PA_{224–233}$ (as well as to other SDDs) increased (Chen *et al.*, 2004b). Based on these findings, the T_{CD8^+} response to $PA_{224–233}$ was thought to be dominated by the $NP_{366–374}$-specific T_{CD8^+}. This immunodomination was neither dependent upon perforin function even though this lysis mechanism accounts for 80–85% of killing activity mediated by CTL and NK cells (Kagi *et al.*, 1994) nor was it dependent upon apoptosis induction via Fas/Fas-L interactions since the secondary immunodominance hierarchy was maintained in both perforin knockout ($pfp^{-/-}$) and Fas-L mutant ($gld^{-/-}$) mice (Chen *et al.*, 2004b). Admittedly, these experiments did not formally exclude the possibility that APC elimination might utilize both perforin and Fas/Fas-L pathways at the same time and thereby have built-in redundancy. Ideally, it is, therefore, necessary to inhibit both these killing mechanisms but knocking-out both perforin and Fas-L in mice causes severe homeostatic perturbation, autoimmunity, and death around 6–8 weeks (Spielman *et al.*, 1998). One other possibility might be to use neutralizing anti-Fas-L Ab (Miwa *et al.*, 1999) in the $pfp^{-/-}$ mice.

In any case, in the IAV model, it is unlikely that the $PA_{224–233}$-specific T_{CD8^+} were dominated by the $NP_{366–374}$-specific T_{CD8^+} due to a lower T_{CD8^+} avidity. Compared to the $NP_{366–374}$ response, $PA_{224–233}$ binds H-2D^b more efficiently (Chen *et al.*, 2004b) and the specific T_{CD8^+} also have higher avidity for their cognate $PA_{224–233}/D^b$ ligand judged by tetramer dissociation (La Gruta *et al.*, 2004). It is most likely that these T_{CD8^+} are dominated because the antigen presentation of $PA_{224–233}$ in a secondary infection is relatively limited (Fig. 1, lower half). There might be other cell types, rather than DCs, infected through the intraperitoneal route (Crowe *et al.*, 2003), and these APCs are either not sufficiently abundant or they are sequestered. Hence, the two IDD-specific T_{CD8^+} most likely access the same APC population and thus their expansion becomes linked. This has been indirectly demonstrated by the generation of robust $PA_{224–233}$-specific T_{CD8^+} responses using either peptide-pulsed APC (Chen *et al.*, 2004b) or IAV carrying $PA_{224–233}$ in the neuraminidase stalk ($PA_{224–233}$-NA) (La Gruta *et al.*, 2006).

Taken together, most immunodominant T_{CD8^+} in the primary infection may efficiently differentiate into memory cells and dominate the subsequent recall responses to the same pathogens. However, some of these T_{CD8^+} may be rendered as subdominant in the secondary response due to less efficient Ag-presentation either as the infection is partially controlled by the antigen-specific antibodies and/or by the dramatically increased antigen-specific T_{CD8^+}.

V. IMMUNODOMINANCE IS SUSCEPTIBLE TO VIRAL ESCAPE

Viruses have coevolved with humans and other species and developed many clever mechanisms to escape the host immune surveillance. There are many general types of T_{CD8^+} escape strategies. For instance, HSV encodes the protein ICP47 to block TAP function; CMV encodes multiple proteins, including US2 and US11, which cause host MHC molecules to exit the ER thus targeting them for cytosolic degradation. In both instances, the result is to limit antigen presentation by infected host cells. HIV and CMV induce Fas-L expression and HCMV induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on infected cells, causing T_{CD8^+} apoptosis (Xu *et al.*, 2001b; Yewdell and Hill, 2002). Of course, viruses also escape detection by determinant mutation (Goulder and Watkins, 2004; McMichael and Phillips, 1997). Here we mainly review evidence on how viruses might escape T_{CD8^+} detection as a result of persistent immunodominant T_{CD8^+} responses. Given the features of immunodominance described already, it is easy to imagine that the immunodominant T_{CD8^+} exert more immune pressure on viruses than subdominant T_{CD8^+} and that viruses would mutate their genomes to avoid them first.

A. Mutating the IDD to Avoid Immune Recognition

In mice, T_{CD8^+} escape mutants were first demonstrated using the LCMV system (Pircher *et al.*, 1990). The TCR Tg-mice with T_{CD8^+} specific to the IDD $GP_{33–41}$ from LCMV were infected with LCMV. The surviving LCMV in these mice were isolated and point mutations in the IDD were found, suggesting the mutated LCMV became invisible to the Tg-T_{CD8^+}. The same group went on to demonstrate this phenomenon *in vitro* (Aebischer *et al.*, 1991). Notably, however, these experiments were somewhat artificial since Tg-T_{CD8^+} were used to examine pressure under *in vitro* conditions.

Phillips *et al.* (1991) reported the first *in vivo* example of T_{CD8^+} escape mutants in humans. They followed six HIV patients longitudinally for their immunodominant HLA-B8–restricted and subdominant HLA-B27–restricted, Gag-specific T_{CD8^+} responses and sequenced the viral isolates for the regions corresponding to these T_{CD8^+} determinants. HLA-B8–restricted determinants accumulated more mutations then the B27-restricted determinants and the virus specific T_{CD8^+} expanded from premutation blood samples did not recognize the mutated peptides.

B. Mutations in IDD Frequently Incur a Fitness Cost to the Virus

Viral mutations in IDDs are not acquired without a cost to the virus. Leslie *et al.* (2004) studied over 300 HIV AIDS patients with or without *HLA-B57/B*5801* alleles for an immunodominant anti-gag response ($Gag_{240–249}$, TSTLQEQIAW). They first demonstrated that mutations at positions 3 (T → N) and 9 occurred in nearly 100% of B57-positive patients but were not evident in any of the HIV patients who did not express either of these two *HLA* alleles. They then showed that HIV viruses transmitted from B57-negative patients into positive patients quickly gained those mutations. Finally, they showed that reversal of these mutations occurred when viruses were vertically transmitted from B57-positive mothers to their non-B57 babies. In one mother who gave birth to multiple babies, when the virus was transmitted to a B57-negative offspring, the mutations were reversed within months; but when the virus was transmitted to offspring who expressed B57 the mutations remained stable for years (Leslie *et al.*, 2004).

Similar observations have been made in simian immunodeficiency virus (SIV)-infected rhesus macaques. Although the IDD coding region was mutated within a few weeks after viral infection in the host with the relevant MHC molecules, revertants quickly selected when the mutated isolates were inoculated into the animals lacking the restricting *MHC* allele (Fernandez *et al.*, 2005; Friedrich *et al.*, 2004a). Taken together, these observations indicate that the mutations accumulated because of immune pressure and their acquisition was at the cost of viral fitness. Importantly, the mutations had functional significance. Thus, the T_{CD8^+} that specifically recognized the wild-type determinant recognized synthetic peptides corresponding to the mutated determinants very poorly or not at all (Fernandez *et al.*, 2005; Leslie *et al.*, 2004). Moreover, the mutated virus did not stimulate the corresponding immunodominant T_{CD8^+} response upon inoculation into naïve hosts bearing the relevant MHC molecule (Friedrich *et al.*, 2004b).

Viral escape from the immunodominant response is also seen in mouse hepatitis virus (MHV) infection. In this system, B6 mice infected with MHV (JHV strain) normally mount an immunodominant T_{CD8^+} response to the surface glycoprotein-derived determinant $S_{510–518}$ and a subdominant response to $S_{598–605}$. Although a few mice clear the viral infection, in the majority of mice the virus quickly develops mutations within the IDD resulting in a progressive demyelinating encephalomyelitis, which manifests as hind-limb paralysis several weeks after infection. The mutations were highly specific as very few appeared within the SDDs or within the flanking regions of the IDD (Pewe *et al.*, 1996). When mutant viruses—bearing either changes to the MHC anchor residues or those predicted to be

involved in TCR contact—were used to infect naïve littermates they caused increased mortality and growth retardation (Pewe *et al.*, 1998).

Some findings remain controversial. Chen *et al.* (1992) followed the immunodominant $Gag_{182–190}$-specific T_{CD8^+} responses in three monkeys infected with SIV over 2.5 years. Although numerous mutations were identified within the Gag coding region, only four point mutations were identified within the determinant, all of which were still recognized by a T_{CD8^+} clone specific to the parental sequence. However, it remains possible that the T_{CD8^+} determinant followed was not immunodominant and that some of the mutations might have been associated with escape from undefined, even more immunodominant, determinants restricted by other MHC molecules. Alternatively, the mutations might be more related to pressure from antiviral antibodies.

VI. IMMUNODOMINANCE IN ANTITUMOR RESPONSES AND TUMOR ESCAPE

An important role for T_{CD8^+} in suppressing some tumors has been demonstrated experimentally and is termed immunosurveillance (Smyth and Trapani, 2001). T_{CD8^+} also play a critical role in shaping tumor development, and this is referred to as immunoediting (Dunn *et al.*, 2002). In many human tumors, a common feature is the loss of MHC-I expression. In at least 80% of such cases, a single HLA molecule is lost (Marincola *et al.*, 2000; Ruiz-Cabello *et al.*, 1991). Haplotype loss occurs much less frequently (Romero *et al.*, 2005). The mechanisms of class I molecule loss are complex, especially when only a single *MHC* allele is affected and other components of the MHC haplotype are intact (Garrido and Algarra, 2001; Marincola *et al.*, 2003). It is possible that for certain tumors this allows escape from immune pressure exerted by immunodominant T_{CD8^+}. In this scenario, tumors that evade destruction from the dominant T_{CD8^+} response by downregulating the relevant MHC molecule are selected for outgrowth. Tumor cells also frequently mutate their genes due to their inherent genetic instability (Khong and Restifo, 2002), which may lead to antigen loss (Van Waes *et al.*, 1996) and change of the established immunodominance hierarchy (Yamshchikov *et al.*, 2005).

Switching-off surface MHC expression on tumor cells not only evades T_{CD8^+} destruction but also eliminates the need to modify tumor antigen expression. Antiviral immunity relies upon DCs for T_{CD8^+} priming (Belz *et al.*, 2004b; Carbone and Heath, 2003; Jung *et al.*, 2002; Norbury *et al.*, 2002; Probst and van den Broek, 2005; Smith *et al.*, 2003), which is also likely to be the case for antitumor immunity. Tumor antigens are generally

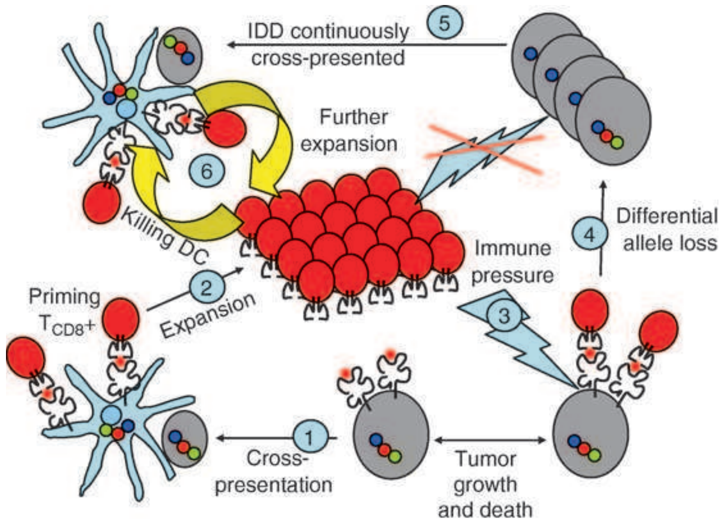

Fig. 3 Immunodominance may enhance tumor escape. DCs are thought to be the major APC responsible for priming T_{CD8^+} cells. When tumor cells first appear, anti-tumor T_{CD8^+} responses to both IDD and SDD are initiated by DCs through cross-priming, thus creating the initial determinant hierarchy. The activated IDD-specific T_{CD8^+} exert significant immune pressure on the proliferating tumor cells ultimately selecting for the loss of the restricting MHC-I molecule (the IDD is depicted as a red dot complexed to black MHC-I). However, cross-presentation of IDD by DCs persists leading to continued stimulation of IDD-specific T_{CD8^+}. These cells further dominate the immune response by competing out activation of SDD-specific T_{CD8^+} cells and by preventing the emergence of novel T_{CD8^+} through elimination of DCs.

cross-presented by DCs, so-called cross-priming and this process can be very robust, leading to immunodominance hierarchy comparable to what is observed in viral infections (Chen *et al.*, 2004a; Schirmbeck *et al.*, 1996). Thus, DCs that acquire antigenic material from dead or damaged, MHC-deficient tumor cells will continue to stimulate established immunodominant T_{CD8^+} through cross-presentation (Fig. 3). This not only will eliminate the possibility of boosting subdominant responses due to killing of DCs by the IDD-specific T_{CD8^+} but also leaves little hope for priming against novel tumor antigens (Seung *et al.*, 1993).

VII. IMMUNODOMINANCE AND CANCER VACCINES

Over the past decade or so, many tumor antigens and their antigenic determinants for both $CD4^+$ and $CD8^+$ T cells have been identified. These serve as the primary focus for many different cancer vaccine strategies both

as therapeutic targets and for immune monitoring. Therapeutic cancer vaccines are different from prophylactic childhood immunizations, which are nearly always conducted in the absence of disease. Most cancer vaccines are used in patients with either *in situ* or resected tumors. In either case, the immune system has been exposed to the tumor already and the goal is clearly therapeutic rather than preventative.

Another key difference from conventional vaccines is that most tumor antigens are self-antigens differentially expressed by tumors and some normal tissues. Such antigens usually induce immune tolerance in normal individuals (so as to avoid autoimmunity) but partial reversal of self-tolerance has been observed in patients with melanoma (Touloukian *et al.*, 2003). Thus, the aim of cancer vaccines in most cases is to prime or boost "anti-self" immune responses in the face of a potentially tolerized T_{CD8^+} repertoire (Spiotto *et al.*, 2003). T_{CD8^+} specific to these self-antigens have been shown to infiltrate, recognize, and clear tumors upon adoptive transfer. Self-reactivity in the form of skin localization and melanocyte destruction (resulting in vitiligo) has also been observed under these conditions (Dudley *et al.*, 2002; Yee *et al.*, 2000). Unlike most tumor antigens, the cancer-testis tumor antigens lack expression in normal tissues (apart from germ cells) and, combined with the fact that many are highly expressed in various tumors, there is optimism that these antigens might be used to stimulate more efficient T_{CD8^+} responses (Scanlan *et al.*, 2002).

A. General Considerations for Effective Cancer Vaccine

Rational vaccine design has also been aided by our increased understanding of other aspects of immunity. Included amongst these are the importance of innate stimulating signals such as ligands for toll-like receptors (Iwasaki and Medzhitov, 2004) from viruses, bacteria, and other pathogens—previously referred to as "danger" signals (Matzinger, 1994). There is also the critical role of $CD4^+$ T cells in generating efficient antigen-specific T_{CD8^+} responses at the various stages of immunity, priming (Bennett *et al.*, 1998), memory cell differentiation (Shedlock and Shen, 2003), and recall responses (Janssen *et al.*, 2003). Thus, it will be important when vaccinating cancer patients to provide danger signals and other immune-enhancing adjuvants along with the immunodominant T helper and T_{CD8^+} determinants to achieve a robust cellular immune response. This might sound relatively trivial but it is likely to be highly complex given the rather limited determinant mapping that has been conducted for the majority of tumor antigens as well as the inherent complexity provided by the polymorphism of HLA molecules within the human population. Principles gleaned from antiviral immunity also suggest that immunodominant T_{CD8^+} will dominate

subdominant T_{CD8^+} and the immunodominant status of a given determinant will be directly linked to the entire repertoire of HLA molecules due to potential allelic immunodomination (Belz *et al.*, 2000a; Schirmbeck *et al.*, 2002; Tourdot and Gould, 2002; Tussey *et al.*, 1995). It will be important to predict which peptides are likely to be the most immunodominant ones in an individual patient based on their HLA phenotype. It is reasonable to believe that many of the peptides thus far used for cancer vaccines do not represent the best IDDs. Alternatively, even if IDDs have been used, the proper T helper determinant(s) might have not always been provided. Both of these factors could have contributed to the relatively poor outcomes observed in most cancer vaccine trials to date (Rosenberg *et al.*, 2004).

B. Targeting Both Tumor and its Stroma

It has also been well-demonstrated in mouse models that cancer vaccines need to consider stromal targeting. Spiotto *et al.* (2004) transferred purified 2C TCR Tg-T_{CD8^+}, which recognize allo-MHC L^d molecules as well as SIYRYYGL complexed to K^b, into B6 mice. The B6-derived MC57 fibroblast sarcoma cell lines were first transfected either with an inducible SIYRYYGL-GFP determinant or L^d then inoculated into B6 mice that received transferred 2C Tg-T_{CD8^+}. The uninduced SIYRYYGL-GFP expressing MC57 clone was well recognized by the 2C T_{CD8^+} *in vitro* but was not rejected *in vivo* and eventually overgrew as a population of antigen loss variants. The induced MC57 clone expressed 26-fold more antigen and was rejected by the transferred 2C Tg-T_{CD8^+}. Tumor rejection in the latter scenario was not due to better killing of the tumor cells directly; rather, it was due to better cross-presentation by bone marrow-derived stromal cells within the established tumor; this did not occur to a sufficient extent when the antigen level was low as in the noninduced transfectants. On the other hand, transfer of tumor cells expressing an allo-MHC L^d molecule, which could only be directly recognized by the 2C Tg-T_{CD8^+}, led to their rapid adaptation by losing the L^d molecule emphasizing the importance of cross-presentation in this model.

Most likely, the bone marrow-derived stroma cells in this study were DCs which constitutively express immunoproteasome (Macagno *et al.*, 2001) and are well equipped to cross-present tumor antigens. It is important to note that tumor cells are likely to express the housekeeping proteasome in the absence of cytokines such as IFNγ and TNFα. It might therefore be important to focus on IDDs that can be generated by both the housekeeping and the immunoproteasome to ensure successful tumor and stroma targeting.

C. Targeting Multiple IDDs

MHV quickly escapes from the immunodominant T_{CD8^+} response to the surface glycoprotein-derived IDD $D^b/S_{510-518}$. However, when Kim and Perlman introduced an LCMV-derived IDD, D^b/GP_{33-41}, into MHV vigorous T_{CD8^+} responses to both IDDs were observed and that was associated with protection from clinical disease and lack of mutation in the $S_{510-518}$ sequence. A control MHV strain with an anchor mutation for the GP_{33-41} determinant not only failed to stimulate any GP_{33-41}-specific T_{CD8^+} but was also associated with selection for viral mutations (Kim and Perlman, 2003), confirming the importance of the extra immunodominant T_{CD8^+} response in controlling the infection. The exact mechanisms why more than one immunodominant T_{CD8^+} response should diminish persistent viral infection, clinical disease, and minimize T_{CD8^+} escape are unclear. However, this was certainly related to T_{CD8^+} function during *early* infection since the inserted *GP_{33-41}* gene fragments, both the wild-type (WT) and the mutated, were deleted by the viruses about 20 days postinfection (Kim and Perlman, 2003). Thus, it might be useful to either vaccinate with multiple immunodominant T_{CD8^+} determinants or adoptively transfer such T cells into tumor-bearing patients, either as a virally transduced autologous T_{CD8^+} population (Hughes *et al.*, 2005; Zhao *et al.*, 2005) or as *in vitro* expanded T_{CD8^+} from tumor infiltrating lymphocytes (Dudley *et al.*, 2002).

D. Avoiding Immunodomination

For tumors that escape immunodominant T_{CD8^+} through the downregulation of the MHC molecules that present IDDs, it might be better to design vaccination strategies that purposely avoid stimulating the immunodominant T_{CD8^+}, which have no function against the tumor cells with MHC loss (Van Waes *et al.*, 1996). This would involve stimulating subdominant T_{CD8^+} restricted by other HLA molecules still expressed on the tumor cells, either using peptide-based vaccines or a modified full-length protein containing mutated IDD(s). Alternatively, one could target other full-length tumor antigens against which little response was previously stimulated. This might lead to the induction of novel immunodominant T_{CD8^+} and the reestablishment of effective tumor immunosurveillance.

Avoiding such immunodomination might also be achieved by effectively stimulating individual immunodominant and subdominant T_{CD8^+}. Such an approach might work better as a peptide-based (or determinant-specific) vaccine in which individual peptides are targeted to separate APC rather than a "polytope" strategy, which could also lead to significant

immunodomination due to delivery of all the IDDs and SDDs to the same APC (Palmowski *et al.*, 2002; Sandberg *et al.*, 1998). The goal under these vaccine conditions is to prime or boost the immunodominant and subdominant T_{CD8^+} equivalently on the grounds that these T_{CD8^+} could simultaneously exert pressure on tumor cells. The strategy might be even better if these T_{CD8^+} are restricted by different MHC molecules making escape more difficult.

Many investigators have gone back to whole tumor vaccine strategies incorporating DCs, either tumor-DC fusions or DCs loaded with tumor lysates (Avigan, 2003; Kao *et al.*, 2005). Because a single tumor cell can express multiple tumor antigens, it is argued that this kind of immunization might be more beneficial because of potentially wider tumor antigen targeting as for fused DC-tumor cells *in vitro* (Parkhurst *et al.*, 2003). However, it is not clear whether this approach might also be influenced by immunodomination *in vivo*, especially if allogeneic tumor cells are used. Additionally, when allogeneic tumor cells are used as an antigen source, it is highly possible that the host tumor cells and the allogeneic tumor cells do not share the same tumor antigens, which could lead to T_{CD8^+} priming against irrelevant tumor antigens and these T_{CD8^+} might dominate the responses to the antigens expressed on the patient's tumor cells. Similarly, allogeneic T_{CD8^+} responses, or responses to MiHAs, might also dominate (Rowley and Stach, 1993).

Another possibility is to actively interfere with immunodominant T_{CD8^+} that no longer eliminate tumor cells due to MHC-I loss but continue to kill DCs cross-presenting the IDD (Crowe *et al.*, 2003; Schreiber *et al.*, 2002). A similar context could arise if the relevant IDDs are poorly presented on tumor cells yet effectively presented by DCs due to their dependence on the immunoproteasome rather than the housekeeping counterpart (Basler *et al.*, 2004; Morel *et al.*, 2000; Schultz *et al.*, 2002; Sijts *et al.*, 2000). Such immunotherapy could be mediated by antigen-specific T_{CD8^+} depletion through bead-coupled multimers, MHC/peptide complexes carrying a mutated $\alpha 3$ domain, or engaging T_{CD8^+} with terminally differentiated macrophages upon activation (Munn *et al.*, 1996; Russell, 1995; Xu *et al.*, 2001a). Such strategies are fraught with risk, however, since it will be difficult to exclude the possibility that the immunodominant T_{CD8^+} might still be exerting a beneficial effect as might be the case if a subpopulation of tumor cells still retains the relevant MHC-I and presents the IDD peptide.

E. Alternating Prime-Boost Vaccines

If viral vectors are used for cancer vaccines, then avoiding repetitive use of the same vector would appear wise since it would avoid not only the formation of neutralizing antibodies to the virus but also immunodomination

of vector-specific T_{CD8^+} over tumor-specific T_{CD8^+} (Harrington *et al.*, 2002; Sharpe *et al.*, 2001). This caution should extend to cancer vaccines that use virally infected tumor cells as a means of providing a "danger" signal (Karcher *et al.*, 2004). In such instances, the anti-viral response could easily become immunodominant over the anti-tumor responses of interest. It has been shown in mouse models that prime-boost strategies with homologous vaccines, including various viral vectors, induced limited numbers of antigen-specific T_{CD8^+}. However, under similar conditions using a heterologous prime-boost approach, activated antigen-specific T_{CD8^+} could be 100-fold greater (Palmowski *et al.*, 2002).

VIII. CONCLUSIONS

The development of effective, cellular immunity-based viral or cancer vaccines will depend upon our general understanding of the mechanisms that determine immunodominance and immunodomination. Taken together, lessons learned from anti-viral immunity and other model CTL systems are highly likely to be relevant in anti-tumor immunity. Optimizing the balance of dominant and subdominant responses will require a detailed understanding of key target antigens and host HLA types. In addition, the technology for analyzing and monitoring cellular immunity will need to become more robust before customized cellular immunotherapy becomes more routine for many laboratories. Ultimately, we need to be able to control immunodomination via various *in vitro* and *in vivo* manipulations both at the APC level as well as at the specific T_{CD8^+} level.

In addition, it is clear that there are many other important considerations that impact on the design of nonviral cancer vaccines. For instance, the schedule and frequency of any vaccine must be optimized. The vaccine route, dose and delivery vector may significantly affect T_{CD8^+} priming (Estcourt *et al.*, 2005; Harrington *et al.*, 2002). Immunosuppression should also be avoided. In this regard, we note that the effect of regulatory $CD4^+$ T cells (Treg) on antigen-specific T_{CD8^+} cells has not been discussed in this chapter. Given, it has been demonstrated that intratumoral depletion of $CD4^+$ T cells restores anti-tumor immunity (Yu *et al.*, 2005) and depletion of Treg cells enhances direct- and cross-priming (Haeryfar *et al.*, 2005), effective strategies to deal with Treg may well be critical for initiating successful anti-tumor immunity. On the other hand, the identification of tumor-Ag-specific Treg cells may indicate that a full-length tumor-Ag-based vaccine could have its own intrinsic limitation (Wang *et al.*, 2004). Finally, vaccine adjuvants will also need to be optimized. Thus, a successful cancer vaccine of the future may rely upon incorporating potent innate danger

signals, or compounds that efficiently target antigen both to the desired APC through specific surface molecules (Hart *et al.*, 2004; Rohrbach *et al.*, 2005) as well as to the optimal antigen-processing pathway (Schnurr *et al.*, 2005).

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NK Cell Receptors as Tools in Cancer Immunotherapy

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Natural killer (NK) cells were identified 30 years ago based on their ability to "spontaneously" kill tumor cells. The basis for NK cell recognition and activation is due to a variety of receptors that bind to specific ligands on tumor cells and normal cells. Some of these receptors have the ability to inhibit NK cell function, and other receptors activate NK cell function. Therapeutic strategies for cancer therapy are being developed based on preventing NK cell inhibition or using NK cell receptors to activate NK cells or T cells. There are intriguing clinical data from studies of bone marrow transplantation that support the idea that preventing NK cell inhibition by human leukocyte antigen (HLA) class I molecules can be a means to promote graft-versus-leukemia (GvL) effects and limit graft-versus-host disease (GvHD) in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients. Experimental findings also support the blockade of NK cell inhibitory receptors as a way to protect against leukemia relapse."f

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It may be possible to use our knowledge of NK cell activating receptors and their ligands to immunize patients with modified tumor cells to promote beneficial NK cell responses and development of host antitumor cytotoxic T lymphocytes (CTLs). Finally, new data support the idea of using modified NK cell receptors as a means to target patients' T cells against their own tumor cells and induce long-term immunity against them. Tumors are essentially tissues that have overcome normal regulation mechanisms, and therefore the ability to distinguish normal cells from abnormal cells is a key part of selectively attacking tumor cells. NK cells have various receptor systems designed to recognize infected and abnormal cells. Understanding NK cell receptors and their recognition mechanisms provides new tools for the development of immunotherapies against cancer.
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I. INTRODUCTION

Natural killer cells, or NK cells, were first described in 1975 as cells found in murine spleen or human blood that "spontaneously" killed tumor cells (Herberman *et al.*, 1975a,b; Kiessling *et al.*, 1975a,b). These findings of natural immune activity against tumor cells generated interest that this killing of tumor cells could be exploited as a potent therapy in the fight against human cancer. In addition to activity against tumor cells, NK cells were responsible for an initial rapid rejection of allogeneic and F1 hybrid bone marrow grafts (Bennett, 1987). NK cells are lymphoid cells that do not rearrange or express T-cell receptor or immunoglobulin genes. NK cell development is dependent upon the bone marrow, and NK cells are found in animals deficient in both T and B lymphocytes (Trinchieri, 1989). Originally NK activity was a function of large granular lymphocytes (LGLs) also referred to as "null cells" due to the lack of specific markers to identify these cells. NK1.1 was identified as a specific marker on murine NK cells in some strains of mice and can be used along with other markers to identify them (Koo and Peppard, 1984). Human NK cells are often identified as $CD56^+$, $CD3^-$ cells, many of which also express CD16. A large number of different cell-surface markers can now be used to delineate specific subsets of human NK cells (Jonges *et al.*, 2001).

NK cells are a part of the innate immune system and are capable of promoting specific responses of the adaptive immune system (Biron and Brossay, 2001; Degli-Esposti and Smyth, 2005; Raulet, 2004; Smyth *et al.*, 2002). They are found in the hematopoietic tissues and in most major organs, including lung, liver, and endometrium. NK cells respond rapidly and do not display an immunological memory response. The response of NK cells is limited, so that a large tumor or bone marrow challenge can overwhelm this line of defense. NK cells produce high amounts of interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) upon

activation. They have also been demonstrated to produce a variety of other cytokines, including TNF-α, IL-8, IL-10, and several chemokines. Due to their production of cytokines and lysis of tumor cells, NK cells promote antigen presentation, Th1 cell development, macrophage activation, and cytotoxic T lymphocyte (CTL) development. Thus, NK cells are a link between rapid innate responses and the development of proinflammatory responses and cytolytic T cells.

Much has been discovered during the last 30 years about how NK cells recognize and attack tumor cells. Initially tumor cells were classified as being either NK susceptible or resistant, although the molecular basis for this difference was unknown. Today, many receptors on NK cells and some of their ligands have been identified (Bottino *et al.*, 2005; Kumar and McNerney, 2005; Lanier, 2005). Some of these receptors activate NK cells, and others inhibit NK cell function. The molecular understanding of how NK cells interact with tumor cells has provided the foundation for development of strategies that use NK cells and their receptors to treat human cancer. This chapter briefly summarizes the discoveries of NK cell receptors and addresses the use of NK receptors and NK cell recognition of tumors in cancer therapy.

II. NK CELL RECOGNITION OF TUMOR CELLS

A. Types of Receptor Recognition

NK cells express a variety of different receptors that, upon binding to specific ligands, have the ability to activate or inactivate NK cells. Some of these receptors are expressed on many other cell types as well, some are restricted to a few cell types (including NK cells) or states of activation, and some are exclusively expressed on NK cells (Kumar and McNerney, 2005; Lanier, 2005; Moretta *et al.*, 2001). Although many of these receptors were first identified on NK cells, they are expressed on other immune cells and may function differently on those cells.

NK cell receptors have the ability to recognize different types of ligands. The *inhibitory receptors* recognize self-proteins and cause the inhibition of NK cell activation. The ligands for these receptors are found on the surface of many normal cells and may be downregulated upon tumor transformation or infection. NK cells express *activating receptors* that bind to self-proteins that are expressed or upregulated upon tumor transformation or infection, thus providing a means to identify aberrant cells. Another type of activating receptor on NK cells recognizes ligands derived from microorganisms themselves. These receptors directly bind to pathogen molecules on the organism or pathogen-encoded molecules expressed on infected cells.

Because cancer is the aberrant growth and loss of control of normal cells, NK receptors that bind to self-proteins are the receptors that are most likely to be of use in cancer therapy.

B. "Missing Self" Recognition

A major breakthrough in understanding NK cell recognition came not only from examining why NK cells killed particular tumor cells, but also from studying why NK cells did not lyse certain tumor cells. Klas Kärre made the astute observation that the ability of NK cells to kill a particular tumor cell was inversely related to the tumor cell's expression of MHC class I (Kärre *et al.*, 1986). He made the hypothesis that NK cells were able to kill many cells readily, but those cells that expressed MHC class I were able to turn NK cells off. Dr. Kärre proposed the "missing self" hypothesis that stated that NK cells can kill any normal or tumor cell, but the expression of MHC class I prevented NK cells from killing normal cells (Ljunggren and Kärre, 1990). This hypothesis was important in that it linked the loss of MHC class I expression on tumor cells, believed to occur as a consequence of T-cell recognition of tumor antigens, to an increase in susceptibility to NK cell lysis. The missing self hypothesis also explained the phenomenon of F1 hybrid resistance. Hybrid resistance is an exception to the Laws of Transplantation where F1 hybrids often reject parental bone marrow, even though the parental bone marrow expresses no foreign antigens (Bennett, 1987). The rejection of parental bone marrow is NK cell dependent, and according to the missing self hypothesis, NK cells reject parental bone marrow because the donor bone marrow cells fail to engage inhibitory receptors on all NK cells due to the fact that they do not express both sets of parental MHC class I molecules. In 1992, Karlhofer and Yokoyama identified a receptor on murine NK cells, now known as Ly49A, which recognized MHC class I (Karlhofer *et al.*, 1992). Inhibitory receptors that bind to MHC class I have also been identified on human and rat NK cells (Moretta *et al.*, 2001; Rolstad *et al.*, 2001).

However, the missing self hypothesis presents one conundrum: although hematopoietic cells express high levels of MHC class I molecules on their cell surface and are resistant to NK cells, many other cells in the body express rather low levels of MHC class I but are also resistant to NK cells. Why is there no NK cell attack against these normal cells that express low levels of MHC class I? One possible explanation is that there are non-MHC molecules that inhibit NK cell function, and another idea is that cells with low-MHC class I expression lack the ability to activate NK cells. As it turns out, both explanations are correct. At that time, the mechanisms for NK cell activation were largely unknown. Many activating receptors have been

identified on NK cells; some of these are structurally related to inhibitory receptors and others are quite different proteins (Bottino *et al*., 2005; Lanier, 2005). There is also a growing list of non-MHC molecules that inhibit NK cell function by binding to receptors on NK cells (Kumar and McNerney, 2005). It is now generally accepted that it is the balance of activating and inhibitory signals that determines whether an NK cell responds upon encounter with another cell. Thus, it is possible to shift responses in favor of NK cell activation by increasing activating receptor signals or by blocking inhibitory receptors. Both of these strategies are being considered for cancer therapy.

C. Clinical Findings

Even though the mechanisms of NK cell recognition and activation were unclear, NK cells have been used in cancer therapy. Passive immunotherapies with activated tumor-infiltrating lymphocytes (TILs) or lymphokine activated killer (LAK) cells have been successful as treatments for some melanoma and renal tumor patients (Dudley and Rosenberg, 2003; Rosenberg *et al*., 1994; Yannelli and Wroblewski, 2004). In the initial studies, effector cells were complex cell populations with broad and usually undefined specificities. The distinct roles of NK cells, tumor-infiltrating T cells, and macrophages in these trials were unclear. Purified NK cells show high-LAK activity after stimulation with IL-2. Both LAK and activated NK (A-NK) cells have been used in clinical trials. In general, LAK cells did not migrate to tumor sites *in vivo*, and severe side effects associated with high-dose IL-2 therapy have limited the use of this approach. In some cases IL-2 therapy has resulted in the regression of established tumors in patients with malignant melanoma or renal cancer (Yannelli and Wroblewski, 2004). Although there were some significant responses in selected patients with advanced melanoma, the overall responses were disappointing in these early clinical trials. However, these initial studies were important steps in the development of better immunotherapies for cancer. It has become clear that the use of tumor-specific lymphocytes, their migration to tumor sites, and their persistence *in vivo* are key for the success of adoptive cell-based therapies (Gattinoni *et al*., 2005).

III. NK CELL RECEPTORS

Since the early 1990s, a large number of molecules have been identified as NK cell receptors. Many of these molecules are now known to be expressed on other cell types, in particular T-cell subsets, but they are often referred to

as NK cell receptors due to their initial identification on NK cells and their association with innate immune responses. We will briefly review both inhibitory and activating receptors found on NK cells. For more extensive details on NK cell receptors and their function in immunity, see these reviews (Kumar and McNerney, 2005; Lanier, 2005; Moretta and Moretta, 2004a; Raulet, 2003, 2004).

A. Inhibitory Receptors

Inhibitory receptors are able to prevent the activation of NK cells and have been thought of as fail-safe mechanisms to prevent an attack on normal cells and tissues. In general, these receptors express one or more immunoreceptor tyrosine-based inhibition motifs (ITIM), and they recruit SHP1, SHP2, and/or SHIP proteins upon binding to their ligands (Lanier, 2005). These phosphatases prevent the activation of cellular signaling cascades by inhibiting phosphorylation of proteins. There are two major categories of inhibitory receptors based on their ligand specificity: MHC class I receptors and non-MHC class I receptors (Table I).

B. MHC Class I-Inhibitory Receptors

MHC class I-inhibitory receptors were predicted by the work of Kärre and colleagues before they were discovered. The first identified inhibitory receptor, Ly49 (now known as Ly49A), is specific for an MHC class I molecule, H-2D^d (Karlhofer *et al.*, 1992). Ly49A is the prototype for an extended family of receptors that are C-type lectin-like proteins, which are encoded in the NK gene complex, a cluster of genes predominately expressed by NK cells (Yokoyama and Plougastel, 2003). Many of the Ly49 proteins contain an ITIM and are expressed on the cell surface as homodimers. Ly49 receptor expression is downregulated on the surface of NK cells in mice that express MHC class I ligands for those receptors (Held and Raulet, 1997; Karlhofer *et al.*, 1994; Olsson *et al.*, 1995). Although expressed at a reduced amount, the number of receptors is sufficient to prevent activation of NK cells against host cells that express their MHC class I ligand and to allow NK cell activation when even a small reduction in MHC class I occurs (Olsson-Alheim *et al.*, 1997). The Ly49 repertoire is dependent upon the genetic background strain, and the Ly49 expression patterns have been defined for a large number of inbred mice (Anderson *et al.*, 2005; Kane *et al.*, 2004; Makrigiannis and Anderson, 2000). Rat NK cells also express Ly49 receptors that bind to MHC class I molecules and function to inhibit NK cell responses (Rolstad *et al.*, 2001). In addition, Ly49 receptors

Table I NK Cell Inhibitory Receptors

Receptor	Species	Receptor expression[a]	Ligand(s)[b]	References
2B4	Human/ mouse	NK cells and $CD8^+$ T cells	CD48	Brown *et al.*, 1998; Garni-Wagner *et al.*, 1993; Valiante and Trinchieri, 1993
CD94/ NKG2A	Human/ mouse	NK cells and T-cell subsets	HLA-E/$Qa\text{-}1^b$	Braud *et al.*, 1998; Vance *et al.*, 1998
CEACAM1	Human/ mouse	Many cell types	CEACAM1, CEACAM5	Markel *et al.*, 2002; Moller *et al.*, 1996
KIR2DL1	Human	NK cells and T-cell subsets	$HLA\text{-}C2^{Lys80}$	Colonna *et al.*, 1993; Moretta *et al.*, 1990b
KIR2DL2/3	Human	NK cells and T-cell subsets	$HLA\text{-}C1^{Asn80}$	Colonna *et al.*, 1993; Moretta *et al.*, 1990a
KIR2DL4	Human	NK cells and T-cell subsets	HLA-G?	Rajagopalan and Long, 1999; Yusa *et al.*, 2002
KIR3DL1	Human	NK cells and T-cell subsets	HLA-Bw4	Cella *et al.*, 1994; Litwin *et al.*, 1994
KIR3DL2	Human	NK cells and T-cell subsets	HLA-A3,-A11	Dohring *et al.*, 1996; Hansasuta *et al.*, 2004
KLRG1	Human/ mouse	Subsets of NK cells, basophils, activated T cells, mast cells	Unknown	Butcher *et al.*, 1998; Corral *et al.*, 2000

(*continued*)

Table I (*Continued*)

Receptor	Species	Receptor expression[a]	Ligand(s)[b]	References
Ly49A	Mouse	NK cells and T-cell subsets	H-2D^d, H-2D^k, H-2D^p	Karlhofer *et al.*, 1992; Olsson-Alheim *et al.*, 1999
Ly49C	Mouse	NK cells and T-cell subsets	H-2K^b (and other H-2K, H-2D alleles)	Brennan *et al.*, 1996; Yu *et al.*, 1996
Ly49G2	Mouse	NK cells and T-cell subsets	H-2D^d, H-2L^d	Mason *et al.*, 1995
NKR-P1B/D	Mouse	NK cells	CLR-B	Carlyle *et al.*, 1999, 2004; Iizuka *et al.*, 2003; Kung *et al.*, 1999
SIGLEC7/9	Human	NK cells, monocytes, and subsets of CD8$^+$ T cells	Sialic acid	Falco *et al.*, 1999; Ito *et al.*, 2001; Nicoll *et al.*, 1999; Zhang *et al.*, 2000

[a]Ly49 and KIR receptors are expressed in a variegated pattern; multiple receptors are expressed by each cell, but not all cells express each receptor (Raulet *et al.*, 2001).
[b]Specificity for C57BL/6 allele of Ly49A, Ly49C, and Ly49G2.

are expressed on NKT cells, memory phenotype $CD8^+$ T cells, and a subset of thymocytes. Ly49 receptors have been implicated in T-cell repertoire development and peripheral T-cell responses, but the role of these receptors on T cells remains unclear (Coles *et al.*, 2000; Fahlen *et al.*, 2000; Kane *et al.*, 2004; Ugolini *et al.*, 2001; Vivier and Anfossi, 2004). They have been shown to inhibit T-cell function, although this inhibition can be overcome by increasing T-cell receptor activation signals (Oberg *et al.*, 2000). Thus, Ly49 inhibitory receptors are believed to be modulators of immune cell function rather than off/on switches.

The human equivalent to Ly49 receptors are the killer cell immunoglobulin-like receptors (KIR). These molecules are members of the immunoglobulin superfamily but function in a similar manner as Ly49 receptors. KIRs have been identified that bind to HLA-C, HLA-B, or HLA-A class I molecules (Moretta and Moretta, 2004a). Most of the studies have focused on KIRs that recognize HLA-C molecules, which are the KIRs expressed at the highest numbers on human NK cells. Polymorphisms in the alpha-1 domain of HLA-C define two major groups of HLA-C molecules that are recognized by different isoforms of the KIR2D (Colonna *et al.*, 1993; Lanier, 2005). KIR haplotypes have been defined in many human populations, and there are wide differences in the number of inhibitory and activating genes between groups (Hsu *et al.*, 2002; Uhrberg *et al.*, 1997; Yawata *et al.*, 2002). These findings have generated intriguing data on how KIRs and MHC class I molecules may have evolved together (Moretta and Moretta, 2004a; Vilches and Parham, 2002).

Not all human NK cells express KIRs, and most of those that do not express CD94/NKG2A dimers. The proportion of NK cells that express KIRs or CD94/NKG2A varies between individuals (Valiante *et al.*, 1997). CD94/NKG2A inhibitory receptors bind to HLA-E, a nonclassic HLA class I molecule (Braud *et al.*, 1998). HLA-E is widely expressed on many cell types and presents peptides derived from signal peptides of HLA class I molecules (O'Callaghan and Bell, 1998). Thus, a cell that expresses HLA molecules will also produce peptides that allow the cell-surface expression of HLA-E. Murine NK cells also express CD94/NKG2A dimers, and these bind to Qa-1, a nonclassic MHC molecule (Vance *et al.*, 1998). *CD94* and *NKG2* genes have little polymorphism, unlike *KIR* or *Ly49* genes. Thus, there are three sets of receptors that inhibit NK cell function and recognize classic and nonclassic MHC class I molecules.

C. Non-MHC–Binding Inhibitory Receptors

The non-MHC–binding inhibitory receptors are a diverse group of proteins; some are encoded by genes within the NK gene complex, and others

are encoded by genes at different loci (Kumar and McNerney, 2005). In general, these receptors are present on NK cells and many other cell types, and their ligands, if known, are widely expressed. The role of these molecules and their ligands in the regulation of NK cell function remains unclear. NKR-P1 molecules are a family of receptors, two of which have an inhibitory function on murine NK cells (Carlyle *et al.*, 1999; Kung *et al.*, 1999). These molecules are mostly restricted in expression to NK cells and some T-cell subsets. NKRP1B and NKRP1D bind to osteoclast inhibitory ligand (Ocil; also called Clr-B) (Carlyle *et al.*, 2004; Iizuka *et al.*, 2003), which is expressed in lymphoid and nonlymphoid tissue (Plougastel *et al.*, 2001). Target cells that express Ocil/Clr-B can inhibit NK cell cytotoxicity, indicating that this is a ligand for inhibitory receptors.

2B4 binds to CD48 in mice and humans (Brown *et al.*, 1998). CD48 is found on all hematopoietic cells and human endothelial cells. 2B4 is expressed on all NK cells as well as memory $CD8^{+}$ T cells, monocytes, granulocytes, and mast cells (Boles *et al.*, 2001; Kubota, 2002; Munitz *et al.*, 2005). 2B4 contains immunoreceptor tyrosine-based switch motifs (ITSMs) in the cytoplasmic tail, which allows 2B4 to interact with SLAM-associated protein (SAP) (Boles *et al.*, 2001). 2B4 has been functionally described as both an inhibitory and activating receptor, and this dual role is now understood to be due to the differential recruitment of signaling components depending on the involvement of other activating receptors (McNerney *et al.*, 2005; Schatzle *et al.*, 1999). Mice deficient in 2B4 eliminate $CD48^{+}$ tumor cells better than wild-type mice indicating that 2B4 likely functions as an inhibitory receptor *in vivo* (Lee *et al.*, 2004b; Vaidya *et al.*, 2005).

Several other inhibitory receptors have been identified on NK cells. Humans have 11 sialic-acid–binding immunoglobulin-like lectins (SIGLECs), and human NK cells express SIGLEC7 and SIGLEC9 (Crocker and Varki, 2001). These molecules bind to sialic-acid residues and can inhibit NK cell function (Falco *et al.*, 1999; Nicoll *et al.*, 1999). KLRG1 is a receptor associated with terminally differentiated NK cells and T cells (Robbins *et al.*, 2004). The ligand for KLRG1 is unknown, but this receptor contains an ITIM and can inhibit NK cell function upon antibody-mediated cross-linking (Robbins *et al.*, 2002). LAIR-1 is expressed by human NK cells, in addition to other cell types (Meyaard, 1999). LAIR-1 contains ITIMs in its cytoplasmic domain and can inhibit NK cell activation, although the ligand for LAIR-1 is not known (Meyaard *et al.*, 1997). There are many inhibitory receptors expressed on murine and human NK cells, including some not mentioned here (Kumar and McNerney, 2005). Due to the expression of these inhibitory receptors on many other cell types, it may be difficult to achieve selective immune activation through inhibition of these molecules. As the ligands for these receptors are identified and their role in

biological responses becomes better understood, new strategies may develop to allow targeting of these molecules. At this stage it remains premature to propose targeting the function of these receptors for therapeutic purposes.

D. Activating Receptors

NK cells require external signals to begin the process of cell activation, which usually occurs via one (or several) triggering receptors. A number of receptors have been identified that allow NK cells to become activated. In some cases, these receptors bind to the product of microorganisms, but many of the activation receptors bind to proteins that are encoded by host genes (Table II).

E. Natural Cytotoxicity Receptors

Natural cytotoxicity receptors (NCRs) are unique for NK cells and account for much of the NK cell-specific recognition of tumor cells (Moretta *et al.*, 2001). Three major proteins that account for natural cytotoxicity have been identified on human NK cells. Two of these molecules, NKp46 and NKp30, are constitutively expressed on human NK cells, while NKp44 is expressed on A-NK cells. These proteins are members of the immunoglobulin superfamily and associate with adapter proteins that contain immunotyrosine-based activation motifs (ITAMs) in their cytoplasmic domains and recruit kinases upon ligand binding (Moretta and Moretta, 2004b). The ligands for the NCRs have not yet been identified, but there is some evidence that NKp46 and NKp44 may interact with viral hemagglutinins (Arnon *et al.*, 2001; Bottino *et al.*, 2005). NK cell cytotoxicity can be blocked by using antibodies against these receptors *in vitro*. Human NK cell clones have been shown to express either high levels of NCRs (NCR^{bright}) or low levels of NCRs (NCR^{dull}), and the extent of lysis of tumor cells correlated with NCR density (Bottino *et al.*, 2005; Moretta *et al.*, 2001). Cytotoxicity of target cells by NCR^{dull} NK cells is due to the use of other receptors, such as NKG2D. One or more of these NCRs may be involved in the recognition and lysis of a particular tumor cell. Thus, to obtain complete blockade of NK cell lysis, antibodies to multiple NCRs are often required. Although the ligands for the NCRs are poorly defined, antibody blockade studies suggest that different tumor cells express different amounts of ligands for these three receptors. In addition to tumor lysis, NKp30 has been shown to be important for the interaction of NK cells and dendritic cells (DCs) (Castriconi *et al.*, 2003; Ferlazzo *et al.*, 2002).

Table II NK Cell Activating Receptors

Receptor	Species	Receptor expression[a]	Ligand(s)	References
CD16	Human/mouse	Most NK cells	IgGs	Perussia *et al.*, 1989; Vivier *et al.*, 1991
CD94-NKG2C/E	Human/mouse	NK cells and T-cell subsets	HLA-E/Qa-1[b]	Borrego *et al.*, 1998; Lazetic *et al.*, 1996; Vance *et al.*, 1999
DNAM-1	Human	NK cells, T cells, monocytes, B cells	PVR and Nectin	Bottino *et al.*, 2003; Shibuya *et al.*, 1996
KIR2/3DS1–5	Human	NK cells and T-cell subsets	HLA-C	Katz *et al.*, 2001; Lanier, 2005; Olcese *et al.*, 1997
Ly49D	Mouse	NK cells and T-cell subsets	H-2D[d]	Mason *et al.*, 2000; Smith *et al.*, 1998
Ly49H	Mouse	NK cells and T-cell subsets	MCMV m157	Smith *et al.*, 1998, 2002
NKG2D	Human/mouse	NK cells, some NKT cells, $CD8^+$ and $\gamma\delta$ T cells in humans, activated/memory $CD8^+$ in mice	MICA, MICB, ULBP1–4, Rae1, H60, MULT1	Bauer *et al.*, 1999; Diefenbach *et al.*, 2000; Houchins *et al.*, 1991
NKp46	Human/mouse	NK cells	Viral HA?	Biassoni *et al.*, 1999; Mandelboim *et al.*, 2001; Sivori *et al.*, 1997
NKp44	Human	NK cells after IL-2 stimulation	Viral HA?	Arnon *et al.*, 2001; Vitale *et al.*, 1998
NKp30	Human	NK cells		Pende *et al.*, 1999
NKR-P1A	Human/mouse	Subset of NK cells and T cells		Lanier *et al.*, 1994; Ryan *et al.*, 1995
NKR-P1C	Mouse	NK cells and NKT cells		Arase *et al.*, 1996; Koo and Peppard, 1984
NKR-P1F	Mouse	Activated NK cells	Clr-g	Plougastel *et al.*, 2001

[a]Ly49 and KIR receptors are expressed in a variegated pattern; multiple receptors are expressed by each cell, but not all cells express each receptor (Raulet *et al.*, 2001).

F. NKG2D

NKG2D is expressed as a homodimer on the cell surface of NK cells and associates with adapter proteins, Dap10 or Dap12 (also called KARAP) (Diefenbach *et al.*, 2002; Gilfillan *et al.*, 2002; Wu *et al.*, 1999). Human $CD8^+$ T cells express NKG2D, but in the mouse, only activated and memory $CD8^+$ T cells express this receptor (Bauer *et al.*, 1999; Jamieson *et al.*, 2002; Raulet, 2003). Most $CD4^+$ cells do not express NKG2D, although data from rheumatoid arthritis patients suggest that $CD4^+$ T cells in synovial fluid may upregulate NKG2D expression (Groh *et al.*, 2003). All $\gamma\delta$T cells in humans express NKG2D, but only specific subsets of murine $\gamma\delta$T cells express this receptor. NKG2D surface expression is dependent upon the expression of Dap10 or Dap12, which mediate activation signals when NKG2D binds to one of its ligands on target cells (Cerwenka and Lanier, 2003; Raulet, 2003). Dap10 is expressed by NK cells and T cells and signals through the PI3-kinase (PI3K) pathway. Thus, signals via NKG2D-Dap10 in T cells are analogous, but not identical, to CD28-mediated signals and can provide costimulation, but not a primary activation signal, to T cells (Ehrlich *et al.*, 2005; Gilfillan *et al.*, 2002; Groh *et al.*, 2001; Maasho *et al.*, 2005; Markiewicz *et al.*, 2005). Dap12 is expressed by NK cells and contains an ITAM that can deliver a direct activation signal to mediate cytotoxicity of target cells (Lanier *et al.*, 1998; Tomasello and Vivier, 2005).

NKG2D in murine cells can be expressed in a short form (NKG2D-S) or a long form (NKG2D-L), differentiated by the size of the cytoplasmic tail (Diefenbach *et al.*, 2002). Both forms of NKG2D bind to the same external ligands. However, NKG2D-S can associate with either Dap10 or Dap12, but NKG2D-L can only pair with Dap10 (Diefenbach *et al.*, 2002; Gilfillan *et al.*, 2002). Human NKG2D does not associate with Dap12 (Rosen *et al.*, 2004) and thus primarily signals via Dap10 activation of PI3K (Wu *et al.*, 1999). However, cross-linking of NKG2D on human blood NK cells activates a variety of signaling pathways (Billadeau *et al.*, 2003; Sutherland *et al.*, 2002). T cells express Dap10, so NKG2D signals provide costimulation rather than a primary activation signal in T cells, although there are reports of TCR-independent cytotoxicity mediated by NKG2D (Maccalli *et al.*, 2003; Verneris *et al.*, 2004). Because at least some NKG2D ligands can be upregulated by infection or transformation, the NKG2D receptor–ligand system provides a means for a cell to use an internal mechanism to flag itself to the immune system when it becomes abnormal (Raulet, 2003).

Ligands for human NKG2D include MHC class I-related proteins A and B (MICA and MICB), various UL16-binding proteins (ULBPs), and lymphocyte effector cell toxicity-activating ligand (Letal/ULBP4) (Conejo-Garcia *et al.*, 2003; Cosman *et al.*, 2001; Pende *et al.*, 2002; Sutherland *et al.*,

2002). Ligands for murine NKG2D include retinoic acid early transcript-1 proteins (Rae1α-ε), a minor histocompatibility antigen (H-60), and mouse UL16-binding protein-like transcript (Mult1) (Carayannopoulos *et al.*, 2002; Cerwenka and Lanier, 2001; Diefenbach *et al.*, 2000, 2003). MICA and MICB are related to MHC class I but do not associate with β2m or require TAP1/2 for expression (Bahram, 2000; Groh *et al.*, 1996). NKG2D ligands have been detected on carcinomas derived from colon, breast, prostate, ovary, lung, brain, liver, and kidney (Conejo-Garcia *et al.*, 2004; Diefenbach *et al.*, 2000; Groh *et al.*, 1999; Jinushi *et al.*, 2003; Pende *et al.*, 2002). MICA and MICB have not been found on most normal tissues, with the exception of differentiated gut epithelium (Cerwenka and Lanier, 2003; Groh *et al.*, 1996; Raulet, 2003). Even when MICA or MICB proteins have been detected, these proteins are found in intracellular vesicles and are absent (or at low levels) on the cell surface. Ligands for NKG2D can also be found on lymphomas, leukemias, myelomas, and melanomas (Carbone *et al.*, 2005; Pende *et al.*, 2001, 2002; Salih *et al.*, 2003). In the mouse, ligands for NKG2D have been found on a large number of tumors derived from different tissues. It is important to note that neither all tumors from these tissues express ligands for NKG2D nor do all tumor cells within a tumor necessarily express NKG2D ligands. Data suggest that, in many cases, the process of transformation often leads to expression of ligands for NKG2D, and the induction of MICA and MICB expression is dependent upon DNA damage in cells (Gasser *et al.*, 2005).

G. MHC-Binding Activating Receptors

Some members of the Ly49, KIR, and CD94/NKG2 receptor families have been identified that lack ITIMs in the cytoplasmic tail (Lanier, 2005). These receptors associate with adapter proteins that enable them to activate cells. KIRs have been demonstrated to bind to specific MHC class I molecules and activate NK cells. An intriguing observation is that individuals often express both inhibitory and activating KIRs for the same MHC class I allele. The advantage of expressing seemingly functionally opposed KIRs or how these function together remain unclear. Ly49D and Ly49H on murine NK cells have the ability to activate NK cell cytotoxicity and cytokine production. Ly49D recognizes H-2D^d and xenogeneic tumor cells (George *et al.*, 1999; Idris *et al.*, 1999; Nakamura *et al.*, 1999). Although Ly49D is a functional receptor that can lead to rejection of bone marrow grafts that express H-2D^d, it is unknown whether MHC class I is the only ligand for this receptor. CD94/NKG2C is an activation receptor expressed on human NK cells. The ligand for CD94/NKG2C is unknown but may be nonclassic HLA molecules (Lanier, 2005).

H. Activating Receptors Specific for Microorganism-Derived Molecules

NK cells express receptors that directly bind to molecules derived from microorganisms. These receptors allow NK cells to become activated directly by the presence of pathogens or pathogen-infected cells. NK cells express a number of different toll-like receptors (TLRs). Human blood NK cells have been shown to express several TLRs and respond to TLR agonists (Chalifour, 2004; Hornung *et al.*, 2002; Schmidt *et al.*, 2004; Sivori *et al.*, 2004). It remains controversial whether NK cells can be activated by TLR ligands alone or whether cytokines or cell–cell contact is also required to induce NK cell activation. Ly49H has been shown to bind directly to a *Cytomegalovirus* (CMV)-derived protein and induce activation of murine NK cells (Arase *et al.*, 2002; Smith *et al.*, 2002). Activation and expansion of Ly49H^{+} NK cells has been demonstrated in murine models of CMV infection (Andrews *et al.*, 2003; Daniels *et al.*, 2001; Dokun *et al.*, 2001). The biology of receptors that recognize microorganisms is of great interest in order to understand the role of NK cells during infection.

Perhaps the best studied and understood NK cell activating receptor is the low-affinity Fc receptor, FcRγIII, or CD16. CD16 is expressed by 90% of human NK cells and provides them with the ability to mediate antibody-dependent cellular cytotoxicity (ADCC). Antibodies bind to native foreign antigens and provide a way to target infected cells directly using the specificity of adaptive immunity. CD16 associates with FcRεRIγ or CD3ζ and, upon cross-linking, activates Zap70 and Syk, which initiate various signaling events (Lanier, 1998; Ravetch and Bolland, 2001).

In the last 15 years, a large number of receptors have been identified on human and murine NK cells. Some of these molecules inhibit NK cell function, and others allow NK cells to recognize malignant cells. One can imagine a variety of strategies to block the inhibitory receptors or induce activation via activating receptors as a means to promote antitumor responses.

IV. BLOCKING INHIBITORY RECEPTORS TO ACTIVATE IMMUNITY

One approach to enhance NK cell activity against tumors is to block inhibitory receptors, which regulate NK cell activity. The idea of this approach is to shift the balance from a lack of responsiveness to activation of effector functions by removal of an inhibitory signal. As discussed in the

earlier section, there are many NK cell receptors that can inhibit NK cell function.

A. Experimental Findings

There have been many *in vitro* studies to define inhibitory receptors, demonstrate ligand specificity, and determine receptor structure. *In vivo* studies have focused on receptor function, regulation, and specificity. However, there has been little *in vivo* data on blockade of these receptors in animal tumor models.

One report has demonstrated that blockade of Ly49 receptors can lead to an increased survival using a murine leukemia model (Koh *et al.*, 2001). In this study, $F(ab')_2$ fragments of anti-Ly49C/I (5E6 mAb) were used to treat C57BL/6 (B6) mice that had been injected with syngeneic C1498 leukemia cells intravenously. Ly49C is specific for MHC class I $H\text{-}2K^b$ expressed in B6 mice (Yu *et al.*, 1996). There was an increased survival in mice treated with blocking antibodies against the self-MHC Ly49 receptor compared to mice treated with control antibodies. Other studies demonstrated that blocking NK inhibitory receptors during purging of leukemia cells from bone marrow with syngeneic NK cells or allogeneic NK cells resulted in greater long-term survival in this murine leukemia model (Koh *et al.*, 2002, 2003). These data provide evidence that NK cell receptor blockade is one means to promote greater antileukemia responses *in vivo*, and they are consistent with a model in which NK cells that have self-MHC inhibitory receptors blocked become able to directly kill tumor cells that express MHC class I.

Greater activation of NK cells may lead to potential autoimmune or inflammatory responses. Human NK cell clones have also been demonstrated to inhibit hematopoietic colony formation from progenitor cells *in vitro* (Bellone *et al.*, 1993). In one murine study, there was a decrease in myeloid cell reconstitution in those animals where bone marrow was treated *in vitro* with activated syngeneic NK cells coated with blocking anti-Ly49 receptor antibodies (Koh *et al.*, 2002). The observed decrease in myeloid cells was transient, and the myeloid cell numbers soon recovered to normal. However, a follow-up study using allogeneic NK cells did not observe suppression of hematopoietic reconstitution (Koh *et al.*, 2003). Any effects of inhibitory receptor blockade will be lost as blocking antibodies are depleted *in vivo*, and this strategy may require high levels of blocking antibodies to mediate biological effects. Small molecular weight inhibitors of Ly49 receptors have been reported (Tajima *et al.*, 2004). These small inhibitors may provide another way to specifically block Ly49 receptor function *in vivo*.

Ly49 and KIR molecules are known to be expressed on cells other than NK cells, mainly T-cell subsets. The role of these T cells in mediating antitumor immunity and whether receptor blockade may affect their function are issues that remain to be addressed. MHC-specific inhibitory receptors on NK cells were once thought to be a mechanism to prevent responses against all host cells, but this inhibition may be most important for protection of hematopoietic cells, which express high amounts of MHC class I, rather than other cell types that have low-basal expression of MHC molecules. Many inhibitory receptors, such as 2B4 and KLRG1, have been identified on NK cells, and only some of these receptors have identified ligands. Mice genetically deficient in 2B4 have reduced tumor growth compared to wild-type mice when challenged with tumor cells that express CD48 (Vaidya *et al.*, 2005). The consequences of blockade of these non-MHC–binding inhibitory receptors *in vivo* and their role in NK cell and other immune cell responses against tumor cells remain largely unknown.

An alternative approach to blocking receptors on the cell surface would be to prevent inhibitory receptor signaling. In the case of KIR or Ly49 receptors, there are roles for SHP-1, SHP-2, and SHIP as mediators of signal transduction (Lanier, 2005). These phosphatases are likely involved in the action of several different receptor signaling cascades; therefore, it is unknown whether inhibition of SHP1 or SHP2 would allow selective action on immune cells that express KIR or Ly49 receptors. In addition, these phosphatases may have some redundant function in NK receptor signaling, so inhibition of more than one could be required to achieve a physiological effect. Thus, it may be possible to inhibit the signal mediated by KIR or Ly49 receptors.

Tumor reduction in these initial studies was most likely due to the direct lysis of tumor cells by NK cells. However, the mechanisms that mediated protection from leukemia after *in vivo* Ly49 receptor blockade remain to be determined. Other cell types, such as T-cell subsets, express Ly49 receptors, and these Ly49^{+} cells may also be involved in the antitumor responses mediated by Ly49 receptor blockade. Future studies are needed to clarify these issues. Overall, data suggest that blockade of NK cell inhibitory receptors may be a good way to shift the balance in favor of antitumor immunity.

B. Clinical Findings

NK cell receptor blockade or receptor activation has yet to be used in humans, but KIR haplotypes have been associated with both positive and negative outcomes in autoimmunity, HIV infection, preeclampsia, and hematopoietic stem cell transplantation (HSCT) (Chan *et al.*, 2005;

Flores-Villanueva *et al.*, 2001; Giebel *et al.*, 2005; Hiby *et al.*, 2004; Martin *et al.*, 2002; Momot *et al.*, 2004; Nelson *et al.*, 2004; Ruggeri *et al.*, 2002; Warrington *et al.*, 2001). These findings provide evidence that treatments altering NK cell receptor function may be of benefit to induce specific responses and change clinical outcomes. In particular, data on human NK receptors and their HLA ligands have been generated in clinical studies to suggest a role for these receptors in bone marrow transplantation. Understanding these data and the underlying biological processes will be important to develop improved clinical therapies.

HSCT in combination with radiation and/or chemotherapy has been used successfully for a number of years to treat leukemia. Graft failure, tumor relapse, and graft-versus-host disease (GvHD) remain major clinical problems during recovery from a transplant. Data from Ruggeri *et al.* (2002) demonstrate that it may be possible to use knowledge of donor KIR specificity and patient HLA expression to improve clinical outcomes in AML. This study demonstrated that mismatching between NK cell inhibitory receptors and HLA class I resulted in an increase in survival, decrease in relapse, lower incidence of GvHD, and a decrease in graft failure in patients transplanted with bone marrow from related haploidentical donors. Although the best clinical outcomes occur in HSC transplants between HLA-matched siblings, there are no appropriate donors in many cases due to the lack of a HLA-compatible donor. Haploidentical transplants, where the donor and recipient share one complete HLA haplotype, are often possible and can include KIR–HLA mismatches (Ruggeri *et al.*, 2005). Mismatching for NK cell KIRs and host HLA-C alleles is thought to work because donor NK cells appear soon after transplant and cannot be "turned off" by host cells, due to the KIR mismatch with host HLA. This allows the donor NK cells to kill residual tumor cells (resulting in a decreased rate of tumor relapse) and host DCs (resulting in a decreased incidence of GvHD). Host DCs are key for priming of donor T cells to host antigens. Thus, the KIR–HLA "mismatch" approach allows matching at the most important HLA molecules for T cells, while mismatching for donor KIR and host HLA class I alleles. About 33% of individuals have MHC alleles that would preclude this approach because they express HLA class I molecules that can block all major NK cell inhibitory receptors.

Since the initial report, additional studies have supported the benefits of KIR mismatching during allogeneic HSCT, while other studies did not find KIR haplotypes associated with favorable clinical outcomes (Bignon and Gagne, 2005). How is this disparity to be resolved? Some of the differences between these studies may be due to patient and/or disease characteristics, and transplant regimens. The most favorable outcomes associated with KIR haplotype mismatching occurred for the treatment of AML and CML (Hsu *et al.*, 2005). There did not appear to be a role for KIRs in acute

lymphoblastic leukemia (ALL), a tumor that is generally resistant to NK cell killing. The use of T-cell depletion of the donor graft and limited use of immunosuppression after transplantation were associated with beneficial effects of KIR mismatching (Bignon and Gagne, 2005; Giebel *et al.*, 2003). In some cases, a KIR–HLA mismatch was associated with inferior clinical outcomes (Bishara *et al.*, 2004; Davies *et al.*, 2002; Schaffer *et al.*, 2004). One possible reason may be the expression of certain activating KIRs on donor cells. The expression of KIRs is donor-specific and not related to HLA expression of the donor or recipient (Leung *et al.*, 2004). These findings suggest that it will be necessary to evaluate both the donor KIR haplotype (both inhibitory and activating KIRs) and the presence of host HLA molecules that can be ligands for KIRs on donor NK cells. A study reported that the presence of two activating KIRs in the donor was associated with a reduced rate of leukemia relapse (Verheyden *et al.*, 2005). The best model to predict clinical outcome with HSCT takes into account the potential interactions between KIRs on donor cells and host HLA ligands (Leung *et al.*, 2004).

Based on the experimental findings in murine leukemia models, it may not be necessary to mismatch for KIR–HLA interactions. It might be possible to block inhibitory KIRs during an autologous HSCT. A relatively short-term KIR blockade protocol could provide the same graft-versus-leukemia (GvL) benefits as a KIR–HLA mismatch. Data from both human and murine experiments suggest that the inhibitory function of NK receptors for MHC class I may be partially overcome by the use of IL-12 and IL-18 (Leung *et al.*, 2004; Ortaldo and Young, 2003). Thus, cytokine therapy combined with short-term receptor blockade may provide a means to boost NK cell activity against autologous tumor cells.

It remains unclear the extent to which modification of KIR function may be used against solid tumors. These tumors typically express low levels of MHC class I, but they may not activate NK cell cytotoxicity. One study reported that HLA–KIR interactions could be important for *in vitro* responses of NK cells, $\gamma\delta$T cells, and CTLs against melanomas (Bakker *et al.*, 1998). Another study showed that allogeneic NK cells that were mismatched for KIR–HLA ligand interactions had increased cytotoxicity against melanoma and renal cell carcinoma cells (Igarashi *et al.*, 2004). These data support the use of allogeneic NK cells with a KIR–HLA mismatch as immunotherapy for solid tumors. Downregulation of MHC class I or loss of specific MHC class I alleles is quite common in human tumors, particularly solid tumors (Chang *et al.*, 2005). In the event that MHC class I is not expressed, KIR blockade would likely provide little benefit to treat those tumors. In contrast, lymphomas and myeloid leukemias often express MHC class I molecules (Chang *et al.*, 2005; Wetzler *et al.*, 2001). This may reflect the fact that these tumors are found in blood and lymphoid tissue,

locations with high numbers of NK cells, so maintaining selective MHC class I expression may provide tumors a means to inhibit NK cell function (Demanet *et al.*, 2004). In one report there was an increased frequency of inhibitory KIRs in patients with leukemia compared to healthy controls, supporting the idea of NK cell inhibition through KIR–HLA interaction as a mechanism of tumor escape (Verheyden *et al.*, 2004). Thus, lymphomas and myeloid leukemias may be the most likely malignancies in which KIR blockade will provide a clinical benefit.

Although tumors may downregulate classic MHC class I molecules, a variety of different tumors have been reported to express nonclassic MHC class I HLA-E, HLA-F, and HLA-G (Chang *et al.*, 2005). The percentage of a given tumor that expresses these molecules varies between different tumor types. HLA-E binds to CD94/NKG2A heterodimers and will inhibit NK cell function (Braud *et al.*, 1998; Vance *et al.*, 1998). A significant number of NK cells, in particular, the $CD56^{bright}$ NK cell subset that is known to produce proinflammatory cytokines, express CD94/NKG2A receptors (Jonges *et al.*, 2001). *In vitro* studies have shown that blockade of CD94/NKG2A leads to an increase in NK cell cytotoxicity and cytokine production, but no studies have examined whether this strategy may promote an antitumor response *in vivo*. A strategy that involves blockade of both CD94/NKG2A and specific KIRs may be a more effective approach against tumors than blockade of KIRs alone.

V. USE OF ACTIVATING NK RECEPTORS OR LIGANDS IN CANCER IMMUNOTHERAPY

In the last several years, data have been reported that indicate that NK cell recognition of tumors may be used to trigger immune responses against tumor cells and to promote the development of T-cell–specific antitumor immunity. Several different approaches to improve cancer therapy have been put forth based on NK cell activating receptors, including: (1) use of NK cell receptor ligands on tumor cells, (2) antibodies that induce ADCC, (3) bispecific antibodies that link NK receptor bearing cells and tumor cells, and (4) activation of endogenous NK receptors that are found on T cells.

(1) One approach for cancer immunotherapy involves inducing or expressing ligands for NK cell activating receptors on tumor cells. These modified tumor cells can trigger NK cells and lead to activation of host antitumor immunity and tumor-specific CTLs. The ligands used are self-molecules that can be recognized by NK cells.

NKG2D is a potent activating receptor on NK cells whose ligands are expressed widely on tumor cells but only to a limited extent on normal

tissue. This restricted tissue expression makes these ligands good candidates for tumor-specific recognition. Although, one should note that the extent of expression of NKG2D ligands on many tissues is not known. It has been reported that tumors that express NKG2D ligands are more easily rejected than tumors that lack these ligands (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001, 2003; Hayakawa *et al.*, 2002; Westwood *et al.*, 2004). The more interesting observation from these studies was that rejection of a tumor (RMA-Rae1) that expressed a ligand for NKG2D resulted in the host being able to reject a subsequent challenge with the parental tumor (RMA) that lacked expression of a ligand for NKG2D. The primary rejection of NKG2D ligand-bearing tumors in these studies was mediated by NK cells and $CD8^+$ T cells and was dependent upon perforin (Diefenbach *et al.*, 2001; Hayakawa *et al.*, 2002). The immunity against the parental RMA tumor cells required $CD8^+$ T cells but not IFN-γ, IL-12, or invariant NKT cells based on studies using mice deficient in these molecules or cells (Hayakawa *et al.*, 2002; Westwood *et al.*, 2004). However, one study failed to show host antitumor immunity following rejection of RMA-Rae1 (Cerwenka *et al.*, 2001). A major difference between these studies was the fact that the latter study challenged mice with tumor cells intraperitoneally whereas the others gave tumors subcutaneously. Thus, tumor location may also be an important factor in the activation of host antitumor immunity. NKG2D on NK cells can be downregulated by TGF-β, and this may limit the use of NKG2D on NK cells in some settings (Castriconi *et al.*, 2003; Lee *et al.*, 2004a).

CD27 is a member of the TNF receptor superfamily and binds to CD70. Cross-linking of CD27 on murine NK cells results in production of IFN-γ but not cytotoxicity of target cells (Takeda *et al.*, 2000). T cells also express CD27, and activation of T cells via CD27 increases proliferation and cytokine production (Hintzen *et al.*, 1994). MHC class I-deficient tumor cells, which expressed CD70 (RMA-S-CD70), were resisted by wild-type and $Rag1^{-/-}$ mice in a manner dependent upon CD70 expression and NK cells (Kelly *et al.*, 2002a). Mice that had resisted the initial challenge with RMA-S-CD70 were able to resist a subsequent challenge with RMA tumor cells due to $CD8^+$ cells. The priming of secondary immunity was dependent upon NK cells and IFN-γ. This study showed that NK cell activation could lead to activation of host antitumor T-cell immunity.

CD80 and CD86 are important costimulatory molecules expressed on antigen-presenting cells. NK cells have been shown to be able to recognize CD80 and CD86 on DCs and tumor cells (Chambers *et al.*, 1996; Geldhof *et al.*, 1998). In a study similar to that using CD27-CD70, RMA-S cells that were transduced with CD80 were rejected by mice in a NK cell-dependent manner, and these mice developed tumor-specific T-cell memory so that they resisted a subsequent challenge with RMA cells that did not express

CD80 (Kelly *et al.*, 2002b). IL-2–activated human NK cells have been shown to have an increased cytotoxicity against tumor cells that express CD40, and cytotoxicity can be induced on activated human NK cells by cross-linking CD40 ligand (CD154) (Carbone *et al.*, 1997). Therefore, it may be possible for these molecules to be used to trigger NK cell antitumor responses.

One possible therapeutic approach based on these findings is to express these ligands on a patient's tumor cells, and then give irradiated gene-modified tumor cells back to the patient to induce tumor-specific immunity. This approach can be viewed as a tumor vaccine that may induce NK cell activation and help drive antigen presentation and tumor-specific CTL formation. However, many of these *in vivo* studies were conducted with MHC class I-deficient tumor cells that, by themselves, induce a strong NK cell response when injected into mice (Glas *et al.*, 2000; Mocikat *et al.*, 2003). It remains to be determined the extent to which MHC class I-positive tumor cells expressing costimulatory molecules induce such NK cell-dependent T-cell antitumor responses.

(2) Most NK cells express FcRγIII on the cell surface, and this receptor is used to mediate ADCC. Antibodies provide a means for the adaptive immune system to induce directed activation of innate immunity. The exact mechanisms by which antibodies have proven effective in humans remain unclear. Antibodies have potential to activate complement, induce phagocytosis, activate PMNs, induce NK cell ADCC, and directly attack the target cell by disruption of signaling or induction of apoptosis. It is possible that more than one mechanism may be responsible for the biological effects of a given antibody *in vivo*. Antitumor antibodies are being tested as treatments for a number of malignant diseases (Adams and Weiner, 2005). Rituximab (anti-CD20) has been approved for use in patients with B-cell lymphoma (Olszewski and Grossbard, 2004). CD20 is expressed on mature B cells but not on immature B cells, plasma cells, or other nonlymphoid tissues. Approximately 93% of patients with B-cell lymphoma express CD20 on their tumor cells, making CD20 a relatively specific target for antibody therapy. CD20 is not internalized or shed by tumor cells, allowing the elimination of $CD20^+$ cells by ADCC and complement activation. Although induction of ADCC by antitumor antibodies has been shown *in vitro*, the extent to which ADCC contributes to clinical efficacy is still not well defined. In FcR- and complement C1q-deficient mice, antibody-mediated antitumor efficacy diminished, suggesting that both ADCC and complement activation may be important for the observed effects of antibody treatment (Adams and Weiner, 2005). Some clinical studies have shown a correlation between better efficacy and "high responder" FcR polymorphisms in patients consistent with a role for ADCC in antibody therapy (Cartron *et al.*, 2002; Weng and Levy, 2003).

(3) Another therapeutic approach is to use bispecific antibodies to link NK cell activating receptors with tumor cells. These antibodies can be specific for molecules expressed on T cells as well as NK cells, and the bispecific antibody approach may lead to activation of both NK cells and T cells. Experimental and clinical data support the idea that bispecific antibodies can be beneficial; however, there is much work to be done to optimize this approach and improve efficacy. Based on the activation of NK cell FcRs, one approach being tried is the use of bispecific antibodies that target NK cell CD16 and an antigen on tumor cells. CD19-CD16 specific antibodies have been developed to target B-cell lymphomas (Bruenke *et al.*, 2005; Schlenzka *et al.*, 2004), and antibodies with specificity of HER2/neu and CD16 have been developed to target solid tumors (Shahied *et al.*, 2004). Patients with Hodgkin's lymphoma treated with a bispecific antibody against CD16 and CD30 showed a positive clinical response to this antibody treatment (Hartmann *et al.*, 2001).

A related approach is to use a bispecific protein, rather than an antibody, to link two cell types together. A fusion protein of ULBP2-BB4 has been used to link together NK cells and myeloma tumor cells (Pogge von Strandmann *et al.*, 2005). ULBP2 is a ligand for NKG2D, and BB4 binds to CD138. In this study, ULBP2-BB4 fusion protein allowed human NK cells to kill multiple myeloma cell lines and primary malignant plasma cells. Furthermore, when a tumor cell line was implanted in nude mice, coadministration of human blood lymphocytes and ULBP2-BB4 protein prevented tumor growth. $CD8^+$ T cells in humans express NKG2D, and this type of treatment may also target this subset of T cells against myeloma cells. Hence, using natural ligands to NKG2D may be a means to direct NK cells and $CD8^+$ T cells against tumor cells.

(4) Several NK receptors are expressed by T-cell subsets, in particular, activated or memory $CD8^+$ T cells. Therapeutic strategies that activate NK receptors on T-cell subsets may prove to be a better approach to treat cancer than activation of NK cells alone. Although NK cells can directly attack tumor cells and promote antitumor immunity, it has been observed that NK cell responses, like other innate immune responses, are limited. For example, it is possible to overcome NK cell effector mechanisms resulting in tumor growth when a large dose of tumor cells is given to mice (Hoglund *et al.*, 1988). Human $CD8^+$ T cells and $\gamma\delta$T cells express NKG2D (Raulet, 2003). NKG2D function may be particularly important in $CD8^+$ T cells that have been activated by cytokines. It may be possible to design a strategy to activate NKG2D on T cells in the presence of cytokines, such as IL-15, in order to promote antitumor effects against tumor cells that express ligands for NKG2D. A report suggests that $CD8^+$, $CD56^+$ T cells can mediate potent cytotoxicity against myeloma cell lines *in vitro* in a NKG2D-dependent manner (Wu *et al.*, 2004). It could be possible to

selectively expand and activate this T-cell subset from patients and use the autologous T cells to treat myeloma.

VI. CHIMERIC NK CELL RECEPTORS AS CANCER IMMUNOTHERAPY

In this section, a new therapeutic approach to cancer is described that employs NK cell receptors as a means to target T cells against tumor cells. This receptor strategy is designed to allow T cells to recognize tumor cells as NK cells do, and to retain the effector functions, survival, and expansion properties of T cells. This is accomplished by producing a chimeric NK receptor that combines the broad tumor specificity of NK cells with signaling elements that directly activate T cells. The NKG2D receptor has been combined with the CD3ζ intracellular domain to produce a chimeric NKG2D (chNKG2D) receptor that mediates antitumor immunotherapy (Zhang *et al.*, 2005).

One of the major challenges posed by the use of adoptive T cells or vaccine approaches to cancer immunotherapy is the need to select a tumor-specific or a highly overexpressed antigen as a target. Identifying and targeting such antigens can be quite difficult in a number of patients. Each antigen may need to be carefully selected for each patient's MHC haplotype and tumor type. In comparison, almost 70% of human cancers are derived from tissues where tumors have been shown to express ligands for NKG2D. Immunotherapy with chimeric NK receptor-expressing T cells offers advantages over the use of NK cell immunotherapy or activated host T cells. These advantages include effector cell longevity, potential for expansion, cell trafficking, and T-cell effector functions, while maintaining the widespread tumor recognition ability of NK cells (Fig. 1). Thus, immunotherapy that uses chNKG2D receptors as a means to target T cells to attack tumors may potentially be useful in a large number of cancers.

NKG2D is a type-II membrane protein where the NH2 terminal is found inside the cell. CD3ζ is a type-I protein with the COOH terminus inside of the cell. To combine these receptors into one functional molecule, CD3ζ was flipped in orientation so that it was expressed on the cytoplasmic terminus of NKG2D in the reverse orientation. This reversed expression of CD3ζ did not appear to alter its ability to function in T cells, and triggering of chNKG2D-bearing T cells by target cells that express ligands for NKG2D resulted in T-cell proliferation, cytotoxicity, and cytokine production (Zhang *et al.*, 2005). Similar to wild-type NKG2D, the chNKG2D receptor required the presence of Dap12 or Dap10 in order for chNKG2D to be expressed at the cell surface. $CD8^+$ T cells express Dap10,

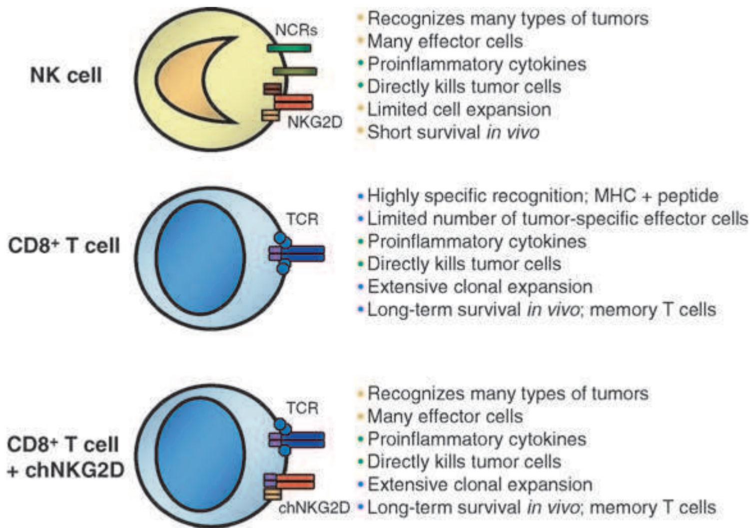

Fig. 1 Comparison of chimeric NKG2D (chNKG2D) receptors on $CD8^+$ T cells with conventional $CD8^+$ T cells and NK cells. chNKG2D receptors provide $CD8^+$ T cells with the broad recognition ability of NK cells and survival and expansion capabilities of T cells.

and it is this expression of Dap10 that allows the chNKG2D receptor to be expressed on the surface of these T cells. In order for naive T cells to become activated, they must receive both a primary signal (via the TCR) and a costimulatory signal (often via CD28). Dap10-mediated signals appear to be able to induce many of the costimulatory signals required for T-cell activation. Because chNKG2D can activate T cells via CD3ζ and Dap10, it is possible, although not yet determined, that triggering by a chNKG2D receptor alone may stimulate multiple signaling pathways in T cells. That is, chNKG2D is directly linked to CD3ζ and can provide a primary TCR-like signal, and chNKG2D's association with Dap10 may also supply a costimulation signal (Fig. 2).

When murine chNKG2D-bearing T cells are cocultured with hematopoietic tumor cells, the cells produce large amounts of IFN-γ (Zhang *et al.*, 2005). These chNKG2D cells also produce chemokines (CCL3 and CCL5), GM-CSF, and some IL-3. They do not produce TNF-α, IL-5, or IL-10, and the production of cytokines is specific for NKG2D ligand expression (Zhang *et al.*, 2005). The extent of production of specific cytokines may depend upon the particular target cell, as data suggest that TNF-α may be produced under certain circumstances (our unpublished data). Murine chNKG2D-bearing T cells mediate cytotoxicity against tumor cells, which express NKG2D ligands in an NKG2D-dependent and MHC-independent manner

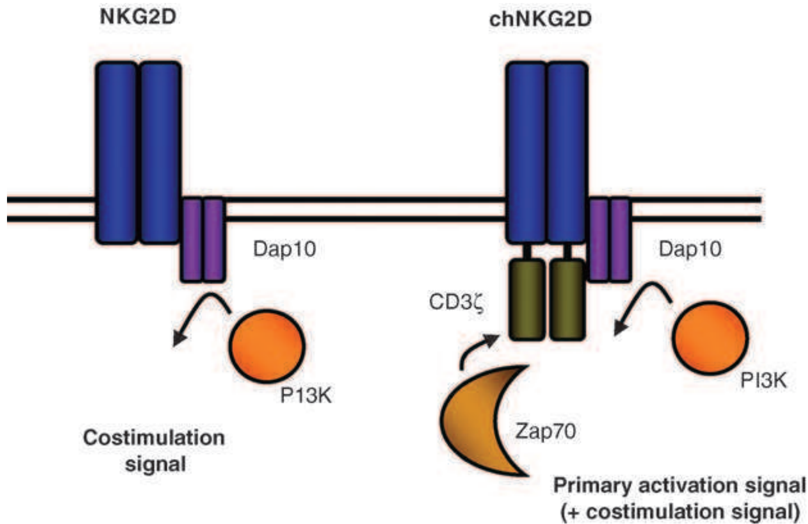

Fig. 2 Chimeric NK receptors allow the induction of both primary and costimulatory signals. chNKG2D receptors consist of NKG2D fused to CD3ζ and are associated with Dap10. CD3ζ activates Zap70, and Dap10 activates PI3K.

(Zhang *et al.*, 2005). We have constructed and tested a human version of the chNKG2D receptor and found very similar activities as the murine chNKG2D receptor (Zhang *et al.*, 2006). Primary human blood T cells that had been transduced with human chNKG2D were able to kill a variety of tumor cells, which expressed endogenous NKG2D ligands, including cell lines derived from solid tumors and leukemias. Similarly, cytotoxicity of tumor cells was NKG2D ligand dependent and MHC independent. Human $CD8^+$ T cells bearing chNKG2D receptors produced IFN-γ and other proinflammatory cytokines upon coculture with tumor cells that expressed NKG2D ligands. Thus, it is possible to create chNKG2D receptors that can provide antitumor responses for both murine and human T cells.

It has been demonstrated that chNKG2D-bearing T cells can eliminate or delay tumor growth *in vivo* (Zhang *et al.*, 2005). These initial studies focused on a model where lymphoma cells and chNKG2D T cells or control T cells were mixed and immediately injected subcutaneously into mice. It was also shown that chNKG2D T cells administered intravenously could delay tumor growth in mice given tumor cells subcutaneously. One important observation from these studies was that mice that had resisted the initial tumor challenge with the NKG2D ligand-expressing tumor cell (RMA-Rae1β) were resistant to a subsequent challenge with the parental tumor cell line that did not express any NKG2D ligands (RMA) (Zhang

et al., 2005). This suggested that the chNKG2D T cells were able to promote the development of host T cells that were specific for other tumor antigens and resulted in resistance to challenge with the parental tumor cell line. This study has been extended to show that chNKG2D T cells induced similar protection from RMA-Rae1β tumor cells in B6 or B6.Rag1$^{-/-}$ mice (Fig. 3A), indicating that host T or B cells were not required for chNKG2D T cells to prevent tumor growth. When those mice that resisted the initial tumor were challenged with RMA tumor cells (Fig. 3B), all of the B6 mice resisted the RMA tumor cells, while all of the B6.Rag1$^{-/-}$ mice developed tumors in a similar manner as naïve B6 mice. Thus, host T and B cells were required for development of resistance to parental RMA cells. This was most likely due to the presence of host T cells specific for RMA tumor cells, although RMA-specific host T cells have not been shown directly. Taken together, these data suggest that chNKG2D T-cell responses to tumor cells can promote host antitumor immunity.

Our working model is that chNKG2D T cells kill NKG2D ligand-expressing tumor cells and secrete proinflammatory cytokines. The likely result of this is activation of host macrophages that attack the tumor and DC presentation of tumor antigens to host T cells. The outcome of treatment with chNKG2D T cells is a shift in the balance between tumor and host in favor of the host and against survival of the tumor. The tumor-effector cell

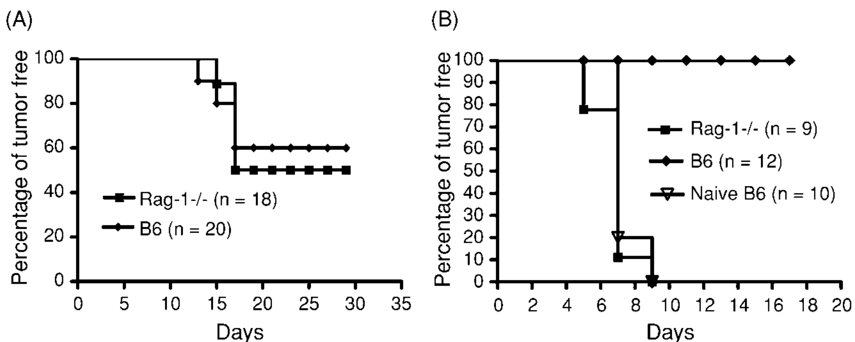

Fig. 3 Host lymphocytes are required for development of resistance to tumors that do not express NKG2D ligands. (A) B6 (diamonds) or B6.Rag1$^{-/-}$ mice (squares) were challenged with 10^5 RMA-Rae1 cells s.c. + 10^6 chNKG2D T cells on day 0. The percentage of tumor-free mice were similar after 30 days, so host T and B cells were not required for chNKG2D T-cell–mediated tumor rejection. (B) Tumor-free mice (from A) were challenged on the opposite flank with 10^4 RMA cells (no NKG2D ligands). All B6 mice (that had survived the initial RMA-Rae1 tumor challenge, diamonds) remained tumor-free, while all B6.Rag1$^{-/-}$ mice (squares) developed tumors in a similar manner as naïve B6 mice (open triangles). Hence, host T and B cells are required for the development of resistance to RMA rechallenge after chNKG2D T-cell immunotherapy.

mixing experiments did not provide a good model for understanding the potential treatment of human tumors *in vivo*. We have begun preliminary studies that indicate that chNKG2D T cells can be used to treat tumor-bearing hosts and lead to greatly diminished tumor growth in models of lymphoma, ovarian carcinoma, and metastatic melanoma (our unpublished data). Thus, it may be possible to use chNKG2D or other chimeric NK receptors as tools to attack tumor cells and promote host antitumor immunity.

A. Future Questions

The use of any immunotherapy based on the use of NKG2D-bearing effector cells will need to address several issues. NKG2D has been shown to be downregulated upon long-term exposure to NKG2D ligand-expressing cells and tissues (Oppenheim *et al.*, 2005; Wiemann *et al.*, 2005). Thus, effector cells that express NKG2D may be effective for a limited time *in vivo*. This may not be unfortunate, as highly activated effector cells that recognize NKG2D may be problematic over the long term. Ligand-driven downregulation of NKG2D expression may provide a means to inactivate these effector cells and limit potential damage to self-tissues. Many cancer patients have soluble MICA in their serum, which may inhibit recognition of MICA on tumor cells (Groh *et al.*, 2002; Holdenrieder *et al.*, 2006; Salih *et al.*, 2003). However, human T cells that express chNKG2D were not able to be inhibited by soluble MICA at amounts found in patient serum (Zhang *et al.*, 2006). The expression of many of the ligands for NKG2D remain undefined in human tissues, so safety issues will need to be addressed with this type of therapy. Disregulation of NKG2D ligand expression and activation of $CD8^+$ IELs have been shown to be associated with celiac disease in humans (Hue *et al.*, 2004; Martin-Pagola *et al.*, 2004; Meresse *et al.*, 2004). In a murine model of type-I diabetes, blockade of NKG2D was able to prevent the onset of disease, suggesting that NKG2D recognition may be involved in the development of diabetes (Ogasawara *et al.*, 2004). Although the involvement of NKG2D or its ligands in human diabetes is unknown and these data may be relevant only to this particular mouse model, it will still be important to consider such data as NKG2D-based therapies move into clinical testing. It may be possible to engineer cells with a "suicide gene" to allow removal of transferred cells in patients. A suicide gene provides modified cells with proteins that make them susceptible to apoptosis in the presence of specific drugs. Long-term effector cell survival, development of host antitumor T-cell memory, and trafficking of effector cells to distal tumor sites are all issues that remain to be addressed for the use of chimeric NK cell receptor-based immunotherapy.

VII. CONCLUSIONS

For immunotherapy of cancer to be successful, therapies must overcome several obstacles. Many of these obstacles are simply mechanisms that prevent host responsiveness against normal "self" tissue, as tumors are basically self tissues that have overcome homeostatic regulation mechanisms. Thus, it is difficult for the immune system to identify tumor cells from normal cells. NK cells express several receptors that recognize self-proteins that are upregulated on tumor cells. Studies suggest that it will be possible to use NK cell receptors as weapons in the fight against cancer. Several different strategies have emerged that can promote destruction of tumor cells and induction of host antitumor immunity (Fig. 4).

Blockade of inhibitory receptors is one approach to allow NK cell responses to autologous tumor cells. Data from HSCT studies suggest that

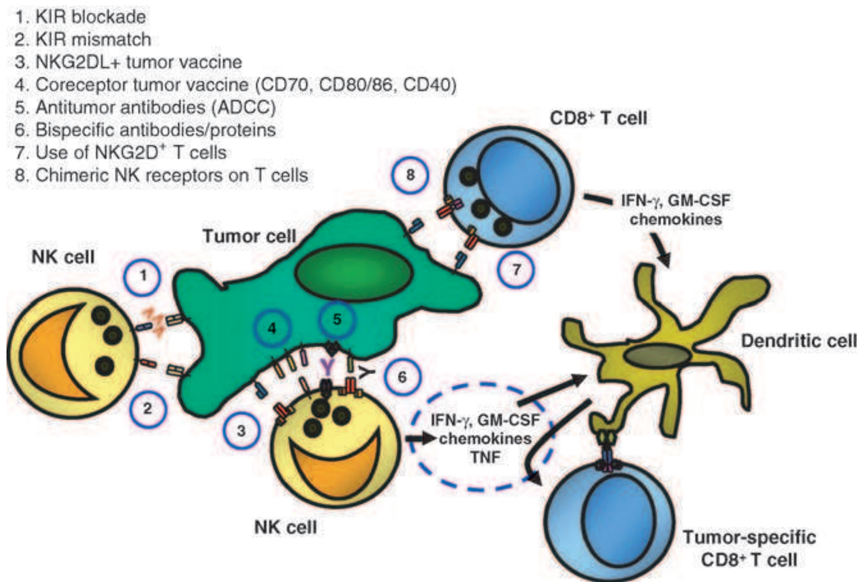

Fig. 4 Strategies to promote antitumor responses through NK cell receptors. Several different strategies have been studied to promote antitumor immunity using NK cell receptors. These include: KIR blockade, KIR mismatch, tumor vaccines with NKG2D ligands on tumor cells, tumor vaccines that use coreceptors on tumor cells, antitumor antibodies that induce ADCC, bispecific antibodies or proteins, use of NKG2D^{+} T cells, and the development of chimeric NK receptors for use on T cells. Activated NK cells and CD8^{+} T cells may attack the tumor directly, but they also produce proinflammatory cytokines and activate DCs. These DCs will produce cytokines (e.g., IL-12) and induce host antitumor specific T cells.

knowledge of donor NK cell KIRs can be used to reduce tumor relapse in AML patients. Tumor vaccine strategies designed to promote NK cell activation and killing of tumor cells may be able to shift the immune balance in favor of antitumor responses. Antitumor antibodies and bispecific antibodies that link NK cells and tumor cells can lead to tumor cell killing. Another promising approach is to use NK receptors expressed or transduced on T cells to target a wide variety of tumor cell types and lead to development of tumor-specific memory T cells. New studies are needed to explore the translational potential of therapies based on the understanding of NK cell receptors and their ligands to cancer immunotherapy. Innate immune signals drive the initial activation of adaptive immunity. It may now be possible to use the innate recognition by NK cells to help promote adaptive T cell immunity against tumors. The identification of new NK cell receptors and their ligands has allowed us to understand how NK cells "spontaneously" recognize and kill tumor cells and provided an opportunity to design novel treatments for cancer therapy.

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Note added in proof: After this review had gone to press, the ligand for KLRG1 was identified as E-cadherin (Grundemann *et al.*, 2006; Ito *et al.*, 2006).

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Innate Immune Recognition and Suppression of Tumors

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I. Tumor Immune Surveillance: An Introduction
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In this chapter, we first summarized the strong evidence that now supports the existence of an effective cancer immune surveillance process that prevents cancer development in both mice and humans. We then focused the remainder of the chapter on methods of tumor recognition that contribute to natural host immune suppression of tumors. In particular, NKG2D is a type II transmembrane-anchored glycoprotein expressed as a disulfide-linked homodimer on the surface of all mouse and human natural killer cells (NK cells). Stimulation of NK cell through NKG2D triggers cell-mediated cytotoxicity and in some cases induces production of cytokines. NKG2D binds to family of ligands with structural homology to major histocompatibility complex (MHC) class I, however, NKG2D ligands often display upregulated surface expression on stressed cells and are frequently overexpressed by tumors unlike conventional MHC class I molecules. Evidence clearly implicate that NKG2D recognition plays an important role in tumor immune surveillance. © 2006 Elsevier Inc.

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I. TUMOR IMMUNE SURVEILLANCE: AN INTRODUCTION

The idea that the immune system regulates cancer development is experiencing a new resurgence. For the past 50 years much of the debate has focused on the validity of the cancer immune surveillance hypothesis originally proposed by Burnet (1957) and Thomas (1982). There have been inherent problems in experimentally revealing whether or not natural immune defense mechanisms do protect the host against the development of cancers. However, a cancer immune surveillance process, which functions as an effective extrinsic tumor-suppressor mechanism, has been demonstrated by an overwhelming amount of definitive experimental data from mouse models together with important clinical data from human patients. The development of gene-targeting and transgenic mouse technologies, and the capacity to produce highly specific neutralizing monoclonal antibodies (mAb) to particular immune components, has enabled the cancer immune surveillance hypothesis to be tested in molecularly defined murine models of immunodeficiency (Dunn *et al.*, 2002, 2004a,b; Schreiber *et al.*, 2004; Smyth *et al.*, 2001b). There has also been greater recognition that tumor elimination represents only one dimension of a complex relationship between the immune system and cancer (Dunn *et al.*, 2002, 2004a,b; Shankaran *et al.*, 2001). When the immune system fails to eliminate all tumor cells, tumors with reduced immunogenicity may emerge that are capable of escaping immune recognition and destruction (Shankaran *et al.*, 2001; Smyth *et al.*, 2000c; Svane *et al.*, 1996; Takeda *et al.*, 2002). This combination of host-protective and tumor-sculpting functions of the immune system throughout tumor development has been termed "cancer immunoediting" (Dunn *et al.*, 2002, 2004a,b). Immunoediting has been viewed as a dynamic process comprising three phases: elimination, equilibrium, and escape. Elimination encompasses the classical concept of cancer immune surveillance, equilibrium is the period of immune-mediated latency after incomplete tumor destruction, and escape refers to the final outgrowth of tumors that have outlasted immunological restraints of the equilibrium phase.

II. THE IMMUNE SYSTEM IS AN EXTRINSIC TUMOR SUPPRESSOR

Cancers develop by an evolutionary process as somatic cells mutate and escape the restraints that normally restrict their untoward expansion. Numerous intrinsic tumor-suppressive mechanisms exist that trigger apoptosis, repair, or senescence, if proliferation is uncontrolled. The major

cell-death program is responsive to the signals of survival factors, cell stress, and injury and is dependent on mitochondria (Cory *et al.*, 2003; Danial and Korsmeyer, 2004; Green and Kroemer, 2004) and terminal activation of executioner caspases. In contrast, a second cell-death pathway is activated through ligation of cell-surface death receptors such as tumor necrosis factor (TNF) receptor, Fas/CD95, and tumor necrosis factor apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2, DR5) (Peter and Krammer, 2003). Upon ligation with corresponding members of the tumor necrosis factor (TNF) superfamily, these receptors form the death-inducing signaling complex (DISC), which activates the apical caspase-8.

A number of cellular proteins (e.g., p53) detect disturbances caused by potentially mutagenic insults, trigger this apoptotic-effector machinery, and thereby function as intrinsic tumor suppressors (Lowe *et al.*, 2004). Oncogenes can also target various pieces of the cell-death machinery independently of p53. Activated oncogenes may also trigger cellular senescence, a state characterized by fixed cell-cycle arrest and specific changes in morphology and gene expression that characterize the process from quiescence (reversible cell-cycle arrest) (Serrano *et al.*, 1997). Escape from oncogene-induced senescence is a prerequisite for transformation. Cancers must acquire cooperating lesions that uncouple mitogenic Ras signaling from senescence to achieve a maintenance state. Generally, both apoptosis and senescence act as potent barriers to the further development of any preneoplastic cell.

On the other hand, at least three general extrinsic mechanisms have been identified by which cells and their adjacent tissues "sense" the presence of transformed cells. The first relies on the basic dependency that cells possess for specific trophic signals in the microenvironment that prevent their innate tendency to suicide (e.g., epithelial cell–extracellular matrix association). The second appears to involve important links between cell polarity genes that control cell proliferation and cellular junctions (Humbert *et al.*, 2003). The third mechanism involves the detection and elimination and/or cytostasis of transformed cells by leukocytes of the immune system (immune surveillance). These effector cells employ extremely diverse mechanisms to kill tumor targets that involve both cell-death receptor and mitochondrial pathways. In combination, these varied intrinsic and extrinsic tumor-suppressing mechanisms are remarkably effective and specific. The remainder of this chapter will focus upon the extrinsic tumor suppression offered by the immune system.

III. SYNPOSIS OF AN EFFECTIVE IMMUNE RESPONSE

We imagine that the immune system manifests its effects only after transformed cells have bypassed their intrinsic tumor-suppressor mechanisms (Macleod, 2000). Yet in reality, infection with potentially oncogenic viruses

may stimulate immunity at the same time as the activation of intrinsic tumor-suppressor mechanisms. Immune surveillance of tumor, as in host defense to microbial pathogens, likely requires an integrated response involving both the innate and adaptive arms of the immune system. Initiation of host immunity occurs when cells of the innate immune system become aware of the presence of a growing tumor due to either the local tissue disruption that occurs as a result of angiogenesis (Carmeliet and Jain, 2000; Hanahan and Folkman, 1996) or tissue invasion (Hanahan and Weinberg, 2000; Sternlicht and Werb, 2001). The structural changes in the stroma induced during these processes may produce proinflammatory molecules that along with chemokines often produced by the tumor cells (Vicari and Caux, 2002), direct innate immune cells to this new source of local "danger" (Matzinger, 1994). Dendritic cells (DC) act as detectors of tissue stress, damage, and/or transformation, and danger signals can take the form of heat-shock proteins (HSPs) and/or proinflammatory factors. Some of these cytokines (e.g., interleukin-1 (IL-1), IL-15, tumor necrosis factor (TNF)-α, type I interferon (IFN), and granulocyte-macrophage colony-stimulating factor (GM-CSF)) may also promote DC differentiation and activity by increasing costimulation between DC and NK cells and later DC and T cells.

The "danger" microenvironment allows the recruitment of NK cells, macrophages, $\gamma\delta^+$ T cells and/or natural killer T cells (NKT) cells, and these cells recognize molecules that have been induced on tumor cells either by cellular transformation or inflammation. Furthermore, T cells and NKT cells may recognize developing tumors via T-cell receptor (TCR) interaction with either MHC-/tumor-associated peptide complexes or glycolipid–CD1 complexes expressed on tumor cells, respectively (Smyth *et al.*, 2002a). These effector cells may then deliver cytotoxic molecules to eliminate the transformed cell(s) and secrete IFNs that both directly control tumor growth and indirectly expand the immune response by a variety of mechanisms already reviewed (Dunn *et al.*, 2004a).

Tumor-specific adaptive immune responses are driven when tumor antigens are liberated by a variety of cell-death pathways in the context of innate immune signals. Immature DCs that have been recruited to the tumor site become activated either by exposure to the cytokine microenvironment created during innate immune attack or by interacting with tumor-infiltrating NK cells themselves (Gerosa *et al.*, 2002). The activated DC may then acquire tumor antigens either directly by ingestion of tumor cell apoptotic bodies or via indirect mechanisms involving transfer of complexes of tumor cell-derived HSPs and/or tumor antigens to DC (Li *et al.*, 2002; Srivastava, 2002). DC acquire a highly activated mature phenotype and, in response to distinct chemokines and/or cytokines, migrate to the lymph nodes (Sallusto *et al.*, 2000), where they trigger the activation of naïve tumor-specific Th1 CD4$^+$ T cells. Th1 cells enable the development of

tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTL) via cross-presentation of antigenic tumor peptides on DC MHC class I molecules (Huang *et al.*, 1994). Peptides derived from tumor-associated antigens are presented to $CD4^+$ or $CD8^+$ T cells in the context of MHC class II or class I molecules, respectively, and on occasion the activation of B cells may also occur.

In the final stage, the development of tumor-specific adaptive immunity may provide the host with the capacity to completely eliminate the developing tumor. Tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site where they kill antigen-positive tumor cells. $CD4^+$ T cells produce IL-2 that, together with host cell production of IL-15, helps to maintain the function and viability of the tumor-specific $CD8^+$ T cells. Tumor-specific $CD8^+$ T cells efficiently recognize their tumor targets and induce tumor cell death by both direct and indirect mechanisms. It is probable that these $CD8^+$ T cells directly kill many tumor cells *in vivo*, however, they will also produce large amounts of IFN-γ thereby halting or killing tumors by IFN-γ–dependent cell-cycle inhibition, -apoptosis, -angiostasis, or -induction of macrophage tumoricidal activity. These two basic mechanisms of killing and cytokine production most likely occur together; however, their relative contributions may be tumor dependent. Hence, the elimination phase is a continuous process that must be repeated each time neoplastic cells with distinct antigens arise.

IV. INNATE AND ADAPTIVE IMMUNE CELLS THAT CONTROL TUMORS

A number of cellular components of both the innate and adaptive immune system have now been implicated in natural tumor immunity (Fig. 1). Lymphocytes protect mice against both spontaneous and chemically-induced tumors, including $\alpha\beta^+$ T cells (Shankaran *et al.*, 2001), $\gamma\delta^+$ T cells, NK cells, and NKT cells.

A. Natural Killer Cells

NK cells are specialized innate lymphocytes capable of distinguishing between normal healthy cells and abnormal cells such as virus-infected cells or transformed tumor cells (Cerwenka and Lanier, 2001b; Smyth *et al.*, 2002b). Unlike CTL, NK cells do not require somatic recombination encoded antigen-specific receptors to recognize target cells. Instead, NK cell function is tightly regulated by the balance of positive and negative signals integrated from a variety of cell surface receptors (Lanier, 2005; Smyth

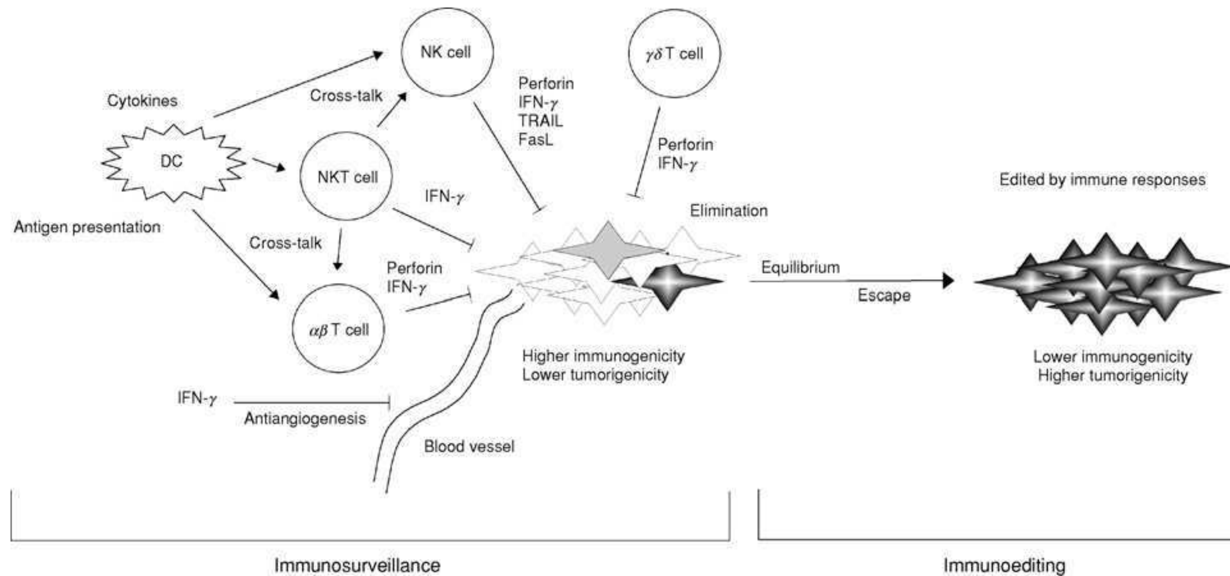

Fig. 1 Schematic illustration of the immune response to tumor. The initial immune response involves innate lymphocytes (NK, NKT, and $\gamma\delta$ T cells) mediating immunosurveillance of transformed cells via direct cytotoxicity (i.e., pfp, TRAIL) or IFN-γ production. These effector cells are either primary mediators responding directly to tumor or receive additional signals by cross talk with dendritic cells, some of which may have been the sentinels, first detecting transformed cells in the tissue. Subsequently, further recruitment of these cell types allows further effector control by fully activated lymphocytes including the regulation of tumor angiogenesis via IFN-γ secretion. Tumor growth is also controlled by adaptive $CD4^{+}$ and $CD8^{+}$ $\alpha\beta$ T cells that are primed by antigen presenting dendritic cells(DC) and are specific for tumor antigens. As a consequence of immunoediting, tumor cells emerge with increased capacity to survive/escape further immune detection/attack. The escaping tumor cells develop reduced immunogenicity and greater tumorigenicity, thereby enabling their uncontrolled expansion in the immunocompetent host.

et al., 2005a). NK cells use a sophisticated and complicated repertoire of germ line encoded cell-surface receptors that control their behavior. NK cells exhibit spontaneous cytotoxicity against MHC class I-deficient target cells, and, in particular, they participate in the innate immune responses against transformed cells and tumor metastases *in vivo* (Smyth *et al.*, 1999, 2002b). The effector functions of NK cells, including cytotoxicity and the capacity to produce a variety of cytokines following activation, are of pathophysiological importance. Granule exocytosis is the major mechanism of cell-mediated killing used by NK cells (Trapani and Smyth, 2002), however, these cells also express members of the TNF superfamily (Smyth *et al.*, 2003; Yagita *et al.*, 2004). Activation requires the action of proinflammatory cytokines in combination with differential engagement of cell-surface receptors. In particular, cytokines, such as IL-2, IL-12, IL-15, IL-18, IL-21, and IFN-$\alpha\beta$ can induce NK cell proliferation as well as promote NK cell cytotoxicity and/or production of IFN-γ (Biron *et al.*, 1999; Nutt *et al.*, 2004; Smyth *et al.*, 2004a).

According to the "missing-self" hypothesis (Ljunggren and Karre, 1990), NK cells appear to be actively inhibited by receptors that recognize MHC class I molecules. In general, normal cells expressing certain levels of MHC class I are generally protected from NK cell-mediated destruction, whereas virus-infected or malignant cells may express reduced levels of MHC class I molecules and, therefore, they become susceptible to NK cell-mediated attack. However, it is a common misconception that NK cells attack any cell lacking MHC class I expression, since NK cell activation requires cell–cell contact including immune synapse formation and subsequent Ca^{2+} mobilization when they encounter target cells lacking MHC class I molecules. Thus, emerging evidence suggests that NK cells clearly require the recognition of an activating structure on target cells, other than simply detecting a lack of MHC class I expression. Triggering of activating receptors, accompanied by signaling through inhibitory receptors that dampen signal, typically determines whether a response occurs following NK cell recognition.

The role of innate immune cells, such as NK cells and $\gamma\delta TCR^+$ T cells, in immune surveillance of tumors remains controversial. Both NK cells and $\gamma\delta TCR^+$ T cells express perforin (pfp) (Nakata *et al.*, 1990; Smyth *et al.*, 1990), mediate spontaneous cytotoxicity, and produce many antitumor cytokines, such as IFN-γ, when they recognize target cells via one or more of several cell-surface receptors (Cerwenka and Lanier, 2001b; Natarajan *et al.*, 2002). NK cells can spontaneously kill MHC class I-deficient tumor cell lines *in vivo* (Smyth *et al.*, 1999, 2000b; van den Broek *et al.*, 1995) and suppress experimental and spontaneous metastasis in mice. B-cell lymphomas arising in mice, deficient in both pfp and β2m, were demonstrated to be rejected by either NK cells or $\gamma\delta^+$ T cells when transplanted into syngeneic wild-type mice (Street

et al., 2004). In contrast, only a few models have been described thus far where NK cells or $\gamma\delta$TCR$^+$ T cells prevent primary tumor formation (Girardi *et al.*, 2001; Smyth *et al.*, 2000b, 2001a,b). C57BL/6 mice, depleted of both NK and NKT cells, by using the anti-NK1.1 mAb, were more susceptible to methyl cholanthrene (MCA)-induced carcinogenesis than wild-type controls (Smyth *et al.*, 2001b). A similar effect was observed in C57BL/6 mice treated with *anti*-asialo-GM1, which selectively depletes NK cells and activate macrophages, but not NKT cells. Mice lacking $\gamma\delta^+$ T cells (TCR$\delta^{-/-}$ mice) are more susceptible to MCA-induced tumor formation than wild-type mice (on either an FVB or C57BL/6 genetic background) (Gao *et al.*, 2003; Girardi *et al.*, 2001). In addition, using a carcinogenesis model involving initiation with 7,12-dimethylbenz [*a*]anthracene (DMBA) and promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), host protection against tumor formation was found to be more dependent on the action of $\gamma\delta^+$ T cells than $\alpha\beta^+$ T cells (Girardi *et al.*, 2001, 2003). While it has not been possible to formally test the importance of these innate lymphocyte subsets in natural human immunity to cancer, several studies on patients receiving HLA haplotype mismatch transplants (Ruggeri *et al.*, 2002), mAbs to human CD20 and epithelial cell adhesion molecule (Ep-CAM) (Liljefors *et al.*, 2003; O'Hanlon, 2004), or the c-kit tyrosine kinase inhibitor, Gleevec (Borg *et al.*, 2004) indicate an important role for NK cells in the human antitumor response.

B. Natural Killer T Cells

Some T-cell subpopulations participate as regulatory cells in immune responses to tumors (Godfrey *et al.*, 2000; Sakaguchi, 2002, 2004; Smyth and Godfrey, 2000; Smyth *et al.*, 2002a). These include NKT cells, other CD1d-restricted T cells, and various subsets of CD4$^+$CD25$^+$ T cells. NKT cells are CD1d dependent and MHC independent and express NK cell receptors in combination with a highly biased TCR repertoire (most TCR-αVα14Jα18) and either Vβ8.2, Vβ2, or Vβ7 (Godfrey *et al.*, 2000; Smyth *et al.*, 2002a). NKT cells also exist in humans and are commonly defined by coexpression of an invariant TCR-α chain (Vα24JαQ) and TCR Vβ11—TCR homologs of those used by mouse NKT cells. While the CD1d-reactive marine sponge glycolipid, α-galactosylceramide (α-GalCer) has been shown to activate mouse and human NKT cells and promote antitumor function (Hayakawa *et al.*, 2004), the potential natural ligand for the TCR of NKT cells, isoglobotrihexylceramide (Godfrey *et al.*, 2004; Zhou *et al.*, 2004), has not been examined in this context. The first clear evidence that NKT cells naturally participate in cancer immunosurveillance was obtained in C57BL/6 Jα18$^{-/-}$ mice, lacking Vα14Jα18-expressing invariant NKT cells (Cui *et al.*, 1997). These mice were also shown to

develop MCA-induced sarcomas at higher frequency than wild-type controls (Smyth *et al.*, 2000b). Subsequently, it was shown that NK cells, CD1d, pfp, and IFN-γ were all necessary in NKT cell-mediated control of the same MCA sarcomas when transplanted into Jα18$^{-/-}$ mice (Crowe *et al.*, 2002). In addition, mice treated with the NKT cell-activating ligand α-GalCer throughout MCA-induced tumorigenesis exhibited a reduced incidence of tumors and displayed a longer latency period to tumor formation than control mice (Hayakawa *et al.*, 2003). We have shown that the CD4^{-} subset of NKT cells has more potent antitumor activity than the CD4^{+} subset (Crowe *et al.*, 2005). It will be important now to examine the broader function of NKT cells in a variety of mouse models of cancer as well as establishing their role in human malignancy. To this end, a study illustrated that human NKT cell dysfunction correlated well with the progression of multiple myeloma (Dhodapkar *et al.*, 2003). Of interest is that other CD1d-restricted T cells may suppress immune responses to some tumors (Terabe *et al.*, 2005).

C. Regulatory T Cells

CD4^{+}CD25^{+} regulatory T cells (Treg) comprise 5–10% of the total CD4^{+} T-cell population and function largely to maintain immune tolerance (Sakaguchi, 2000, 2004; Shevach, 2002). Treg are critical in host suppression of organ-specific autoimmune diseases and they promote a dominant state of tolerance during infections and allogeneic transplantation. Treg also suppress immune responses to tumors at both the priming and effector phases (Onizuka *et al.*, 1999; Shimizu *et al.*, 1999; Steitz *et al.*, 2001; Sutmuller *et al.*, 2001; Turk *et al.*, 2004) and improve T-cell–based tumor clearance. Cancer patients have increased numbers of peripheral and tumor-infiltrating Treg cells that functionally inhibit tumor-specific T cells and predict poor survival (Curiel *et al.*, 2004; Liyanage *et al.*, 2002; Woo *et al.*, 2001). Although these regulatory T cells are thought to recognize self-antigen peptides in the context of MHC class II, the molecular profiles of the antigens they respond to remain poorly defined. A study using TCR Jα18$^{-/-}$ and wild-type mice responding to an MCA-induced sarcoma and immunized with the serological expression cloning technique (SEREX) defined autoantigens has suggested that CD8^{+} T and NKT cell antimetastatic activity may be suppressed by CD4^{+}CD25^{+} cells (Nishikawa *et al.*, 2003). Studies by Ghiringhelli *et al.* (2005) and Smyth *et al.* (2006) have also suggested Treg controlled NK cell-mediated antitumor immunity. These studies highlight the potential complex immunoregulation of the response to tumor.

D. Other Innate Leukocytes

Other leukocyte populations, such as neutrophils, may also play important roles in promoting immunity to tumors (Curcio *et al.*, 2003; Di Carlo *et al.*, 2001; Haliotis *et al.*, 1985). A provocative study by Cui *et al.* (2003) provided further evidence that innate immune cells comprise an important arm of the immunosurveillance network. We have a lot more to learn about innate mechanisms of immunity toward tumors, but first we need to generate mice with specific deficiencies in defined leukocyte subsets (e.g., NK cells, DC, and monocyte subsets) on pure genetic backgrounds. There is also very good evidence that, once mobilized, eosinophils can destroy tumor metastases (Mattes *et al.*, 2003) and now that eosinophil-deficient mice have been created (Lee *et al.*, 2004), their natural role in tumor immunosurveillance can be studied. In addition, more specific function modifying mAbs are needed in order to rapidly dissect out the antitumor role of innate immune components in wild-type mice. Thus far, there have been no reports of spontaneous tumor formation in mice, deficient in one or more of the toll-like receptor (TLR) pathways or heat-shock proteins, but many of these studies remain to be undertaken.

V. IMMUNE MOLECULES THAT RECOGNIZE AND CONTROL CANCER

Pivotal studies have shown that deficiencies in key immunologic effector molecules enhanced host susceptibility to both chemically induced and spontaneous tumors, in large part substantiating the cancer immune surveillance hypothesis (Dighe *et al.*, 1994; Kaplan *et al.*, 1998; Shankaran *et al.*, 2001; Smyth *et al.*, 2000b,c; Street *et al.*, 2001, 2002; van den Broek *et al.*, 1996). Effector mechanisms mediating tumor immune surveillance have been reviewed extensively. These include most notably: IFN-γ (Dighe *et al.*, 1994; Kaplan *et al.*, 1998; Shankaran *et al.*, 2001; Street *et al.*, 2001, 2002); type I interferons (Affabris *et al.*, 1987; Belardelli *et al.*, 2002; Dunn *et al.*, 2005; Gresser and Belardelli, 2002; Gresser *et al.*, 1983, 1988; Smyth, 2005); pfp (Smyth *et al.*, 1999, 2000c; Street *et al.*, 2001, 2002, 2004; van den Broek *et al.*, 1996); TRAIL (Cretney *et al.*, 2002; Smyth *et al.*, 2001a, 2003; Takeda *et al.*, 2001, 2002; Zerafa *et al.*, 2005); and Fas ligand (Davidson *et al.*, 1998; Straus *et al.*, 2001).

A. Tumor Antigens

We now know of a number of recognition systems that immune cells use to detect transformation. A systematic survey of the cellular and humoral

immune responses of patients to their own tumors was initiated in the 1970s using an approach termed autologous typing (Old, 1981). Using this approach, a small subset of patients was identified who had specific antibody to cell-surface antigens (Carey *et al.*, 1976; Ueda *et al.*, 1979) or who had autologous tumor-specific T cells (Knuth *et al.*, 1984). The use of autologous typing to characterize tumor antigens recognized by $CD8^+$ T cells was made possible by applying the gene cloning and expression systems developed by Boon and colleagues (Traversari *et al.*, 1992; van der Bruggen *et al.*, 1991). Similarly, Pfreundschuh and colleagues discovered antibody-defined tumor antigens (Sahin *et al.*, 1995). It has been possible to identify MHC class II-restricted tumor antigens recognized by $CD4^+$ T cells (Wang and Rosenberg, 1999).

The first human tumor antigen was identified in 1991 (van der Bruggen *et al.*, 1991), and since then many tumor antigens have been cloned and can be segregated into the following categories: (1) mutational antigens, for example, abnormal forms of p53; (2) overexpressed/amplified antigens, for example, HER-2/neu; (3) differentiation antigens, for example, melanocyte differentiation antigens, Melan-A/MART-1, tyrosinase, and gp-100; (4) viral antigens, for example, EBV and HPV; and (5) cancer-testis (CT) antigens, for example, MAGE and NY-ESO-1 (Boon and van der Bruggen, 1996; Old, 2003; Rosenberg, 1999). CT antigens are of particular interest since they have unique characteristics (Scanlan *et al.*, 2002). In adult normal tissues, their expression is limited to germ cells in the testis, whereas in cancer, different proportions of various tumor types express CT antigens. The SEREX created by Pfreundschuh and colleagues (Sahin *et al.*, 1995) to detect the humoral response to human cancer has greatly expanded the list of CT antigens, including NY-ESO-1 (Chen *et al.*, 1997) as well as other categories of tumor antigens, and there are now more than 20 CT antigens or antigen families recognized in human cancer (Scanlan *et al.*, 2002). The search for immunogenic human tumor antigens continues using current methods (https://www2.licr.org/CancerImmunomeDB/).

B. Proinflammatory Signals

In addition to tumor antigens presented on MHC molecules, transformed cells may overexpress other molecular signals that can function as recognition targets in tumor immune surveillance. In the past, it was argued that cellular transformation did not provide a sufficient cue to alert the immune system to the presence of a developing tumor (Matzinger, 1994; Pardoll, 2003). However, it is now clear that tumor–stromal interactions may provide the necessary proinflammatory signals. For example, danger signals, such as uric acid (Shi *et al.*, 2003), may arise from the inherent biology of

the tumor itself (Seong and Matzinger, 2004). The induction of proinflammatory responses via the generation of potential TLR ligands, such as HSPs (Srivastava, 2002), or extracellular matrix derivatives, such as hyaluronic acid (Termeer *et al.*, 2002) or heparin sulfates (Johnson *et al.*, 2002), may mimic the events that underlie activation of innate immune responses to microbial pathogens (Janeway, 1989). Similarly, tumor-derived ligands for peptidoglycan recognition receptors or CD1 molecules may also trigger early immune responses to tumors. The function of a large number of innate immune recognition molecules remains to be discovered in mammals. It also remains unknown whether leukocytes like macrophages, neutrophils, and eosinophils can directly recognize tumors. Even the role that indirect FcR-mediated activation by tumor-reactive antibodies plays in natural host immunity to cancer remains a mystery.

C. Innate Immune Recognition by NKG2D-Stress Detection

NKG2D is a key homodimeric activation receptor expressed on the cell surface of almost all NK cells, $\gamma\delta^+$ cells, some cytolytic CD8$^+$ $\alpha\beta^+$ T cells and NKT cells, and a small subset of CD4$^+$ $\alpha\beta^+$ T cells (Bauer *et al.*, 1999; Houchins *et al.*, 1991; Lanier, 2005; Raulet, 2003; Wu *et al.*, 1999). NKG2D plays a key role in immune responses, including those against tumor (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001; Moretta *et al.*, 2001; Raulet, 2003), and thus serves as a definitive activating receptor of NK cells. It was shown that NKG2D functions on mouse NK cells via two distinct signaling pathways following its association with two distinct adapter proteins, DAP10 and DAP12 (Diefenbach *et al.*, 2002; Gilfillan *et al.*, 2002). By contrast, the transmembrane segment of human NKG2D can only associate with DAP10 (Andre *et al.*, 2004; Billadeau *et al.*, 2003; Wu *et al.*, 2000). Nonetheless, in both species NKG2D associates with adapter proteins that provide an activating signal.

Several ligands, which bind to NKG2D, are members of the MHC class Ib family (Table I). In humans, the polymorphic MHC class I chain-related molecules MICA and MICB can be recognized by NKG2D (Bauer *et al.*, 1999; Stephens, 2001). Although MIC molecules have not been found in mice, the retinoic acid early inducible-1 (*Rae-1*) gene products, UL16-binding protein-like transcript 1 (MULT-1), and a distantly related minor histocompatibility Ag, H60, have been reported as NKG2D ligands in mice (Carayannopoulos *et al.*, 2002; Cerwenka *et al.*, 2000; Cosman *et al.*, 2001; Diefenbach *et al.*, 2000). Unlike conventional MHC class I, the MHC class Ib MIC proteins are upregulated on the surface of stressed cells

Table I Human and Mouse Ligands for NKG2D

Ligand	Expression	Induction	Structure
Human			
MICA, B	Gut epithelium, epithelial and nonepithelial tumors, tumor cell lines	Heat shock, oxidative stress, tumor transformation, *Mycobacterium tuberculosis*/*Escherichia coli*/ Human cytomegalovirus (HCMV) infection	Transmembrane protein, MHC class-I–related $\alpha1$ and $\alpha2$ domains, unlike other ligands contains $\alpha3$ domain
ULBP1, 2, 3	Tumor cell lines	Tumor transformation	GPI-anchored proteins
Mouse			
Rae-1α, β, γ, δ*, ϵ*	Embryonic tissues, tumor cell lines, not expressed in most normal adult tissues *strain-restricted expression (C57BL/6)	Retinoic acid, carcinogens, tumor transformation	GPI-anchored proteins, MHC class-I–related $\alpha1$ and $\alpha2$ domains
H60	Strain-restricted expression (BALB/c), activated peripheral blood leukocytes and splenocytes	Carcinogens, tumor transformation	Transmembrane protein, MHC class-I–related $\alpha1$ and $\alpha2$ domains
MULT-1	mRNA was detected in a wide variety of tissues, including thymus and spleen, lymph nodes, and to a lesser extent liver, heart and lung, tumor cell lines	?	Transmembrane protein, MHC class-I–related $\alpha1$ and $\alpha2$ domains

and are frequently overexpressed by tumors (Groh *et al.*, 1999; Vetter *et al.*, 2002).

The MICA/B proteins are highly polymorphic, nonclassical MHC cell-surface glycoproteins that do not associate with β2m or require transporter associated protein (TAP) 1 for expression (Groh *et al.*, 1996).While MIC expression in normal tissues has only been documented on the gastrointestinal epithelium of the stomach and large intestines, MICA/B proteins are often expressed in primary carcinomas of the lung, kidney, prostate, ovary, colon (Groh *et al.*, 1996) and liver (Jinushi *et al.*, 2003) as well as in melanomas (Vetter *et al.*, 2002). In addition, UL16 binding proteins (ULBPs) (Pende *et al.*, 2002) and Letal (Conejo-Garcia *et al.*, 2003) are also frequently expressed on tumor cells. Importantly, two data sets link NKG2D recognition to immune surveillance. First, Groh *et al.* (1998) demonstrated that MIC-expressing cells were recognized and killed by the Vδ1 $\gamma\delta$ T-cell subset, and observed a strong *in vivo* correlation ($p < 0.0001$) between MICA/B expression on tumors and tumor infiltration by Vδ1 $\gamma\delta^{+}$ T cells (Groh *et al.*, 1999). Second, data demonstrated a correlation between downregulation of NKG2D on tumor-infiltrating lymphocytes (TILs) and the expression of MICA/B in the tumor (Groh *et al.*, 2002). Compared with NKG2D expression in lymphocytes from patients with MIC^{-} tumors, NKG2D expression was reduced on tumor-infiltrating CD8^{+} T cells, $\gamma\delta^{+}$ T cells, and NK cells and also on peripheral blood mononuclear cells (PBMCs) from individuals with MIC^{+} tumors. Further analysis revealed a correlation between the presence of soluble MIC proteins in the circulation of 7/14 cancer patients and a downregulated expression of NKG2D on lymphocytes. Results of a separate study suggested that shedding of MIC proteins from tumor cell surfaces was the result of the actions of an unknown matrix metalloproteinase (MMP) (Salih *et al.*, 2002). The finding that soluble MIC proteins may attenuate the expression/function of NKG2D on host immune cells provides one explanation for how a growing tumor could escape cancer immune surveillance.

In mice, NKG2D binds to the Rae-1 family proteins, the minor histocompatibility antigen (MiHA) H60 (Cerwenka and Lanier, 2001a; Diefenbach *et al.*, 2000), and mouse MULT-1 (Carayannopoulos *et al.*, 2002; Diefenbach *et al.*, 2003). NKG2D ligand expression has been observed on a wide range of murine tumors (Diefenbach *et al.*, 2000). In mice, the expression of Rae-1 and H60 was shown to be induced by skin painting with the carcinogens DMBA/TPA and the importance of the NKG2D pathway in host protection from skin carcinoma was implicated, but not directly proven (Girardi *et al.*, 2001). Importantly, a study by Raulet and colleagues demonstrated that the DNA-damaged pathway regulates NKG2D ligand expression (Gasser *et al.*, 2005). Mouse and human NKG2D ligands were upregulated in nontumor cell lines by genotoxic stress and stalled DNA replication conditions known to activate an expression of major

DNA damage checkpoint pathway, therefore, implicating that NKG2D ligand may be generally upregulated during cellular transformation and may be an attractive target for tumor recognition by NK cells.

VI. NKG2D TRIGGERS TUMOR CELL KILLING

Natural or induced expression of NKG2D ligands markedly enhances the sensitivity of tumor cells to NK cells *in vitro* (Bauer *et al.*, 1999; Cerwenka *et al.*, 2001; Cosman *et al.*, 2001; Diefenbach *et al.*, 2001; Hayakawa *et al.*, 2002; Pende *et al.*, 2002). Expression of NKG2D ligands by tumor cells also results in immune destruction *in vivo* and the ectopic expression of NKG2D ligands, Rae-1, and H60 in several tumor cell lines results in the rejection of the tumor cells expressing normal levels of MHC class I molecules (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001). Immune depletion and other studies showed that rejection was dependent on NK cells and/or $CD8^+$ T cells (Diefenbach *et al.*, 2001; Hayakawa *et al.*, 2002). There still exists some controversy over whether NKG2D-mediated tumor rejection can generate adaptive immunity to NKG2D ligand-negative tumors (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001; Westwood *et al.*, 2004).

A. NKG2D: A Cytotoxicity Receptor

Despite the fact that NKG2D activates NK cells to induce both cytotoxicity and cytokine production under normal conditions (Hayakawa *et al.*, 2002; Jamieson *et al.*, 2002), previous studies showed that NKG2D ligation triggers cytotoxicity but not cytokine production in the absence of DAP12 or Syk family kinases (Billadeau *et al.*, 2003; Zompi *et al.*, 2003). Such evidence clearly supports the belief that the NKG2D receptor acts as a primary cytotoxicity receptor for NK cells (Fig. 2). Consistent with these molecular studies, tumor cell lines with ectopic NKG2D ligand expression are rejected *in vivo* by NK cell pfp-mediated cytotoxicity, but not NK cell IFN-γ production, suggesting the importance of NKG2D as a primary pfp-mediated cytotoxicity receptor for NK cells in the context of antitumor immune responses (Hayakawa *et al.*, 2002). Alternatively, NK cell IFN-γ secretion plays a regulatory role in antitumor immune responses and potentially following NK cell costimulation by stimulating adaptive immunity. Nevertheless, such biased dependence on pfp-mediated cytotoxicity is unique feature of NKG2D recognition compared with other NK cell-activating structures, such as CD27 or CD28, that require both cytotoxicity and IFN-γ production for their antitumor potential (Kelly *et al.*, 2002a,b),

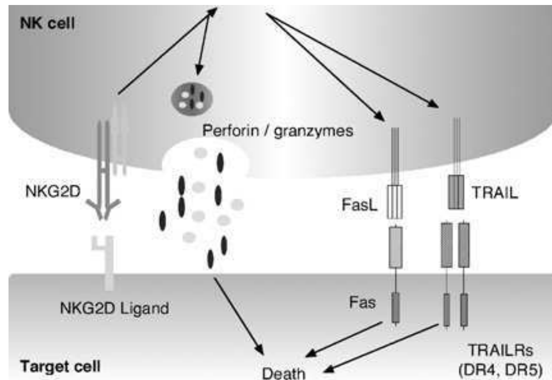

Fig. 2 NK cells mediate two pathways of cell death and the role of NKG2D as primary granule-mediated cytotoxicity. The direct killing of tumor or pathogen-infected cells is a crucial component of the NK cell response. NK cells mediate cell killing through a variety of mechanisms, including pfp/granzyme granule-mediated exocytosis or signaling through the TNF death receptor family members. The granule exocytosis pathway involves the release of cytotoxic granule contents (e.g., pfp, granzymes) into the intercellular space (between NK cell and target cell). By the recognition of NKG2D ligands expressed by tumor cells, NK cells receive an activation signal and preferentially deliver pfp-mediated cytotoxicity to kill target cells. Members of the TNF super family, FasL and TRAIL, are expressed by NK cells and are important mediators of caspase-dependent target cell apoptosis via their corresponding receptors (Fas and TRAIL receptors) on tumor cells.

suggesting the importance of NKG2D as a primary receptor for NK cell granule-mediated cytotoxicity (Fig. 2). Moreover, the importance of the NKG2D pathway in *de novo* tumorigenesis has been determined in a carcinogen-induced sarcoma formation model by blocking NKG2D-specific mAb (Smyth *et al.*, 2005b). Given that mice treated with *anti*-NKG2D mAb had a greater incidence of fibrosarcoma, NKG2D recognition clearly involved host protection in carcinogen-induced tumor formation. Host NK cells and T cells contributed as effector cells in host NKG2D-mediated protection from MCA sarcoma. Although in general, the control of MCA-induced sarcoma is mediated by a combination of pfp-, TRAIL-, and IFN-γ–effector molecules, the importance of the NKG2D pathway was additionally illustrated in mice, deficient for either IFN-γ or TRAIL, while mice deficient for pfp did not display any detectable NKG2D phenotype. This evidence showed that the NKG2D pathway was operating primarily to activate host pfp-mediated cytotoxicity to protect the host from carcinogen-induced tumor formation. Further study of the transformation process will be necessary to detail when—and how—in the course of tumorigenesis a cancer cell first expresses NKG2D ligands or otherwise becomes immunogenic.

B. Immunoediting and Escape

In addition to suppressing tumor formation, the immune system may also select for tumor variants during tumor formation that better survive in an immunocompetent host (Dunn *et al.*, 2002, 2004b). Previous studies of transplantable tumors passaged in immune intact hosts have generated tumor variants with reduced immunogenicity. Several new studies have compared the immunogenic characteristics of tumors originally generated in wild-type mice and gene-targeted mice lacking a specific component of the immune system (Crowe *et al.*, 2002; Shankaran *et al.*, 2001; Smyth *et al.*, 2000c; Street *et al.*, 2004; Takeda *et al.*, 2002). Generally, but not always, it has been observed that tumors formed in the absence of an intact immune system are more immunogenic than tumors that arise in immunocompetent hosts. Therefore, tumors may be shaped by the immune environment in which they are generated. This process was originally termed "immunoediting" by Schreiber and colleagues (Dunn *et al.*, 2004a,b) and was illustrated with a report that type I IFNs contribute to this process (Dunn *et al.*, 2005). Thus, the immune system may play multiple roles in tumor initiation (microenvironment, tissue architecture, and inflammation), suppression (immunosurveillance and immunoediting), and progression (immunoevasion).

Given the potential of the immune system to edit tumors, including sarcomas (Shankaran *et al.*, 2001; Takeda *et al.*, 2002), it was expected that some gene-targeted mice might have an increased proportion of tumors displaying an unedited phenotype and expressing NKG2D ligands. Various sarcoma cell lines derived from wild-type mice demonstrated that Rae-1 expression was variable or absent and a similar frequency was observed in sarcomas derived from another "NKG2D-edited" host such as TRAIL-deficient mice (Smyth *et al.*, 2005b). By contrast, sarcomas derived from "non-NKG2D-edited" mice, such as those treated with *anti*-NKG2D mAb, were shown to universally express medium to high levels of Rae-1 (Fig. 3). Importantly, sarcomas derived from pfp-deficient mice were also shown to express universal expression of Rae-1, implicating the importance of this effector pathway in the process of tumor immunoediting following NKG2D recognition (Smyth *et al.*, 2005b). Therefore, it is possible that the NKG2D-NKG2D ligand pathway only plays a tumor suppressive role very early in the host response to transformation. Once established, some tumors may express NKG2D ligands and yet continued to grow, perhaps by inducing immune suppression by directly downmodulating NKG2D on local immune-effector cells. Systemic NKG2D downmodulation and considerable impairment of NK cell effector function can be observed in a series of transgenic mice constitutively expressing NKG2D ligand either ubiquitously or in a conditional and tissue-specific manner (Ogasawara

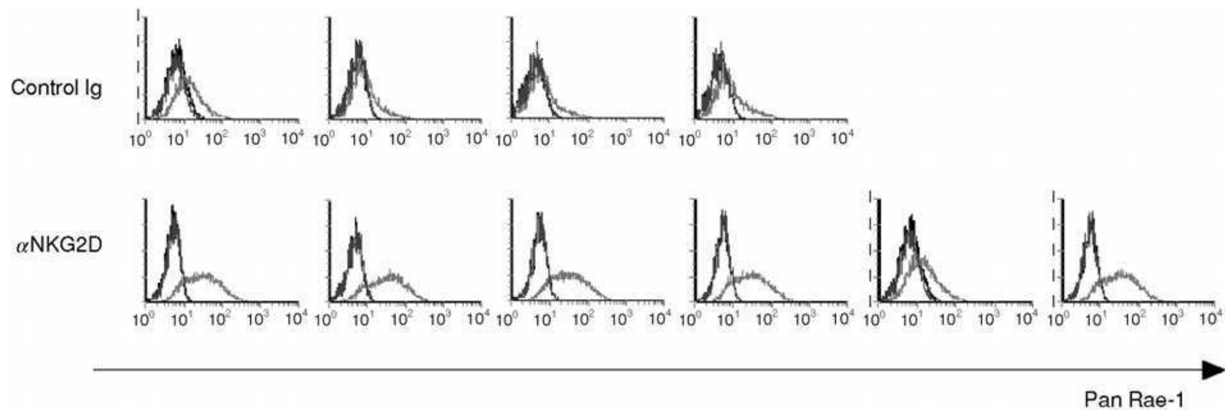

Fig. 3 Expression of NKG2D ligands on tumors indicates immunoediting. MCA-induced sarcoma cell lines were derived from control Ig- or anti-NKG2D–treated mice and assessed for Rae-1 expression (Isotype control–gray lines, Rae-1–solid black lines). As a consequence of the immunoediting process, the sarcoma cell lines derived from "non-NKG2D-edited" mice (treated with *anti*-NKG2D mAb) were shown to universally express medium to high levels of Rae-1. Importantly, sarcomas derived from pfp-deficient mice were also shown to express universal expression of Rae-1 (Smyth *et al.*, 2005b), implicating the importance of this effector pathway in the process of tumor immunoediting following NKG2D recognition.

et al., 2005; Oppenheim *et al.*, 2005). Such mice showed higher susceptibility to tumor development suggesting that constitutive expression of NKG2D ligand on transformed tumor cells and subsequent NKG2D downregulation on NK cells by chronic exposure to its ligand may allow tumor cells to evade NKG2D-mediated immune surveillance (Coudert *et al.*, 2005; Groh *et al.*, 2002; Wiemann *et al.*, 2005).

C. Tumor NKG2D Ligands Determine Response to Immunotherapy

Cytokines have played an important role in tumor immunology and new immunotherapies (Rosenberg, 2001; Smyth *et al.*, 2004a). The use of IL-2 in patients with metastatic melanoma and renal cell cancer has demonstrated that manipulation of the immune system is capable of mediating the durable regression of established metastatic tumors (Rosenberg, 2001). The mechanism of antitumor efficacy of IL-2 is closely related to its ability to expand and activate NK and T cells that express IL-2 receptors. Other promising cytokines in cancer immunotherapy, including IL-12 (Smyth *et al.*, 2000a), IL-18 (Hashimoto *et al.*, 2003), and IL-21 (Brady *et al.*, 2004) have also been shown to mediate their antitumor activities in mice to a large extent via NK cells. Consistent with a natural role of the NKG2D pathway in NK cell pfp-mediated cytotoxicity, the antitumor activities of cytokines triggering NK-cell pfp-mediated cytotoxicity, but not death receptor–ligand interactions (such as FasL or TRAIL), were largely dependent on the NKG2D recognition (Smyth *et al.*, 2004b; Takaki *et al.*, 2005). Therefore, the status of NKG2D ligand expression on target tumor cell might be critical in predicting a pfp-mediated mechanism of NK cell-based immunotherapies. Thus, selective induction of such ligands on tumor cells may be an attractive approach to enhance the efficacy of NK cell-based immunotherapy. Alternatively, it has been reported that *in vitro* cytokine stimulation can reverse NKG2D expression of NK cells that has been downregulated by chronic ligand exposure and subsequently restore through NKG2D (Coudert *et al.*, 2005; Groh *et al.*, 2002; Ogasawara *et al.*, 2003).

VII. CONCLUSIONS

The innate immune system encompasses NK cells, macrophages and granulocytes, the complement system, and antimicrobial peptides. Recognition pathways of the innate immune system include microbial nonself recognition, missing-self-recognition, and induced-self-recognition. Because

tumors develop from self-cells, in general, tumors are poorly immunogenic and are often not recognized effectively by the adaptive immune system. The NKG2D-DAP10 receptor complex activates NK cells and $\gamma\delta^{+}$ T cell subsets upon engagement of ligands that can be conditionally expressed under physiologically harmful conditions such as microbial infections and malignancies (induced-self-recognition). These ligands are frequently expressed on a number of human and mouse tumors and thus potentially serve as one of several types of danger signals to alert the immune system to the presence of transformed cells. This system complements the ability of NK cells to detect an absence or alteration of MHC class I molecules via their inhibitory receptors (i.e., missing-self-recognition). The NKG2D receptor primarily acts to trigger pfp-mediated apoptosis of target tumor cells. Tumors emerging from NKG2D-mediated surveillance generally lack NKG2D ligands, suggesting immunoediting pressure has been exerted by this pathway during the development of the tumor. It remains unclear what functional role such a spectrum of NKG2D ligands might play in tumor elimination, editing, and escape. Nonetheless, often, immune inhibitory and inflammatory mechanisms physiologically outbalance and counteract immune activity and thereby limiting innate immune-mediated destruction of transformed cells. Tumors often create an immunosuppressive milieu, where immune inhibitory cell-surface molecules or soluble ligands and cytokines (e.g., TGF-β) inhibit most types of cellular immunity, including that mediated via the NKG2D pathway. Not surprisingly, tumors additionally interfere with DC maturation and cross talk with other innate leukocytes, inhibiting the expression of self-MHC molecule/antigen complexes and thereby preventing effective antitumor adaptive immunity. Future revelation of other stress-induced recognition systems will only serve to increase our opportunities to stimulate natural immunity in strategies that both treat and prevent malignancy.

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Inhibitors of the HSP90 Molecular Chaperone: Current Status

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The molecular chaperone heat shock protein 90 (HSP90) has emerged as an exciting molecular target for cancer therapy. It operates as part of a multichaperone complex and is essential for the conformation, stability, and function of several key oncogenic client proteins such as mutant p53, ERBB2, B-RAF, C-RAF, and CDK4. The HSP90-based chaperone machine is driven by the hydrolysis of ATP and ADP/ATP nucleotide exchange. Many of the inhibitors of HSP90 interrupt the intrinsic ATPase activity, causing degradation of the client proteins via the ubiquitin-proteasome pathway. The first-in-class HSP90 inhibitor in clinical trials is the geldanamycin analog, 17-allylamino, 17-demethoxygeldanamycin (17-AAG). The results that have emerged from these trials have been encouraging, with stable disease observed in two melanoma patients. Pharmacodynamic endpoints, such as induction of HSP70 and downregulation of C-RAF and CDK4 in peripheral blood mononuclear cells and tumor biopsies from treated patients, provided evidence of HSP90 inhibition at well-tolerated doses. The toxicity of 17-AAG has been mild. Several preclinical studies have shown that 17-AAG may enhance the efficacy of a variety of chemotherapeutic agents. Phase II clinical trials in various cancers have been initiated as well as Phase I trials of combined therapy with 17-AAG. However, there are several limitations with 17-AAG such as solubility, stability, and hepatotoxicity. Thus, it is not surprising that new HSP90 agents are under development against this novel target for cancer therapy and several show promise. © 2006 Elsevier Inc.

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I. INTRODUCTION

The molecular chaperone HSP90 is responsible for controlling the conformation, stability, activation, intracellular disposition, and proteolytic turnover of numerous important proteins that are involved in cell growth, differentiation, and survival (Maloney and Workman, 2002; Richter and Buchner, 2001). Many proteins responsible for the six hallmarks of cancer (Hanahan and Weinberg, 2000) are HSP90 dependent (Fig. 1). They include several kinases (ERBB2, B-RAF, C-RAF, and CDK4), hormone receptors (androgen and estrogen receptors), and other proteins (mutant p53, catalytic subunit of telomerase hTERT) (Isaacs *et al.*, 2003; Maloney and Workman, 2002). An updated list of the ever-growing number of proteins that HSP90 interacts with can be found at http://www.picard.ch. HSP90 has emerged as a promising target for the development of cancer chemotherapeutics because multiple oncogenic proteins can be simultaneously disrupted by inhibition of the HSP90 protein chaperone machinery.

Studies have shown that the HSP90 superchaperone complex that predominates in cancer cells is able to bind an HSP90 inhibitor about 100 times more tightly when compared to the HSP90 from normal cells (Kamal *et al.*, 2003). Hence, it is of no surprise that HSP90 has generated great interest as a novel target for cancer therapy. The majority of cancers that are driven by multiple molecular pathways may be treated using HSP90 inhibitors as the downstream effects of HSP90 inhibition affect a wide range of signaling processes that are crucial for the malignant properties of cancer cells. HSP90 inhibitors may, therefore, exhibit a broad spectrum of anticancer activity.

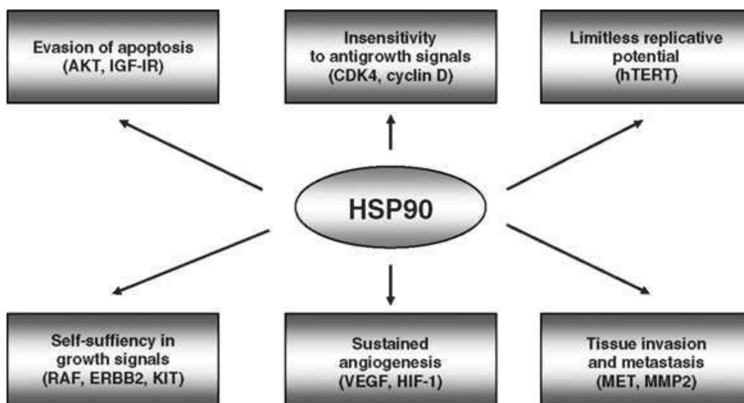


Fig. 1 Examples of HSP90-dependent client proteins involved in the six hallmarks traits of cancer.

There are several HSP90 family members that are localized in different parts of the cell and which are thought to bind to different client proteins. The two major cytoplasmic isoforms are HSP90α (inducible/major form) and HSP90β (constitutive/minor form) (Hickey *et al.*, 1989). Other major isoforms are GRP94 in the endoplasmic reticulum (Argon and Simen, 1999) and TRAP1/HSP75 in the mitochondrial matrix (Felts *et al.*, 2000). A report has described HSP90N as another isoform, which is associated with cellular transformation (Grammatikakis *et al.*, 2002). Details of the function and clinical relevance of the various HSP90 isoforms have been reviewed (Chen *et al.*, 2005; Sreedhar *et al.*, 2004).

The dimerization of HSP90 is essential for its function, together with the binding of a plethora of cochaperones and other proteins that make up the multichaperone complex (Pearl and Prodromou, 2001). The role of these cochaperones, including AHA1 (activator of HSP90 ATPase) protein (Panaretou *et al.*, 2002), remains to be elucidated fully. HSP90 consists of three domains: the N-terminal ATPase domain, a middle domain which is implicated in client protein binding, and a C-terminal dimerization domain (Pearl and Prodromou, 2001). The ATPase activity of HSP90 is essential for the chaperone cycle and controls client protein binding and fate (Obermann *et al.*, 1998; Panaretou *et al.*, 2002).

The HSP90 chaperone–client protein cycle involves the association and dissociation of several cochaperones to form various multimeric protein complexes (Table I) and is dictated by the ATP binding state of HSP90. A client protein initially associates with an HSP70/HSP40 complex (Hernandez *et al.*, 2002) and is then bound to HSP90 via HOP (an HSP90/HSP70 organizing protein), when the chaperone is in its ADP bound state, to form the intermediate chaperone complex (Fig. 2) (Hernandez *et al.*, 2002; Maloney and Workman, 2002). One model of the chaperone cycle suggests that when ADP is hydrolyzed to ATP, the conformation of HSP90 is altered, thus releasing HSP70/HSP40 and HOP, which then allows other cochaperones (e.g., P23, CDC37, or immunophilins depending on the client proteins) to associate with HSP90 to form a mature complex. CDC37 is involved specifically in loading kinase clients onto HSP90 (Roe *et al.*, 2004). At this stage, the conformation and stability of client proteins are maintained, thus allowing them either to bind to ligands (as with steroid hormones) or to be phosphorylated and activate signal transduction pathways (as with kinases such as AKT). It has been reported that another novel protein HARC (HSP90-associating relative of CDC37), which is related to CDC37 both structurally and functionally, is also involved in the HSP90-mediated protein folding, potentially facilitating the binding of HSP90 to early HSP70–client protein complexes (Fig. 2) (Scholz *et al.*, 2001).

Table I Major Components Involved in the HSP90 Chaperone Machinery

Protein	Function
HSP90	A chaperone—to maintain the conformational stability, shape, and function of a range of important proteins
HSP70	A chaperone—involved in the association of client proteins to the multiprotein complex of HSP90 machinery, also has chaperone activity independent of HSP90
HSP40	A cochaperone—increases the ATPase activity of HSP70 for association with HSP90
HIP, HOP (P60)	A HSP90/HSP70 interacting and organizing protein—involved in mediating the interaction between HSP90 and HSP70
AHA1	A cochaperone—increases the HSP90 ATPase activity by causing a conformational change in the activation loop of HSP90
P23	A cochaperone—maintains the stability of the HSP90 in the "mature complex" and subsequently as a client protein release factor
CDC37 (P50)	A cochaperone—involved in the interaction with client proteins, specifically kinases; inhibits HSP90 ATPase activity to facilitate client protein loading onto HSP90
HARC	Structurally and functionally related to CDC37, potentially targeting HSP90 to HSP70-client protein heterocomplexes
Immunophilin	Protein involved in the interaction with client proteins, specifically hormone receptors; increases the ATPase activity to a limited extent

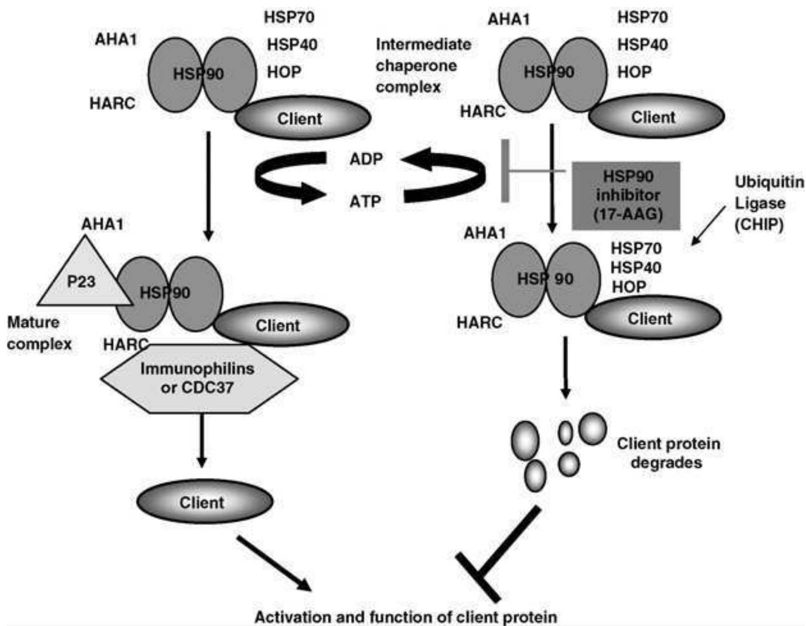


Fig. 2 The HSP90 chaperone machinery.

The majority of HSP90 inhibitors to date act by docking in the N-terminal nucleotide binding site, thereby inhibiting the intrinsic ATPase activity and thus blocking the formation of the mature complex. Consequently, a ubiquitin ligase (e.g., CHIP) is recruited to the complex and the client proteins are then targeted for degradation via the ubiquitin-proteasome pathway (Fig. 2) (Connell *et al.*, 2001).

The first modulator of HSP90 to enter clinical trials is 17-allylamino, 17-demethoxygeldanamycin (17-AAG) and several other inhibitors are now in development. This chapter will focus on the current status of HSP90 inhibitors. Many of these inhibitors have been shown to cause selective degradation of important signaling proteins involved in cell proliferation, cell cycle regulation and apoptosis in a wide range of tumor models. Table II lists the various HSP90 inhibitors that have been reported.

II. HSP90 INHIBITORS

A. Natural Product-Based Agents

1. RADICICOL

Radicicol (Fig. 3) is a macrocyclic antibiotic produced by the fungus *Monosporium bonorden* and was originally thought to act as a kinase inhibitor (Kwon *et al.*, 1992; Zhao *et al.*, 1995). It is structurally unrelated to the ansamycin antibiotics geldanamycin and herbimycin A (see later). However, like geldanamycin, studies have shown that radicicol potently inhibits HSP90 function by binding tightly to the conserved N-terminal domain of HSP90 and competes with ATP binding (Roe *et al.*, 1999; Schulte and Neckers, 1998). Client proteins, such as C-RAF, mutant p53, and ERBB2, have been shown to be downregulated in cells treated with radicicol (Schulte and Neckers, 1998; Soga *et al.*, 2003). Despite the highly promising cellular activity *in vitro*, its *in vivo* antitumor activity in animal models has been disappointing, which may be due to the reactive epoxide moiety and other adverse structural features (Agatsuma *et al.*, 2002; Soga *et al.*, 2003). Japanese researchers from Kyowa Hakko Kogyo Company (http://www.kyowa.co.jp) have synthesized several oxime derivatives of radicicol (KF55823 and KF58333) (Fig. 3) that exhibited potent antiproliferative activities *in vitro*, and *in vivo* studies have reported good antitumor activity at well-tolerated doses in human tumor xenografts, including human breast and colon carcinomas, with no serious liver toxicity (Agatsuma *et al.*, 2002; Kurebayashi *et al.*, 2001; Soga *et al.*, 2003). Treatment with KF58333

Table II Current Status of HSP90 Inhibitors

Chemical class	Drug or lead compound	Binding to HSP90 ATP-binding pocket	Current status	Company/institution
Benzoquinone ansamycin	17-AAG	N-terminal	17-AAG: Phase I completed, Phase I in combinations and II clinical trials commenced	Kosan Biosciences/National Cancer Institute
Benzoquinone ansamycin	17-DMAG	N-terminal	Phase I clinical trial	Kosan Biosciences/National Cancer Institute
Hydroquinone form of 17-AAG	IP-504	N-terminal	Phase I clinical trial	Infinity Pharmaceuticals
Benzoquinone ansamycin	CNF1010	N-terminal	Preliminary Phase I clinical trials showed that CNF1010 is well tolerated at 175 mg/m^2, dose escalation is continuing	Conforma Therapeutics
Antibody	Mycograb	Unknown	Phase II clinical trial for invasive candidiasis	NeuTec/Manchester University

Pyrazole	CCT018159 VER49009	N-terminal	Preclinical development, VER49009 exhibits high enzyme and cellular potency *in vitro*	Vernalis/Novartis/Institute of Cancer Research
Purine	PU24F-Cl	N-terminal	Preclinical evaluation	Memorial Sloan-Kettering Cancer Center
Radicicols	Radicicol and oxime derivatives	N-terminal	Preclinical development	Kyowa Hakko Kogyo
Geldanamycin dimer	Geldanamycin dimer	N-terminal	Preclinical development	Conforma Therapeutics
Platinum compound	Cisplatin	C-terminal	Phase I combination clinical trial with 17-AAG	Generic
Coumarin	Novobiocin analogs	C-terminal	Analog development	Yu *et al*., 2005
Adenosine analog	NECA	GRP94	Analog development	Soldano *et al*., 2003
taxane	paclitaxel	Unknown	Phase I combination clinical trial with 17-AAG	Bristol Myers Squibb
Histone deacetylase inhibitor	SAHA	Unknown	Phase I clinical trials	Merck

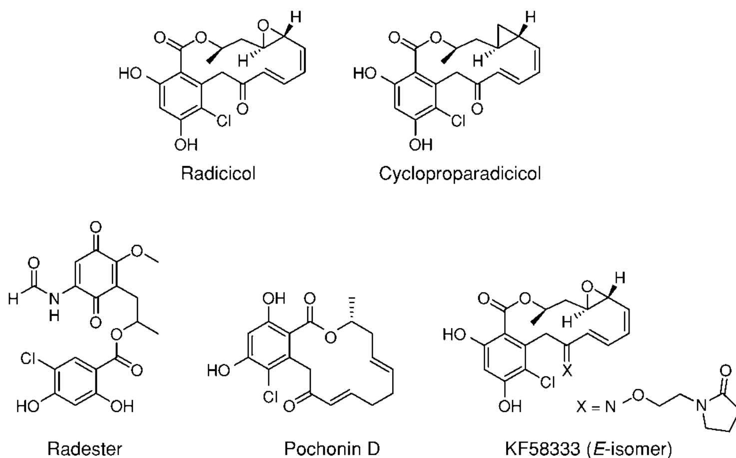


Fig. 3 Radicicol and analogs.

downregulated client protein depletion consistent with HSP90 inhibition, and induction of apoptosis and potent antitumor activity in the KPL-4 breast xenograft that expressed high levels of ERBB2 were observed (Soga *et al.*, 2001). Other *in vivo* studies have demonstrated the therapeutic potential of these radicicol derivatives for the treatment of BCR-ABL expressing chronic myelogenous leukemia (CML) (Shiotsu *et al.*, 2002). However, radicicol oxime derivatives have yet to enter clinical trials, possibly related to reports of toxicity to the eye (Janin, 2005).

In an attempt to address the reactive epoxide moiety, cyclopropyl analogs of radicicol have been synthesized (Fig. 3). These have shown similar cellular potency to radicicol, with potential for improved *in vivo* activity (Yamamoto *et al.*, 2003; Yang *et al.*, 2004). Radester is a hybrid composed of radicicol's resorcinol and geldanamycin's benzoquinone part structures and shows respectable cellular activity against MCF7 breast cancer cells, together with degradation of ERBB2 and C-RAF, which is consistent of HSP90 inhibition (Shen and Blagg, 2005).

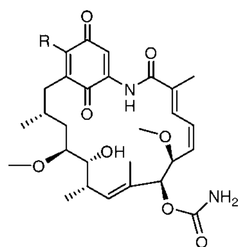
Studies based on conformational similarity to radicicol have led to the identification of pochonin D (Fig. 3). The affinity of one analog of pochonin D for HSP90 (80 nM) is close to that of radicicol (20 nM) (Moulin *et al.*, 2005). It also lacks the reactive epoxide moiety.

A series of zearalenol compounds have been developed by Conforma Therapeutics (http://www.conformacorp.com), with the most potent compound (β-zearalenol) demonstrating low micromolar activity in MCF7 cells.

2. BENZOQUINONE ANSAMYCINS: GELDANAMYCIN, 17-AAG, AND 17-DMAG

The benzoquinone-containing antibiotic geldanamycin (Fig. 4) was isolated from *Streptomyces hygrocopicus* (DeBoer *et al.*, 1970). Like radicicol, geldanamycin was originally believed to be a kinase inhibitor but later studies have shown that geldanamycin inhibits HSP90 function *in vitro* at low micromolar concentrations via the interaction with the N-terminal ATP binding domain of HSP90, which results in the destabilization of oncogenic client proteins, such as ERBB2, EGFR, C-RAF, and CDK4, via the proteasomal degradation pathway (Prodromou *et al.*, 1997; Whitesell *et al.*, 1994). Another benzoquinone ansamycin antibiotic, herbimycin A, was isolated and found also to inhibit v-SRC and BCR-ABL tyrosine kinases *in vitro* (Fukazawa *et al.*, 1994). Geldanamycin and herbimycin A have been proved to be effective against cancer cells *in vitro*. However, despite their cellular potency, they were not considered to have a sufficient therapeutic window for clinical development due to hepatotoxicity; they also have limited metabolic stability (Supko *et al.*, 1995). It is possible that hepatotoxicity in this class of compounds may be caused by the metabolism of the benzoquinone moiety rather than being HSP90 mechanism-based.

Accordingly, many analogs of geldanamycin have been evaluated (Schnur *et al.*, 1995a,b). The 17-allylamino derivative of geldanamycin 17-AAG (Fig. 4) was found to have increased biological activity and metabolic stability and still retains all the HSP90-related therapeutic characteristics (Schulte and Neckers, 1998). 17-AAG has been shown to cause apoptosis and cell cycle arrest in cancer cells *in vitro* (Hostein *et al.*, 2001). Cells with high levels of ERBB2 have been shown to be particularly sensitive to 17-AAG *in vitro* (IC_{50} of <10 nM in BT474 and SKBr-3 breast cancer cell



17-DMAG R=$NHCH_2CH_2N(CH_3)_2$

17-AAG R=$NHCH_2CH{=}CH_2$

Geldanamycin R=OCH_3

Fig. 4 Benzoquinone ansamycin family of HSP90 inhibitors.

lines (Munster *et al.*, 2001). 17-AAG has been shown to interfere with the multiple functional compartments of tumor angiogenesis (Sanderson *et al.*, 2006). Preclinical studies have shown that the hepatic metabolism of 17-AAG by cytochrome P450 enzymes leads to the formation of 17-amino, 17-demethoxygeldanamycin (17-AG) (Egorin *et al.*, 2001), which retains its HSP90 inhibitory action (Kelland *et al.*, 1999). 17-AAG was found to be highly potent *in vitro*, which may be due to increased uptake and accumulation in cancer cells (Chiosis *et al.*, 2003; Workman, 2003). This agent has been shown to possess antitumor activity at nontoxic doses in various animal models, including human melanoma, breast, prostate, colon, and non-small cell lung cancer xenografts (Kelland *et al.*, 1999; Smith *et al.*, 2005; Solit *et al.*, 2002). It has entered clinical trials in the United States and UK and the results of the Phase I clinical trials will be discussed later.

The initial intravenous formulation of 17-AAG used in clinical trials incorporates a large amount of DMSO, together with egg phospholipid. This is cumbersome and may have adverse effects. More acceptable formulations for 17-AAG have been developed by Conforma Therapeutics (e.g., CNF1010) and Kosan Biosciences (http://www.kosan.com; e.g., KOS 953), which have resulted in clinical trials. Preliminary Phase I clinical trial results showed that CNF1010 was well tolerated at doses <175 mg/m^2 and dose escalation is continuing (Dragovich *et al.*, 2005). There are ongoing efforts to develop additional geldanamycin analogs that have advantages over 17-AAG. One of these, the more soluble 17-demethoxy, 17-(2-dimethylamino) ethylamino geldanamycin (17-DMAG) (Fig. 4) (Egorin *et al.*, 2002), has entered Phase I clinical trials. 17-DMAG has similar activity to 17-AAG both *in vitro* and *in vivo* but is more water soluble and orally bioavailable (Burger *et al.*, 2004; Kaur *et al.*, 2004; Smith *et al.*, 2005). Like 17-AAG, 17-DMAG has been shown to distribute widely in tissues but was retained for longer in tumors than normal tissues (Eiseman *et al.*, 2005).

Quinolinedione-based geldanamycin analogs have been designed using X-ray crystallography and showed geldanamycin-like activity (Hargreaves *et al.*, 2003). Studies have reported a novel dimeric ansamycin (EC5), designed to link both amino-terminal binding sites on the HSP90 dimer (Yin *et al.*, 2005). EC5 has been found to be more potent than 17-AAG, both *in vitro* and *in vivo*, and inhibited tumor cell proliferation in several lines that were resistant to 17-AAG. Additional geldanamycin analogs modified at the 17-position include amides, carbamates, and ureas and 17-arylgeldanamycins have shown selectivity for HSP90 derived from tumor cells using a novel cell lysate binding assay (Le Brazidec *et al.*, 2004).

With respect to the quinone moiety in 17-AAG and related benzoquinone ansamycins, the levels of bioreductive enzymes are involved in the bioreductive metabolism. The enzyme that has the greatest influence on 17-AAG metabolism is the NAD(P):quinone oxidoreductase 1 (NQO1;

DT-diaphorase) (Kelland *et al.*, 1999). An excellent correlation was observed between 17-AAG sensitivity and NQO1 expression. A subsequent study showed that 17-AAG can be reduced to 17-AAGH_2 by NQO1 and that this hydroquinone may be a more potent HSP90 inhibitor than 17-AAG (Guo *et al.*, 2005). Infinity Pharmaceuticals (http://www.ipi.com) have developed the hydroquinone form of 17-AAG, IPI-504, for clinical evaluation. In preclinical animal studies, IPI-504 demonstrated utility, as a single agent as well as in combination, in models of a wide variety of hematological malignancies as well as solid tumors (http://www.ipi.com). A report has shown IPI-504 causes inhibition and degradation of KIT in imatinib-resistant gastrointestinal stromal tumors (GISTs), which may provide a compelling rationale for the upcoming clinical trial (Bauer *et al.*, 2005).

B. Pyrazoles

High-throughput screening at the Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, has led to the discovery of novel diarylpyrazole compounds that inhibited the ATPase activity of the yeast HSP90 (Rowlands *et al.*, 2004). These were exemplified by the hit compound designated as CCT018159 (Fig. 5) (Cheung *et al.*, 2005). Using the malachite green assay to measure the ATPase activity of yeast HSP90 (Rowlands *et al.*, 2004), the ATPase IC_{50} values of CCT018159 and 17-AAG were found to be 7.1 and 6.6 μM, respectively. In addition to the yeast enzyme, human HSP90 enzyme and the novel cochaperone AHA1 (Panaretou *et al.*, 2002) were used to measure the ATPase inhibitory IC_{50} of CCT018159 (3.2 μM) and 17-AAG (3.6 μM). Growth inhibition assays showed that CCT018159 had a cellular GI_{50} value of 4.1 μM in the HCT116 human colon cancer cell line (Cheung *et al.*, 2005). The induction of HSP70 and downregulation of client proteins, such as C-RAF, CDK4, and ERBB2, were demonstrated in various human cancer cell lines treated with CCT018159 (Sharp *et al.*, 2003). AHA1, the novel cochaperone of HSP90, which was discovered at The Institute of Cancer Research (Panaretou *et al.*, 2002), was also shown to be induced following

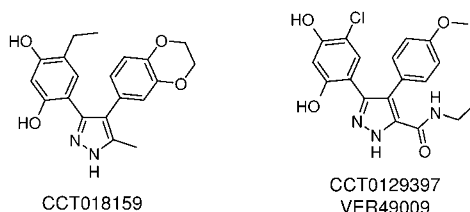


Fig. 5 Pyrazole class of HSP90 inhibitors.

treatment with CCT018159 (Holmes *et al.*, personal communication). Treatment of cancer cells with CCT018159 resulted in cytostasis and apoptosis (Sharp *et al.*, 2003).

As mentioned earlier, previous studies have shown positive correlation between DT-diaphorase activity and sensitivity to 17-AAG (Kelland *et al.*, 1999). For example, this was seen in the NCI panel of 60 human tumor cell lines. There is also evidence that HSP90β is associated with the multidrug resistance protein, P-glycoprotein (Bertram *et al.*, 1996). Unlike 17-AAG, the cellular activities of CCT101859 have been found to be essentially independent of DT-diaphorase and P-glycoprotein (Sharp *et al.*, 2003).

The X-ray cocrystal structure of CCT018159 bound to the N-terminal ATP site of the yeast HSP90 identified the key binding features, including the critical water molecules (Cheung *et al.*, 2005). The structural data also indicated how the potency could be improved and identified a region on the CCT018159 molecule where substitutions could be made to improve solubility and pharmacokinetic properties. Based on this, a structure-based design approach was carried out by The Institute of Cancer Research and Vernalis. Driven by X-ray crystallography with human HSP90α this led to the identification of CCT0129397/VER49009 (Fig. 5) (Dymock *et al.*, 2005). The IC_{50} value for inhibition of yeast HSP90 ATPase of CCT012937 as measured by malachite green was found to be 140 nM and the cellular GI_{50} as determined by SRB assay was 260 nM. The accompanying cellular biomarker changes were consistent with HSP90 inhibition. CCT012937 exhibited similar cellular properties to 17-AAG and displayed the potency and potential for clinical development (Dymock *et al.*, 2005).

C. Purines

The synthetic purine small molecule HSP90 inhibitor PU3 (Fig. 6) was designed on the basis of molecular modeling by Chiosis *et al.* (2002). Structures of HSP90α and -β N-terminal domains complexed with PU3 and some of its analogs revealed a conformational change in the top 'lid' of the

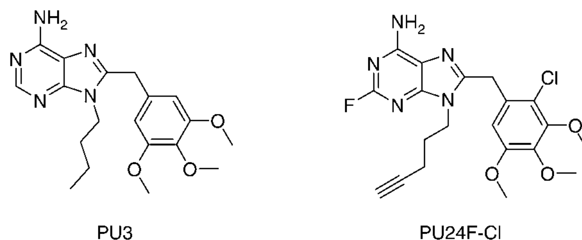


Fig. 6 Purine class of HSP90 inhibitors.

HSP90 pocket (Wright *et al.*, 2004). PU3 exhibits the characteristic molecular signature of HSP90 inhibition, including degradation of ERBB2, as demonstrated in breast cancer cells (Chiosis *et al.*, 2002). Although PU3 is more soluble than 17-AAG, it is significantly less potent. Optimization led to the identification of PU24F-Cl (Fig. 6), which has a much higher affinity for the N-terminus of HSP90 than the parent compound PU3 (Vilenchik *et al.*, 2004). The biological effects of PU24F-Cl were demonstrated in a 2–6 μM concentration range (Vilenchik *et al.*, 2004). Studies have shown that PU24F-Cl exhibits high potency against a wide range of tumor cells, including those resistant to 17-AAG. Furthermore, its affinity for HSP90 in tumor cells is at least 10–50-fold higher than is the case for HSP90 normal cells (Vilenchik *et al.*, 2004). *In vivo* antitumor activity was achieved in MCF-7 xenograft tumors at nontoxic doses, accompanied by downregulation of client proteins (ERBB2, AKT, and C-RAF). A more recent study has disclosed synthesis of several water soluble 8-arylsulfanyl, 8-arylsulfoxyl, and 8-arylsulfonyl adenine derivatives of the PU class, exhibiting approximately 50-nM potency in cellular and animal models (He *et al.*, 2006). Conforma Therpeutics have reported the first orally active purine-based inhibitors of HSP90 *in vivo* but high doses are currently necessary for antitumor activity (Biamonte *et al.*, 2006).

D. Novobiocin

The family of coumarin antibiotics binds to the bacterial DNA gyrase, thus inhibiting bacterial DNA synthesis. Unlike all the other HSP90 inhibitors mentioned in this chapter so far, the coumarin antibiotic novobiocin (Fig. 7) binds to a second proposed ATP binding site within the C-terminus of HSP90 instead of the N-terminal domain (Marcu *et al.*, 2000a) and disrupts the interaction of both the cochaperones P23 and HSC70 with the HSP90 chaperone complex. As with N-terminal HSP90 binding agents, treatment with novobiocin results in the degradation of HSP90 client proteins *in vitro*, including ERBB2, C-RAF, mutant p53, and v-SRC (Langer *et al.*, 2002;

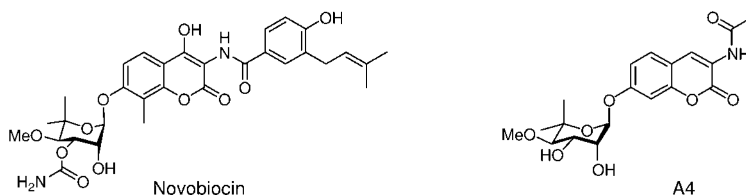


Fig. 7 Novobiocin and its analog A4.

Marcu *et al.*, 2000a,b; Yun *et al.*, 2004). *In vivo* treatment in murine splenocytes with novobiocin also demonstrated downregulation of C-RAF (Marcu *et al.*, 2000b). The C-terminal region mediates HSP90's dimerization (Wegele *et al.*, 2003; Yamada *et al.*, 2003). Studies have shown that there is an important interaction between the HSP90 N- and C-terminal domains as binding of novobiocin to the chaperone inhibits geldanamycin binding (Soti *et al.*, 2002). The synthesis of photolabile analogs of novobiocin may be useful to further elucidate the C-terminal of the HSP90 ATP binding pocket (Shen *et al.*, 2004).

Due to the relatively weak ability of novobiocin to degrade HSP90 client proteins (700 μM in SKBr3 cells) (Marcu *et al.*, 2000b), more potent analogs have been identified from a library of novobiocin derivatives with compound 4A (Fig. 7) being the most active (Yu *et al.*, 2005). Studies are now underway to identify improved analogs of 4A.

E. Other HSP90 Inhibitors

Other companies are also synthesizing novel HSP90 inhibitors for the treatment of cancer. These include Synta Pharmaceuticals (http://www.syntapharma.com) and Sirenade Pharmaceuticals (http://www.sirenade.biz). The latter have developed orally available compounds with micromolar potency that are still at the early preclinical stage.

Shepherdin, a novel anticancer peptidomimetic, interacts with the ATP pocket of HSP90 and causes destabilization of client proteins, including survivin (Plescia *et al.*, 2005). It demonstrated selectivity against cancer cells and tumors *in vivo* and does not appear to affect the viability of normal tissues. Further shepherdin analogs are being evaluated.

F. Inhibitors of Specific HSP90 Isoforms

There are reports that have demonstrated that expression of specific isoforms of HSP90 (HSP90α, HSP90β, GRP94, and TRAP1) is associated with some tumor types. Overexpression of HSP90α is associated with a poor prognosis of breast cancer, pancreatic cancer, and leukemia (Gress *et al.*, 1994; Jameel *et al.*, 1992; Yufu *et al.*, 1992). HSP90β expression has been implicated in P-glycoprotein multidrug resistance (Bertram *et al.*, 1996). It is possible that selective inhibitors of particular HSP90 isoforms could have some advantages. The majority of the current HSP90 inhibitors bind to the N-terminal ATP binding domain. It is not surprising that there is no major difference in the inhibition of the HSP90 isoforms by the inhibitors as the N-terminal nucleotide binding pocket is fairly conserved in the HSP90α and

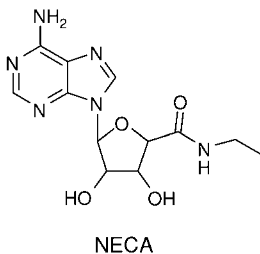


Fig. 8 GRP94 inhibitor.

HSP90β. HSP90N has been shown to lack the N-terminal ATP domain but still retains chaperone activity (Grammatikakis *et al.*, 2002). Therefore, it is important to consider designing inhibitors of both the N- and C-terminal of HSP90.

An adenosine analog, 5′-*N*-ethylcarboxamideadenosine (NECA) has been shown to be active against one of the HSP90 isoform, GRP94, but not against HSP90, with a K_D of 200 nM (Fig. 8) (Soldano *et al.*, 2003).

Studies have reported that the HSP90α isoform alone is expressed extracellularly, where it interacts with the matrix metalloproteinase 2 (MMP2). The inhibition of HSP90α has been shown to decrease both MMP2 activity and invasiveness in HT-1080 fibrosarcoma cells *in vitro* (Eustace *et al.*, 2004). A study has reported that the cell-impermeable, geldanamycin derivative (which has HSP90 binding affinity between 0.5 and 1 μM) exhibited strong anti-invasive activity with low cytotoxicity (Tsutsumi *et al.*, 2005).

A human recombinant antibody directed against fungal HSP90 (Mycograb), which is being developed by NeuTec under license from the University of Manchester, is considered as a potential treatment for invasive cancers (Matthews *et al.*, 2003). Phase II clinical trial in patients with systemic candiasis demonstrated encouraging overall response rates (84%) with no drug-related toxicity (Matthews and Burnie, 2004).

G. Miscellaneous

1. CISPLATIN

Cisplatin, *cis*-diamminedichloroplatinum (II), is a widely used anticancer drug, particularly in the treatment of human ovarian, testicular, bladder, and head and neck cancers (Loehrer and Einhorn, 1984; Wong and Giandomenico, 1999). The ability of cisplatin to react with DNA to form intra- and interstrand cross-links is generally considered to be the basis of its therapeutic effect

(Kartalou and Essigmann, 2001a,b). The formation of platinum-DNA adducts results in cell cycle arrest and cell death, primarily by apoptosis and in some cases by necrosis (Chu, 1994; Gonzalez *et al.*, 2001). Cisplatin has been reported to bind to HSP90 at a site that overlaps the putative ATP/novobiocin-binding site (Itoh *et al.*, 1999), near to the C-terminal of HSP90, which is different from the geldanamycin-binding site (Soti *et al.*, 2002). This has been shown to affect its chaperone function, specifically disrupting the binding of androgen and glucocorticoid receptor to HSP90, with complete inhibition at concentrations of 100-μM cisplatin (Rosenhagen *et al.*, 2003). However, other HSP90 client proteins, such as C-RAF, LCK, were not affected, nor did cisplatin induce any stress response.

Whether cisplatin interaction with HSP90 contributes to anticancer activity remains unknown at this time. Inhibition of the ERK pathway and other signal transduction pathways has been shown to increase cisplatin sensitivity and this may provide an opportunity for combination therapy with the clinical HSP90 drug 17-AAG (Persons *et al.*, 1999). There is a Phase I trial underway to evaluate the combination of 17-AAG with gemcitabine and cisplatin in advanced solid tumors (Ivy and Schoenfeldt, 2004).

2. PACLITAXEL

The antitumor action of the plant-derived agent paclitaxel is thought to be involved in the binding and stabilizing microtubules, hence blocking mitosis (Schiff and Horwitz, 1980; Wani *et al.*, 1971). Studies using geldanamycin have shown that paclitaxel has the ability to bind to HSP70 and HSP90 proteins (Byrd *et al.*, 1999). However, the significance of this is unclear. Because HSP90 inhibitors, such as 17-AAG, are able to simultaneously decrease a number of key proteins involved in oncogenic signal transduction pathways and the importance of combination chemotherapy in cancer therapeutics, several studies have evaluated whether 17-AAG could synergize with other anticancer drugs. Many researchers have found synergism between 17-AAG and paclitaxel, particularly in cells overexpressing ERBB2 (Munster *et al.*, 2002) or with an active PI3 kinase pathway (Sain *et al.*, 2006). Combination Phase I trial with 17-AAG and paclitaxel is ongoing in advanced, metastatic cancer (http://www.nci.nih.gov/clinicaltrials).

3. HISTONE DEACETYLASE INHIBITORS

The acetylation of HSP90 has been shown to modulate its activity and several histone deacetylase (HDAC) inhibitors (e.g., depsipeptide FK228 and LAQ824) have been reported to inhibit HSP90 because they give the same molecular signature as HSP90 inhibition. These effects have been seen *in vitro* and in a Phase I clinical trial (Fuino *et al.*, 2003; Kristeleit *et al.*,

2004; Yu *et al.*, 2002). HDAC inhibitors induce acetylation of HSP90, which leads to inhibition of ATP binding. Consequently, the association of client proteins with HSP90 is inhibited. Studies have reported synergistic interactions when HDAC and HSP90 inhibitors (LBH589 and 17-AAG, respectively) were combined *in vitro* in chronic and acute myeloid leukemia cell lines (George *et al.*, 2005). Several HDAC inhibitors are in various stages of clinical trials, with one study showing partial response in melanoma and disease stabilization in other cancers including melanoma and colorectal (Gore *et al.*, 2004).

III. CLINICAL EVALUATION OF 17-AAG AND 17-DMAG

The first-in-class HSP90 inhibitor 17-AAG has completed a series of Phase I clinical trials, which have examined various dose and scheduling strategies. These have been summarized (Pacey *et al.*, 2006). Clinical studies with 17-AAG have shown that the well-tolerated doses given resulted in good pharmacokinetic exposures and demonstrated the molecular signatures of HSP90 inhibition such as upregulation of HSP70 and downregulation of C-RAF and CDK4 proteins (Banerji *et al.*, 2005; Goetz *et al.*, 2005). Toxicity was tolerable and consisted of liver transaminitis diarrhea, nausea, vomiting, fatigue, anorexia, and anemia. Stable disease in two patients with melanoma has been reported (Banerji *et al.*, 2005). There are also unpublished reports of activity in prostate, breast, and multiple myeloma. Stable disease seen in melanoma is consistent with the cytostatic responses observed *in vitro* and *in vivo*. As a result of these favorable Phase I trials, 17-AAG is now in Phase II single agent therapy in various tumor types including melanoma and breast cancers (http://nci.nih.gov/clinicaltrials). In addition, trials are also underway to evaluate 17-AAG as a treatment for pediatric malignancies. Phase I combination trials with 17-AAG have been initiated (http://www.nci.nih.gov/clinicaltrials). Agents chosen for combinations may include those that are standard therapy, or those based on preclinical evidence, or the selection of drugs which do not have overlapping toxicities with 17-AAG. In particular, Kosan Biosciences has shown promising results in a combination trial, demonstrating activity in multiple myeloma of their formulation of 17-AAG (KOS-953) in combination with bortezomib (http://www.kosan.com). Two patients exhibited stable disease after two cycles.

There are several factors that could potentially influence the sensitivity to 17-AAG in the clinic. Studies have shown that 17-AAG response was potentiated by the reductase enzyme NQO1 or DT-diaphorase in a variety of human ovarian and colon cancer cell lines *in vitro* and *in vivo* (Kelland *et al.*, 1999). Furthermore, the major route of metabolism of 17-AAG is

CYP3A4 metabolism (Egorin *et al.*, 2001). The polymorphic expression of both these metabolic regulators may have implications in clinical trial studies. The sensitivity of 17-AAG has also been associated with the multi-drug resistant protein, MDR1, or P-glycoprotein (Kelland *et al.*, 1999). But most limiting in clinical trials is that 17-AAG has complex and cumbersome formulation, poor solubility, and lack of oral bioavailability.

The analog 17-DMAG overcomes some of these limitations in particular has much greater aqueous solubility. Preclinical studies have shown similar HSP90 inhibitory actions, therapeutic activity, and spectrum of toxicity to 17-AAG (Burger *et al.*, 2004; Kaur *et al.*, 2004; Smith *et al.*, 2005). Phase I clinical trials of 17-DMAG investigating a variety of schedules have started in advanced cancer and are being undertaken by Kosan Biosciences in collaboration with the National Cancer Institute. The 17-AAG hydroquinone IPI-504 is entering clinical trials and many other agents are in preclinical development.

IV. CONCLUDING REMARKS

Cancer cells are genetically unpredictable and unstable. They can become resistant due to environmental factors, such as hypoxia, or as a result of chemotherapy and/or radiation treatment. In addition to genetic instability, other factors in drug resistance may be involved such as cellular heterogeneity, as well as the multiple abnormalities that drive malignant progression, and the numerous signaling pathways that may play redundant roles. The majority of the oncogenic proteins that influence the six hallmarks traits are client proteins of HSP90 and inhibition/modulation of multiple pathways by HSP90 inhibitors, therefore, makes this class of anticancer agents unique. Simultaneous combinatorial depletion of multiple oncogenic proteins by HSP90 inhibitors is a major advantage of this class of agents and is likely to make the development of drug resistance relatively difficult. This property should also lead to broad spectrum antitumor activity in multiple tumor types.

The first-in-class HSP90 inhibitor 17-AAG has established the proof-of-principle for target modulation at well-tolerated doses. Evidence of clinical activity has also been seen. Although HSP90 accounts for 1–2% of a cell's total protein and is involved in chaperoning many proteins that are essential for the function and survival of healthy cells, 17-AAG has proved to be quite well tolerated. The reasons for the therapeutic selectivity of HSP90 inhibitors against tumor versus normal cells are not entirely clear. One possibility is that HSP90 inhibitors overcome 'oncogenic addiction,' whereby malignant cells develop a greater dependency than normal cells on

oncogenic pathways. In the case of HSP90 inhibitors, one of the attractions is the ability to overcome 'combinatorial oncogene addiction,' whereby HSP90 inhibitors can simultaneously take out the effects of multiple oncogenic driving forces. As mentioned earlier, this has the advantage of preventing the emergence of resistant clones that could otherwise have arisen by activation of alternative pathways. Although the combinatorial action of HSP90 inhibitors is a major advantage, this does not exclude a role for the action of HSP90 inhibitors against a specific oncogene product in particular tumors, for example, ERBB2 in breast cancer, phospho-AKT in ovarian or prostate cancer, or HIF-1α in renal cancer. Another example is the discovery that various mutant forms of B-RAF, including the common V600E variant, are much more dependent on HSP90 than the wild-type B-RAF, which is actually quite resistant (da Rocha *et al.*, 2005; Grbovic *et al.*, 2006). Thus, HSP90 inhibitors may have activity in melanomas and other cancers driven by B-RAF mutations. In fact, HSP90 inhibitors will also be effective in melanomas and other cancers where the ERK1/2 pathway is activated by RAS mutations or by upstream receptors, since wild-type C-RAF is also depleted by HSP90 inhibitors (Kamal *et al.*, 2003).

Another factor to consider in the cancer selectivity of HSP90 inhibitors is the stress response. Cancer cells are under greater stress than normal cells. This is due to combination of factors, including the molecular stress of overexpressed or mutated oncogenes and microenvironmental stresses of hypoxia, acidosis, and nutrient deprivation in solid cancers. This is likely to drive HSP90 in cancers into the superchaperone complex that is highly sensitive to HSP90 inhibitors (Kamal *et al.*, 2003).

Doses of 17-AAG used were sufficient to inhibit HSP90 function and this has encouraged Phase II clinical trials to be initiated as single agent therapy in various cancers, including melanoma, breast and pediatric leukemias and solid tumors. Preclinical data have generated great interest in combining 17-AAG treatment with radiation therapy, cytotoxic agents, or new molecularly targeted agents. Several Phase I combination trials are now underway. These results are very encouraging and it is of no surprise that the development of second and third generation of HSP90 inhibitors with potential better pharmacological properties while retaining target specificity have progressed so rapidly (Dymock *et al.*, 2004). These include HSP90 inhibitors based on the pyrazole or purine scaffold which are often optimized by structure-based design. The next year or so will be very exciting as a range of new HSP90 inhibitors enter the clinic, while the more advanced compounds progress into more later stage therapeutic studies both alone and in combination. The intriguing possibility of drugging the cancer chaperone for therapeutic benefit is now a tangible reality. Careful clinical studies, including detailed molecular biomarkers, will be essential so that the full therapeutic potential of HSP90 inhibitors can be realized.

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