Immunotherapy for Gastrointestinal Cancer

David Kerr
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Editors

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Chapter 1
Introduction to Modern Immunology

Rachel Kerr

A Brief History of Cancer Immunology

More than a century ago, the Nobel Prize for Physiology or Medicine (1908) was awarded jointly to Ilya Mechnikov and Paul Ehrlich "in recognition of their work on immunity" and it was around this time that Ehrlich expounded his hypothesis that the immune system may play a role in the control of tumours [1]. However his suggestion was actually preceded by work carried out by a young New York bone surgeon, William Coley (1862–1936) who had read about a patient who underwent dramatic regression of a neck tumour after developing erysipelas, a skin infection caused by streptococcus pyogenes. Coley subsequently observed that his own patients who developed post-operative infection after surgery seemed to gain some improvement in outcome with respect to their underlying sarcomatous tumours. He believed that these infections may have stimulated the immune system in a way that rendered it more capable of recognising and attacking the cancer. He developed Coley's toxin comprising killed bacteria, provided by Robert Koch, and he injected this into his patients, reporting a complete regression rate in inoperable sarcomas of approximately 10 % [2]. Although the use of Coley's toxin declined rapidly in the 1950s with the flourishing of cytotoxic drugs and radiotherapy, there are still clinics today that use a variation of this agent comprising *Streptococcus pyogenes* and *Serratia marcescens*. Despite some scepticism about Coley's methods and a general feeling in the early part of the twentieth century that recognition and rejection of 'self' tumours by the immune system would be impossible, this work formed the basis of the subsequent development and use of bacille Calmette-Guerin in the treatment of superficial bladder cancer in the 1970s, which is still recognised as a very effective form of treatment today.

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There is no doubt that, since these early observations, the status and 'fashionability' of cancer immunotherapy has fluctuated hugely. When Burnet and Medawar in the 1940s [3, 4] expanded the theory of immunological tolerance which describes the process by which 'self' proteins (and hence tissues) are protected from recognition and attack through a process of deletion of immune cells specific for such proteins during fetal life, it seemed like an almost fatal blow to this fledgling therapeutic field. However just a few years later *in vivo* mouse experiments were indicating that vaccination against syngeneic tumours, produced by carcinogenic exposure, could be achieved [5]. Burnet subsequently built upon these observations and described immunosurveillance as a process whereby circulating lymphoctyes could recognise and destroy malignant cells [6].

Over the next 20 years the development of cancer immunotherapy suffered further setbacks when scientists described the process of thymic deletion and suggested that all auto-reactive (including tumour reactive T-cells) would be deleted *in utero* [7]. Furthermore it was noted that athymic mice did not have an increased frequency of spontaneous tumours, thus supporting the view that T cells may be of no importance in combating carcinogenesis [8].

Immunotherapeutic research remained in the doldrums until the 1980s when it became clear that that some auto-reactive T cells can escape deletion. Furthermore researchers were starting to delineate T cells that may recognise proteins specifically found on transformed cells, and these were now termed tumour associated antigens (TAAs) [9]. Other areas of cancer research were helpful in this regard, with the demonstration of the inherent genetic instability of tumours explaining the generation of a multitude of neo-antigens on cancer cells, all of which could be potential targets for immune effector cells.

Over the last two decades there has been exponential growth in our understanding of the immune system and of the carefully orchestrated balance between immunity and auto-immunity and how this may relate to carcinogenesis from transformation through invasion to metasasis. The critical importance of both inflammation and immunity were highlighted in the seminal paper "The Hallmarks of Cancer" by Hanahan and Weinberg in 2011 [10]. This accumulated knowledge has driven the development of a broad repertoire of potential cancer immunotherapeutic approaches that are now in experimental or therapeutic use. This book will explore how these approaches are being assessed in the field of gastrointestinal malignancies. This introductory chapter aims to outline our present understanding of modern immunology and how it relates to broad immunotherapeutic strategies.

What Is the Evidence That the Immune System Is Important in the Genesis and Persistence of Cancer?

In addition to the developments in basic immunological research outlined above, a number of clinical and pathological observations have convinced the cancer community about the central role of the immune system in preventing cancer development and metastasis. Firstly, patients who are immunocompromised either

iatrogenically, through immunosuppressive agents post-transplant, or through HIV infection, are noted to be at increased risk of developing cancer [11, 12]. Although this elevated incidence was initially felt to be a consequence of the greater propensity for such patients to acquire, or lose immunological control of, latent viral infections (EBV, HPV etc.) it soon became apparent that the incidence of *non-viral* related cancers, such as colorectal cancer (CRC) and pancreatic cancer, is also increased. Thus for organ-transplant recipients in the UK, the Hazard Ratio for developing CRC in kidney transplant patients is 1.8 relative to the incidence in patients who have not had a transplant [13].

Further evidence for a central role of the immune system in controlling the spread of cancer comes from histopathological studies which have demonstrated an inverse correlation between the frequency of infiltrating lymphocytes in tumours (TILs) and the chance of recurrence post operatively. In fact the density of TILs has been reported to be more predictive of overall survival than all other usual TNM prognostic classifiers and this effect held true in multivariate analysis [14]. These observations have helped to reignite interest in the potential role of cancer immunotherapy.

A Quick Reminder of the Basic Components of the Immune System

In order to understand the spectrum of cancer immunotherapeutic approaches, an understanding of modern immunology, and of the interplay between the various elements, is required. All immune cells begin as immature stem cells in the bone marrow. In the presence of certain cytokines such as interleukins and interferons, they differentiate via myeloid or lymphoid progenitor cells into B cells, T cells, NK cells or phagocytes (see Fig. 1.1). The immune system has traditionally been split into two broad branches termed humoral (with antibodies as the main immune effector) and cellular (where the effectors are the cells themselves). However functionally, the immune system can also be categorised according to whether the immune response under question is innate or adaptive.

CD Markers

In order to understand immunological nomenclature, it is necessary to be aware of the cluster of differentiation (often abbreviated as CD) marker system which has been in use since 1982 [15]. It is used for the identification and investigation of cell surface molecules and allows the immuno-phenotyping of cells. It has become a very important way to identify and categorise cells of the immune system, not only to allow label classification, but also because CD markers/molecules can act in a variety of ways, and are often found to be receptors or ligands that are crucial to

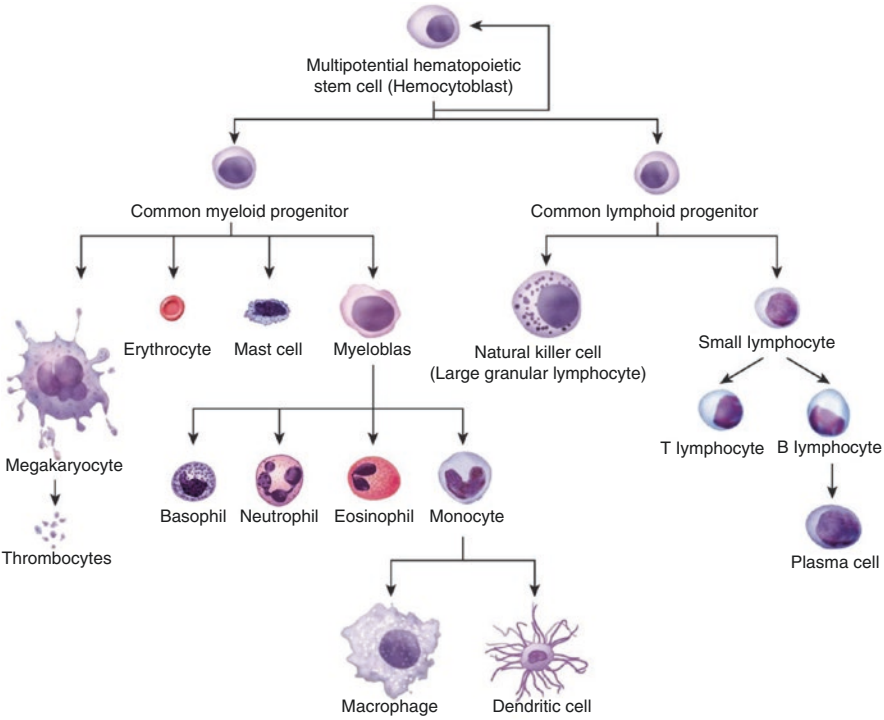

Fig. 1.1 Immune cell differentiation from a common progenitor (This file is licensed under the Creative Commons Attribution 4.0 International license) (Source: https://commons.wikimedia.org/wiki/File:0337_Hematopoiesis_new.jpg)

cellular function. They are frequently part of a cell signalling cascade and they may be critical for cell adhesion and other cell: cell interactions. They are currently numbered up to 371. Table 1.1 gives the broad differentiating CD repertoire for the most important cells of the immune system.

B Cells

The 'humoral' immune system is comprised primarily of B cells. B cells are part of the adaptive immune system and are able to recognise antigens (including tumour antigens), which are frequently concentrated in the lymph nodes, spleen and other lymphoid tissues, and they transform and multiply in to plasma cells and memory B cells. The plasma cells produce antibodies (that are essentially a secretory form of the B cell's specific recognition receptor), which form antigen-antibody complexes that are either destroyed in the spleen or engulfed by antigen-presenting cells allowing surface presentation by major histocompatibility complex (MHC) proteins and further stimulation of other effectors of the immune system. Memory B cells

Table 1.1 Brief table of important CD markers for effector cells of the immune system

Type of cell	CD markers
Stem cells	CD34+, CD31-, CD117+
All leukocyte groups	CD45+
Granulocyte	CD45+, CD11b+, CD15+, CD24+, CD114+, CD182+
Monocyte	CD4+, CD45+, CD14+, CD114+, CD11a+, CD11b+, CD91+,CD16+
T lymphocyte	CD45+, CD3+
T helper cell	CD45+, CD3+, CD4+
T regulatory cell	CD4+, CD25+, Foxp3+
Cytotoxic T cell	CD45+, CD3+, CD8+
B lymphocyte	CD45+, CD19+, CD20+, CD24+, CD38+, CD22+
Thrombocyte	CD45+, CD61+
Natural killer cell	CD16+, CD56+, CD3-, CD31+, CD30+, CD38+

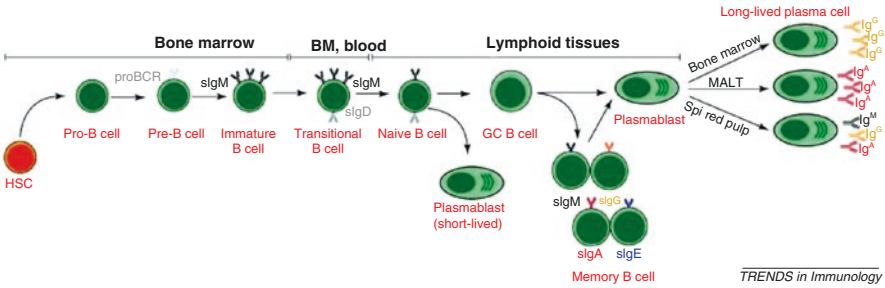

Fig. 1.2 B cell differentiation on stimulation. Following an encounter with antigen, naïve B cells that express receptors specific for the antigen can differentiate into short-lived plasmablasts which secrete predominantly IgM antibody. These cells usually provide a first line of defence against infection. Other activated naïve B cells will seed a germinal centre in a lymph node, where affinity maturation and differentiation into long-lived memory and plasma cells will occur. Following their generation, memory cells and plasma cells can then migrate to distinct sites (mucosal-associated lymphoid tissue (*MALT*), bone marrow, splenic red pulp). Plasma cells continue to produce antibody and (*IgG*, *IgM*, *IgA*) and memory cells will remain quiescent until subsequent exposure to the same antigen (Taken from http://www.cell.com/trends/immunology/fulltext/S1471-4906(11)00149-9 [84])

migrate to the bone marrow where they can reside for many years providing a more accelerated response to any subsequent exposure to the same antigen (Fig. 1.2). The ways in which antibodies can be utilised in the immunotherapy of gastrointestinal cancer are explored in detail in Chaps. 4, 5 and 9.

T Cells

T cells, in contrast to B cells, are members of the 'cell-mediated' immune system which, for full effective stimulation and activation, require tumour antigens to be presented to them by specific "self" major histocompatibility complex (MHC)

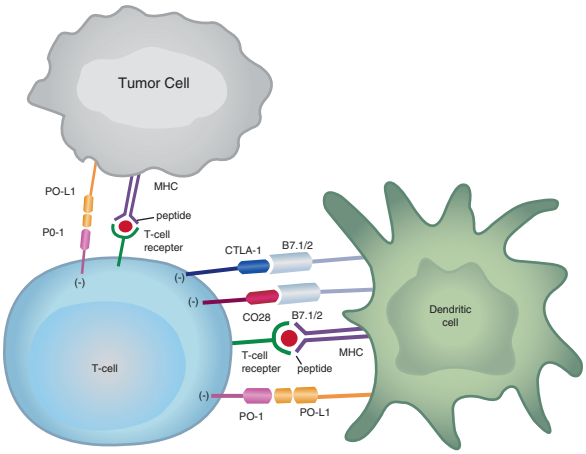

Fig. 1.3 The 'immunological synapse' required for effective T cell activation and subsequent inhibition (production please redraw this figure as it is protected by copyright). The tumour antigenic (TAA) peptide is complexed to the MHC class I within the DC and is recognised by the TCR on the cytotoxic T cell (CTL) surface. To effectively activate TAA-specific T cells, strong co-stimulation is needed which depends on the interaction between positive activatory on the DC with their corresponding receptors on the T cell. In addition the effective activation is dependent upon stimulatory cytokines, derived from either the DCs or T helper cells. In order to curtail the stimulation, inhibitory interactions between the CTL and APC also exist, which help to diminish the risk of auto-immunity

proteins, preferably on antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs). CD8+ (cytotoxic) T cells (CTLs) recognise antigen via their T cell receptor (TCR), when the antigen is presented in association with MHC Class I antigens; and CD4+ (helper) T cells (T_h) recognise antigen presented with MHC Class II antigens.

To effectively activate CTLs, in addition to the binding of the TCR to the antigen/MHC complex, a number of other interactions between the presenting cell and the CTL are required within the so-called 'immunological synapse' and these occur through co-stimulatory molecules (Fig. 1.3). Thus CD80 and CD86, otherwise known as B7.1 and B7.2, on the antigen presenting cell bind to CD28 on the CTL, strengthening the interaction and increasing activation. Finally stimulatory cytokines are required, produced by CD3+ CD4+ helper T cells and DCs. These cytokines include IL6, interferon (IFN)-α, IFN-γ and tumour necrosis factor (TNF) -α. Once CTLs are appropriately activated, they should be able to kill a microbe-infected cell or, in the case of immunotherapy, destroy the cancer cell. A subset will become memory T cells, ready to reactivate quickly if they encounter the same pathogen again. The marker profile of the memory T cells is CD8+ CD45R0+ CCR7+.

In the non-oncogenic situation, when a CTL is stimulated, it will release the cytokine interferon-γ, which acts via the STAT pathway in the APC to upregulate APC expression of PD-L1 (programmed cell death 1 ligand) which ligates the PD-1 receptor expressed on the surface of the CTL and abbreviates or curtails the CTL

stimulation. This is an important homeostatic inhibition, which prevents over-stimulation of a specific clone of T cells, and limits the risk of auto-immune disease. In addition to the PD-L1/PD-1 interaction, the CD80/CD86 on the APCs can bind another inhibitory receptor, cytotoxic T-lymphocyte–associated antigen 4 (anti-CTLA-4), also expressed on the CTL surface (Fig. 1.3). Interfering with these CTL inhibitory interactions forms the basis of immune checkpoint inhibition therapy (see Chap. 6).

Conventional T helper cells, as described above, are necessary in the coordinated activation of a CTL response. However naive CD3 + CD4+ T cells can differentiate towards at least 4 distinct fates and their specific final functionality is determined by the pattern of signals they receive during their initial interaction with antigen. The most well studied populations are T_h1, T_h2, T_h17, and regulatory T (T_{reg}) cells. Mossman and Coffman [16] first described the T_h1 and T_h2 phenotypes in the 1980s after studying long term T-cell lines. T_h1 cells were defined as critical for immunity to intracellular microorganisms (such as viruses and intracellular bacteria) and found to produce interleukin (IL)-2 and IFN-γ and to stimulate CTLs, natural killer (NK) cells and macrophages. The skewing and augmentation of CTL effect by T_h1 cells is felt to be particularly important in the immune response against cancer. T_h2 cells were found to be critical in the immune response to many extracellular pathogens, including helminths, and are now known to produce IL-4, IL5 and IL-13, acting on eosinophils, and skewing immunity towards a B cell humoural response.

More recently a third group of T_h cells has been defined, so-called T_h17 cells, which produce IL-17a, IL-17 F, IL-21 and IL-22; cytokines which skew immunity towards an anti-microbial tissue *inflammatory* response, acting on epithelial and endothelial cells, neutrophils and fibroblasts [17] . There is evidence that such an inflammatory response can actually promote tumourigenesis and can prevent the effective generation of an anti-tumour cytotoxic immune response (see Chap. 8).

Finally T_{reg} cells are a population of CD3 + CD4+ T cells which inhibit the effector functions particularly of B and T cells. They exhibit other cell surface markers including CD25 (also known as interleukin-2 receptor subunit-α (IL-2Rα)) and the transcription factor forkhead box protein P3 (FOXP3), neither of which is exclusively restricted to T_{reg} cells. These T_{reg} cells can dampen the T_h1 driven CTL response felt to be critical in an effective anti-tumour immune response, and many reports have suggested that a high density infiltration of tumour with T_{reg} cells correlates with poor survival across a range of tumours [18, 19]. However this correlation is by no means consistent across all studies and some report better survival with higher densities of FOXP3 positive cells [20]. The apparent contradiction in these findings may be explained by heterogeneity in methods for characterisation and quantification of T_{reg} cells and by the variability in FOXP3 expression across different immune cell populations. An alternative explanation for the conflicting findings could be that T_{reg} cells in some tumours may have the potentially beneficial effect of suppressing an overzealous pro-inflammatory response and therefore allowing a T_h1 directed anti-tumour CTL response to thrive. It is possible that during different stages of carcinogenesis, T_{reg} cells can have completely opposing actions, depending on the tumour microenvironment.

Table 1.2 The association of specific immune cell infiltrates with prognosis in gastrointestinal cancers

	Effect on prognosis				
	CD8 + CD45RO+ T cells (memory CTLs)	T_h1 cells	Th_2 cells	T_h17 cells	T_{reg} cells
Oesophageal cancer	Good [21, 22]	Good [23]		Good [24]	
Gastric cancer		Good [25]	Poor [25]	Good [26]	
Colorectal cancer	Good [14, 27, 28]	Good [14, 27, 28]	None [27]	Poor [27, 29]	Good [27] None [30]

Table 1.2 outlines the published associations between specific immune cell infiltrates and prognosis in gastrointestinal cancers and reflects the reported inconsistencies across tumour types and between studies [14, 21–30].

NK Cells

In addition to T and B cells, which are elements of the adaptive immune system, other populations of *innate* lymphoid cells (ILCs) have been identified. NK cells are a type of cytotoxic ILC that secrete cytokines such as IFNγ, which help to shape the adaptive immune response. A central feature of NK cells is their ability to differentiate cells under stress (through infection, transformation, injury) from healthy cells. In fact their initial identification was a consequence of their ability to kill tumour cells [31, 32]. *In vitro* and *in vivo* models have proven that NK cells can eliminate many transplantable and spontaneous tumours [33, 34]. And an epidemiologic study has demonstrated that low peripheral blood NK cell activity correlates with increased cancer risk, testifying to their importance in immunosurveillance [35]. In addition NK cell infiltration into solid tumours, for example colorectal cancer, is associated with better prognosis [36]. NK cells recognise their cellular targets (whilst sparing healthy cells) through the expression of a repertoire of receptors which can be activating, inhibitory, adhesion or cytokine receptors. Probably the most important of these is the inhibitory receptor expressed on NK cells that is specific for MHC class I molecules (HLA molecules in humans), which in humans are the killer cell immunoglobulin-like receptors (KIRs) [37]. Activation of these inhibitory receptors when an NK cell engages a healthy 'self' cell induces tolerance to self. However if an NK cell engages with a tumour cell where there is frequent loss of MHC class I expression, it will become activated in response, as the activity is no longer inhibited by the inhibitory signal (see Fig. 1.4). This is known as 'missing-self' activation. In addition to using inhibitory receptors to recognise self, NK cells also display activating receptors at their surface, some of which are specific to recognition of microbes, but others such as the receptor NKG2D, detect changes that occur in

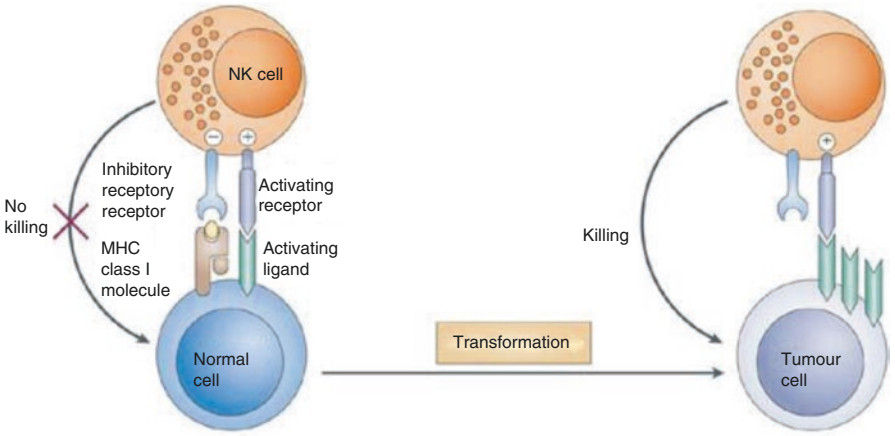

Fig. 1.4 Activating and inhibitory receptors on NK cells important in distinguishing normal from tumour cells. All DCs present protein via MHC class I and MHC. MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol, usually endogenous proteins (synthesised by the cell itself). MHC class II molecules acquire peptides generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous components. $CD8^+$ DCs can also deliver exogenous antigens to the MHC class I (cross-presentation) pathway. The MHC class II and the MHC class I cross-presentation pathways may 'compete' for exogenous antigens in $CD8^+$ DCs (Taken from: http://www.nature.com/nri/journal/v7/n5/fig_tab/nri2073_F1.html [86])

damaged or cancerous host tissues and this is known as 'stress induced self recognition' [38]. In humans, the ligands for NKG2D include stress-induced proteins such as MICA (MHC class I polypepetide-related sequence A), MICB and members of the RAET1 family. It appears that DNA damage (through injury or through transformation) activate a DNA damage response which upregulates NKG2D ligands and stimulates NK cells to attack the damaged cell [39]. There are other activating receptors on NK cells but these are beyond the scope of this chapter.

NKT Cells

Natural killer T (NKT) cells are a heterogeneous group of T cells that share properties of both T cells and natural killer cells, co-expressing an αβ T-cell receptor, but also expressing a variety of molecular markers that are typically associated with NK cells, such as NK1.1. They are functionally polymorphic and are able to act as directly cytotoxic cells, or to act as cytokine producers, directing an orchestrated immune response to a particular pathogen.

The best described subset of NKT cells expresses an invariant T-cell receptor (TCR) α chain. These are referred to as type I or invariant NKT cells (iNKT) cells [40]. They are can respond rapidly to danger signals and pro-inflammatory cytokines. Once

activated, they engage in effector functions, such NK transactivation, T cell activation and differentiation, B cell activation, dendritic cell activation and cross-presentation activity, and macrophage activation.

iNKT cells recognise lipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like antigen presenting molecule. The highly conserved TCR is specific for glycolipid antigens. The best known antigen of iNKT cells is α-galactosylceramide (αGalCer), which is a synthetic form of a chemical purified from the deep sea sponge *Agelas Mauritanius* [41]. iNKT cells develop in the thymus, and distribute to the periphery. Currently, there are five major distinct iNKT cell subsets. These subset cells produce a difference set of cytokines once activated. The subtypes iNKT1, iNKT2 and iNKT17 mirror T_h cell subsets in cytokine production. Once activated iNKT cells can impact the type and strength of an immune response. iNKT cells can also be indirectly activated through cytokine signalling and in this setting the iNKT cells respond more like NK cells, and themselves produce mostly T helper (T_h1 type) cytokines.

There are convincing data from some mice models for a direct role of iNKT cells in tumour immunosurveillance [42] but other models suggest that the influence of iNKT cells is via the control CD1d-expressing tumour-associated macrophages, thereby preventing the latter from promoting angiogenesis, and therefore producing an anti-invasion, anti-metastatic effect [43].

Manipulation of NK and NKT cells for cancer treatment is an evolving strand of immunotherapy.

Myeloid Cells

Three groups of terminally differentiated myeloid cells are critical for the normal functioning of the innate and adaptive immune systems. These are dendritic cells (DCs), macrophages and granulocytes. In the non-cancerous situation these cells protect from pathogens, phagocytose dying cells and promote tissue remodelling and regeneration. Their role in tumourogenesis has only become better described over the last 20 years. It has become increasingly apparent that the tumour itself can alter the local myeloid sub-population distribution, converting the cells into an immunosuppressive army, capable of protecting the tumour from immune attack and promoting angiogenesis, invasion and metastasis. However, all myeloid lineages also have the potential to play a protective role against tumour development.

Dendritic Cells

DCs have been mentioned already. They are terminally differentiated myeloid cells that specialise in antigen processing and presentation. They can arise from various progenitors in the bone marrow, but monocytes are the major precursors of DCs in humans [44]. DCs can be broadly split in to conventional and plasmocytoid DCs with separate differentiation pathways, morphologies, markers and functions.

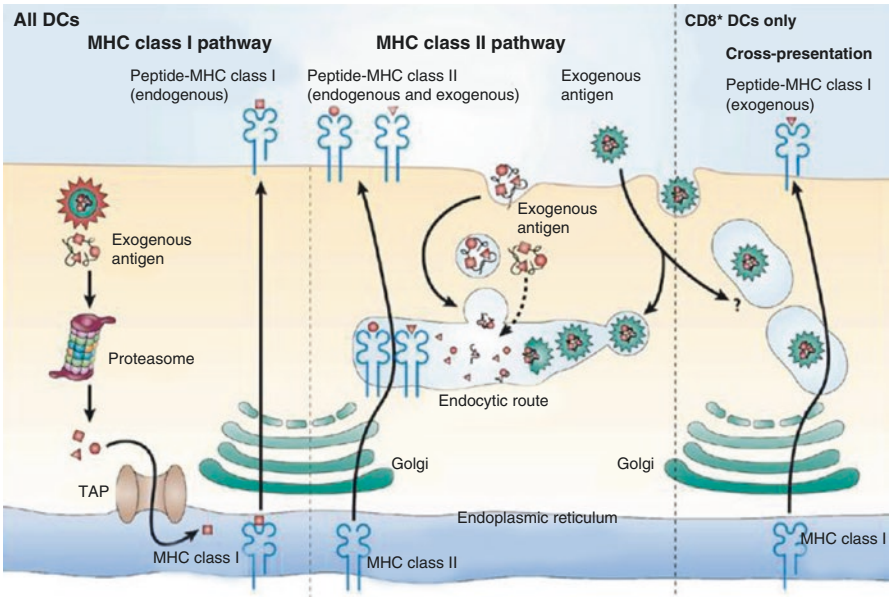

Fig. 1.5 Antigen processing/presentation by dendritic cells in association with MHC class I and II (Taken from: http://www.nature.com/nri/journal/v7/n7/full/nri2103.html [85])

Immature DCs reside in peripheral tissues and actively phagocytose antigens and degrade their proteins into small pieces, using the proteasome (to chop up the protein in to relevant bite-sized epitopes). However in this state are poor antigen presenters and do not activate T cells effectively. Conventional DCs (cDCs) are activated or matured through ligation of their pattern recognition receptors (PRRs), such as Toll Like Receptors (TLRs) 2 and 4, by signals associated with pathogens or damaged tissues, commonly referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Upon maturation, cDCs can transport the class I epitopes of the phagocytosed antigen (usually 8–11 amino acids in length), via the TAP 1 and TAP 2 (transporter-associated proteins) into the endoplasmic reticulum where they are bound to MHC class I molecules and are finally transported via the golgi to be presented at the DC cell surface. In addition, class II epitopes (9–22 amino acids long) are complexed with class II in endosomes via the endocytic route to be presented at the cell surface (see Fig. 1.5) [44]. Simultaneously, the cDCs upregulate cell-surface receptors that act as co-receptors in T-cell activation such as CD80 (B7.1), CD86 (B7.2), and CD40, greatly enhancing their ability to activate T-cells. They also upregulate CCR7, a chemotactic receptor that induces the dendritic cell to travel through the blood stream to the spleen or through the lymphatic system to a lymph node. Here they act as antigen-presenting cells and secrete cytokines such as IL-12: they activate helper T-cells and cytotoxic T-cells (CTLs) as well as B-cells by presenting them with antigens derived from the pathogen, alongside non-antigen specific costimulatory signals. Dendritic cells can also induce T-cell tolerance (unresponsiveness). Certain C-type lectin

receptors (CLRs) on the surface of dendritic cells, some functioning as PRRs, help instruct dendritic cells as to when it is appropriate to induce immune tolerance rather than lymphocyte activation.

Plasmocytoid DCs represent only a minority population of DCs and morphologically they look somewhat like plasma cells, and they express Toll-like receptor (TLR)7 and TLR9 which, when bound by viral nucleic acids of self DNA, produce large amounts of IFN-α.

Macrophages

Macrophages are closely related to DCs. They are derived from monocytes and reside in tissues. Macrophages actually include a broad population of cells, whose cell surface markers and functions are frequently directed by the environment in which they sit. In the non-cancer situation they function to phagocytose and eliminate infectious agents, to promote wound healing and to have some impact upon adaptive immunity [45].

Traditionally, and for simplicity, macrophages are divided into two functional states, M1 and M2, although it is much more likely that there is a continuum of states rather than just these two polarised entities [46]. However, broadly speaking M1 macrophages are 'classically activated' by IFNγ and the products of bacteria, express high levels IL-12 and low levels of IL-10 and are potentially tumouricidal. In contrast, M2 are 'alternatively activated' by IL-4, IL-10 and IL-13 and steroid glucocorticoid hormones, and they express high levels of IL-10 and low levels of IL-12, and can potentiate tissue inflammation and tumour progression.

The role of these subtypes in the failure of oncological immune surveillance will be discussed later.

Granulocytes

Granulocytes are myeloid cells that contain cytoplasmic granules and a specific nuclear morphology; the most common type in humans being the neutrophil. These cells have complex machinery to engulf and destroy bacteria and they are usually not released from the bone marrow until they are fully mature although in states of inflammation their precursors will be released. In human tumours there is some evidence of a correlation between neutrophil infiltration and cancer recurrence [47]. Granulocytes, which are attracted to the site of the tumour by secretion of neutrophil-attracting CXC chemokines, express MMP9, which induces VEGF expression in the tumour and thereby promotes angiogenesis. Interestingly though, in some mice tumour models neutrophils can inhibit the formation of tumour metastases through direct antitumour effects mediated through reactive oxygen species [48]. It seems then that, like macrophages, neutrophils can exhibit a split personality when it comes to cancer and can switch between N1 (antitumour) to an N2 (protumour) phenotype under certain environmental conditions. The switch

from N1 to N2 appears to be the dependent on high levels of TGFβ, and is characterised by ARG1 expression and low levels of TNF, CCL3 and ICAM (intercellular adhesion molecule)1 [49].

What Should Constitute a Coordinated Immune Response Against Cancer; and Where Does It All Go Wrong?

With our knowledge of the individual components and of the functions and interactions of the effectors of the immunity, one can imagine how this system, which may have initially evolved to primarily deal with pathogenic invasion, could also be harnessed by the human host to provide tumour immunosurveillance and anticancer cytotoxicity. One can imagine an ideal scenario in which cells that are showing early signs of DNA damage or chemical or physical insult or transformation, are recognised by NK cells and cleared immediately. Subsequently, one could speculate that if a tumour did become established, that the proteins/neo-antigens generated could be taken up by dendritic cells, be processed and presented in association with MHC class I and class II antigens to produce a coordinated T_h/CTL response to clear that tumour before it could become too established. Furthermore one could speculate that carbohydrate neo-antigens on tumours could be recognised by antibody targeting tumour cells for phagocytosis or cytotoxicity. Finally one could imagine that even if this immune barrier failed, that subsequent invasion and metastasis could be blocked by the presence of an M1/N1 microenvironment around the tumour, preventing angiogenesis and other critical pathways of invasion.

Unfortunately, the fact that one in three people will develop cancer at some point in their lifetime, and that one in four people will die of cancer means that the immune system fails to adequately control cancer at multiple stages of this pathway. This concept of the tumour undergoing ‘immune-editing’, which results in escape from the host defence immunity is widely accepted, and evasion of immune surveillance is considered one of the emerging hallmarks of cancer. Different mechanisms are used by tumour cells to differentiate towards cells with reduced immunogenicity and to skew the host response towards a pro-tumour environment. Understanding these complex mechanisms is a focus of much ongoing research and we will outline the major strands of evidence that have so far been delineated.

NK Cell Function in Cancer

Firstly, with respect to NK cells, which may be seen as the ‘first line of defence’ against cancer, studies have shown recently that NK cell function and reactivity is impaired in an array of tumour types. Thus in prostate cancer, Pasero et al. examined the frequency, phenotype, and functions of NK cells infiltrating control and tumour prostate tissues. NK cell infiltrates in control prostate tissues were mainly

CD56 positive and displayed an unexpectedly immature, but activated, phenotype with low or no cytotoxic potential. TGFβ1 concentrations were high in the prostate environment and partly mediating the immunosuppressive effects on NK cells. In addition to this basal level of immunotolerance to NK cells, the prostate environment became further resistant to NK cell-mediated immunity upon cancer cell infiltration. Co-culture experiments revealed that prostate cancer cells induced the expression of inhibitory receptor (ILT2/LILRB1) and downregulated the expression of activating receptors NKp46 (NCR1), NKG2D (KLRK1), and CD16 (FCGR3) by NK cells, thus preventing their recognition of tumour cells. Notably, blood levels of NKp46 were also decreased in prostate cancer patients and were inversely correlated with levels of prostate-specific antigen, the main prognostic factor in prostate cancer [50]. In colorectal cancer, it is known that cancer-associated fibroblasts (CAFs) are critical components in the process of cancer progression. Li et al. demonstrate that purified colorectal carcinoma-derived fibroblasts exhibit activated phenotypes characterised by substantial α-smooth muscle actin expression. These CAFs sharply suppressed natural killer (NK) cell functions in co-culture experiments. In contrast, normal skin fibroblasts had only a minimal effect on NK cell phenotype and function. Moreover, they demonstrated that prostaglandin E2 (PGE2) was released by fibroblasts in co-culture experiments and the authors suggested that this functional modulation of NK cells by CAFs may represent a novel mechanism linking the pro-inflammatory response to immune tolerance within the tumour milieu.

DC Function in Cancer

DCs in hosts bearing tumours do not stimulate immune responses in a normal fashion which could contribute to immune evasion. This is a result of abnormal myelopoesis in these patients with decreased production of mature competent DCs, increased accumulation of immature DCs at the tumour site, and increased production of immature myeloid cells. Studies have demonstrated DCs of decreased frequency and function in patients with many different types of cancer including breast cancer, hepatocellular cancer and pancreatic cancer [51–53].

Multiple factors are involved in driving this DC impairment including tumour released VEGF, macrophage colony-stimulating factor (M-CSF), and IL6. Other local factors within the tumour can impair DC function including the hypoxic state and increased levels of adenosine. In the presence of hypoxia, DCs upregulate hypoxia-inducible factor (HIF)1α, which in turn induces the expression of the adenosine receptor A2B, which then drives the development of T_h2 cells rather than that of T_h1 cells, the latter of which would have more potent antitumoural effects [54]. *In vitro* DCs differentiated in the presence of adenosine suffered impaired allostimulatory activity in a mixed leukocyte reaction, and expressed higher levels themselves of VEGF, pro-inflammatory cytokines IL-6 and IL-10, COX-2 and TGFβ [55]. In addition, research has indicated that some DCs in hosts with tumours

can actually be conditioned to produce immune tolerance, suppressing CD8+ T cells and T cell responses through arginase 1 (ARG1) production, a mechanism previously thought to be restricted to tumour associated macrophages [56].

Dysfunction in the Antigen Processing/Presentation Pathways in Cancer Cells

A number of different mechanisms by which the tumour itself can prevent antigen presentation on its cellular surface in order to evade the immune system have been described in each of the components of this pathway.

Firstly, most proteins that are to be presented in association with class I molecules at the cell surface for CD8+ T cell recognition require to be ubiquitinated and then to enter the proteasome (which has fixed and inducible subunits) in order for them to be cleaved to the right size to fit in to the class I molecule binding groove. The only exception to this is when exogenous proteins are cross presented by CD8+ DCs to CTLs through the endosomal/lysosomal pathway (see above under DCs). The proteasome has been reported to be dysfunctional in many tumour types, either through downregulation of one or more of the 20S proteasome's β subunits δ, MB1, and Z which is observed in colorectal, bladder, and ovarian carcinomas, as well as medulloblastoma; or through downregulation of one or more of the inducible subunits (LMP2, LMP7, and LMP10) which appears to be more prominent in acute myeloid leukaemia, in carcinoma of the head and neck, oesophagus, stomach, colorectum, kidney, bladder, prostate, cervix, ovary, and breast, and in astrocytoma, medulloblastoma, neuroblastoma, and melanoma [57]. The molecular basis for the downregulation is not fully described. However mutations at coding microsatellites of genes encoding LMP7 have been detected in gastric cancer [58]. Treatment of myeloma cells with decitabine, a potent DNA methyltransferase inhibitor, restored the expression of several proteasome subunits, suggesting that promoter methylation alterations and epigenetic regulation are likely to be involved in some cases [57].

Secondly, the cleaved protein then needs to be actively transported from the cytosol into the ER by the transporter associated with antigen processing (TAP). This heterodimeric complex is composed of the two half-transporters, TAP1 and TAP2, which form a transmembrane pore in the ER membrane whose opening and closing depend on ATP binding and hydrolysis. Low to undetectable levels of TAP1 and/or TAP2 mRNA and/or protein have been reported in primary cells and cell lines from several tumours, including but not restricted to carcinomas of the head and neck, oesophagus, stomach, pancreas, colorectum and breast [57]. In some tumour cell lines in which TAP is downregulated, its levels are restored by IFN-γ treatment. At the genetic level, mutations in *TAP* genes that resulted in loss of expression or in expression of a nonfunctional protein have been observed in multiple tumour types, and methylation of the *TAP1* gene promoter has also been found in cervical carcinoma [59].

Thirdly there may be downregulation or loss of chaperone proteins, which are required for peptide loading onto nascent MHC class I molecules once the former have been transported by TAP to the ER. These proteins are calnexin, the thiol oxidoreductase ERp57, calreticulin, and tapasin. A substantial downregulation of calnexin, calreticulin and tapasin have all been demonstrated in colorectal carcinomas [60, 61].

Finally, across many different cancer types, tumours may show reduced expression of MHC class I and II molecules themselves. For instance, class I molecules are downregulated in more than 70 % of colorectal tumours [62]. In some rare cases there is complete loss of class I, usually resulting from inactivation of beta2-microglobulin (a class I subunit) in microsatellite positive tumours [63]. Downregulation of HLA molecules can result from loss of haplotypes due to mutations, chromosomal non-dysjunction or mitotic recombination. On the positive side, loss of the MHC class I expression, which often occurs early in cancer development, could theoretically make these cells more sensitive to NK cytotoxic activity. Interestingly, in a large cohort of CRC cases, patients whose tumours demonstrated *low* expression of HLA class I suffered a significantly shorter mean disease-specific survival time (41 months compared to 68 months) with intact class I expression; but patients whose tumours had complete loss of HLA class I expression had a similar prognosis (60 months) to the control group, raising the possibility that partial loss impairs immunogenicity but does not stimulate NK activity, whereas total loss of class I may be able to engage NK activity through loss of the inhibitory function that class I molecules usually have upon NK cells through the KIR receptors [64].

Tumour Related Local Immunosuppression

Other changes can occur locally in the tumour that favour immunosuppression. One such is the downregulation of important co-stimulatory molecules that are usually found within the immunological synapse. Thus in non-inflammatory colorectal cancer, CD80 (B7.1) mRNA levels were significantly lower in the non-inflammatory dysplastic colonic mucosa of patients with one or more methylated genes and levels inversely correlated with patients' methylation scores ($\tau = -0.41$, $p = 0.05$ and $\tau = -0.37$, $p = 0.05$, respectively). Treatment with 5-Aza-2′-deoxycytidine significantly increased CD80 expression both in terms of the level of CD80 mRNA ($p = 0.007$) and of CD80+ cells ($p = 0.003$). The authors concluded that the results indicate that the failure of immune surveillance mechanisms in non-inflammatory colon carcinogenesis may be linked to genomic methylation directly or indirectly affecting CD80 expression [65]. The downregulation of T cell costimulatory molecules in solid tumours can be compounded by a simultaneous increase in T cell inhibitory signals. Thus the ligands that normally curb or limit T cell expansion through interaction with receptors on the T cell as a standard homeostatic mechanism to prevent autoimmunity, can be aberrantly overexpressed in tumours to induce immunosuppression. In this regard PD-L1 (otherwise known as B7-H1) is strongly expressed in CRC [66] and is associated with poor prognosis [67].

T_{reg} cells can impair the immune response against tumours through cytokine-dependent or cell-cell contact mechanisms as they secrete the immunosuppressive cytokines IL-10 and TGF-β and immunosuppressive metabolites such as adenosine. Many studies have indicated that elevated blood and tumour T_{reg} cells are found in many cases of CRC [68], and, although we recognise some heterogeneity in the results as described previously, most investigators believe that these could play a role in local immunosuppression in many solid tumours. Accumulation of T_{reg} cells in tumours could be a result of local switching from conventional T_h cells to T_{reg} cells in response to a tumour related signal such as high levels of TGFβ. Alternatively it could be that pre-formed T_{reg} cells are preferentially recruited to the tumour site due to high concentrations of specific chemokines for example CCL17, CCL22 and CCL28 [69]. It is therefore tempting to believe that strategies to reduce local or systemic T_{reg} frequencies may reverse immunosuppression. Indeed in human models, *in vitro* T_{reg} depletion from peripheral blood of CRC patients does induce CD4+ and CD8+ T-cell responses against tumour-associated antigens [70].

It is imperative to acknowledge the impact of other cell populations within the tumour in terms of the overall local immunological milieu. We have previously mentioned that macrophages are traditionally segregated in to two divergent populations coined M1 and M2 depending on their route of activation and their expression of cytokines. There is now extensive literature which suggests that tumour associated macrophages (TAMs) are recruited during carcinogenesis and tumour growth and that their presence correlates with poor outcome [71]. TAMs tend to be phenotypically M2 rather than M1 (probably driven by the tumour itself), and produce immunosuppressive cytokines such as IL10, driving the development of T_h2 helper T cells as opposed to T_h1 and CTL cells and, as a consequence, the IL-4 secreted from the T_h2 cells drives the further development and recruitment of more TAMs [72]. TAMs are ineffective at antigen-presentation and produce chemokine ligand CCL22 which draws in T_{reg} cells which can add to the immunosuppressive milieu [73]. TAMs can also secrete prostaglandin E2 and more TGFβ which further aggravates this anti-cyotoxic environment [74]. In addition TAMs can also express PDL1 (see above) and can therefore directly suppress any reactive CTL that has managed to gain entry into the tumour space [75]. Finally TAMs can also produce matrix metalloproteinases (MMP7 and MMP9), which will promote invasion [76]. Thus there is a complex multidirectional immunosuppressive web within the tumour where each of the constituent cells promotes the generation and recruitment of the others.

In parallel, although this is less well described, there appears to be a skewing of the intratumoural granulocyte environment to an N2 rather than N1 phenotype: It is likely that these granulocytes facilitate the angiogenic switch by expressing MMP9, promoting metastasis [77] and, via release of elastase, and promotion of interactions between PI3K and platelet derived growth factor receptor (PDGFR) promote tumour cell proliferation [78].

Monocyte derived suppressor cells (MDSCs) are very immunosuppressive, immature myeloid cells that are found at high frequency in tumours and which are categorised as either monocytic MDSCs or polymorphonuclear MDSCs [79]. An

exhaustive exploration of these cells is outside the scope of this chapter but they are important in terms of the aggressive immunosuppression they induce. They deplete nutrients required by lymphocytes such as arginine and L-cysteine which leads to downregulation of the zeta chain in the TCR and proliferative arrest of CTLs [80]. They generate oxidative stress which leads to loss of zeta chain expression and interference with IL-2 receptor signalling [81]. They decrease CD62L expression on the surface of naïve CD4+ and CD8+ cells and limit lymphocyte trafficking [82]. And finally, they stimulate the activation and expansion of T_{reg} cells [83]. All in all, these cells make it very difficult to produce an effective CTL response against tumours!

What Should Immunotherapy Strive to Achieve?

For all the reasons described above it has become increasingly clear that successful immunotherapy will require not only the activation of a specific immune response against a tumour, but will also (perhaps even more importantly) require the limitation and reversal of the immunosuppressive effects of the tumour milieu, wrought by the infiltration of dysfunctional myeloid populations, recruitment of other suppressor cell populations and secretion of immunosuppressive cytokines and subsequent promotion of invasion and metastasis.

This will no doubt require some thought with respect to the best immunological manipulation available which might tip the scales towards a more T_h1, M1, N1 cytotoxic response: And should perhaps also direct us to consider the best positioning of immunotherapy in the therapeutic armamentarium. Should the traditional paradigm of trialling a novel therapeutic in heavily pre-treated huge volume disease prior to consideration for transition to the adjuvant arena be scrapped, given that the immunological milieu and our potential for success with immunotherapy is likely to be at its most amenable, malleable and potentially successful in the minimal residual disease setting after surgery before isolated micrometastases have had the opportunity to recruit their immunosuppressive army and build their anti-cyotoxic barricade?

In the following chapters we hope to give you a flavour of the variety of potential immunotherapeutic strategies available and this introduction to modern immunology should arm you with the complex lexicon required to understand this rapidly evolving field.

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Chapter 2
Cell Based Therapy: Modified Cancer Cells

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Introduction

The incidence of colorectal cancer (CRC) is on the rise, and patients with recurrent or metastatic colorectal cancer (mCRC) still have a poor long-term survival. Although the development of multi-disciplinary management has improved the survival of CRC, conventional treatments such as chemotherapeutic interventions and radiation therapy only marginally improved longevity [1, 2]. Hence, improved treatment options that selectively target cancer cells and their microenvironment with little or no toxicity to normal tissues are urgently needed [3]. Immunotherapy offers an appealing addition to traditional chemotherapy, with possible long-term protection against tumour recurrences through immunological memory [4]. The requirement for an immune based strategy against cancer is the induction of an effective tumour specific immunity in order to break immunological tolerance to the tumour

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and generate anti-tumour immunity [5]. New insights into the functional dialogue between cancer cells and immune cells and in the hierarchical status of different tumour-immune escape mechanisms at different stages of tumour development have been provided more recently, guiding the design of novel therapeutic strategies [5]. Although immunotherapy in CRC overall has been the subject of several previous reviews [1, 6–10], however this chapter will focus on autologous cell based immunotherapy including adoptive T cell transfer, dendritic cell based vaccines and autologous tumour cell derived vaccines. Also the possibilities of combining immunotherapy with conventional treatment strategies will be briefly touched upon.

Antitumour Immune Response; Key Players

As described previously [8], the immune system is capable of promoting an effective immunological reaction to tumour-specific neo-antigens leading to the elimination of cancer cells before clinical expression. However, this immune surveillance period can be followed by a latency period where there is a balance between the immune system and the cancer cells, ultimately tilting towards a phase of immune escape allowing tumour progression and clinical expression. Anti-tumour immune surveillance is highly coordinated and requires specifically primed lymphocytes to eradicate aberrant cells [1]. Hence, this calls for a close collaboration between cells of the innate immune system and cells of the adaptive immune system [8].

T Cells

Rudimentary anti-tumour T cell responses are driven by anti-proliferative interferon gamma (IFN-γ) and interleukin 2 (IL-2), which are secreted upon proteasomal degradation of tumour associated antigens (TAAs) and subsequent presentation to T cell receptors in the context of the major histocompatibility complex (MHC) class I and II molecules. Cytotoxic $CD8^+$ T cells (CTLs) recognise TAAs exposed on the tumour cells in association with MHC class I molecules for which their αβ-T-cell receptor (TCR) is specific, leading to a clonal expansion and tumour cell killing through effector molecules such as perforin and granzyme B [1, 3]. Although most CTLs die through apoptosis following effectuation of their killer function, some become long-lived memory cells [8]. $CD4^+$ T cells respond only to antigens presented by the MHC class II proteins expressed by antigen presenting cells (APCs) and autoregulate their proliferation by releasing and binding IL-2. Depending on the cytokine profile produced by the effector cells, $CD4^+$ T cells are subdivided into different T helper (Th) cells, each secreting specific cytokines [8]. $CD8^+$ CTLs require cooperative interactions with type 1 polarised $CD4^+$ T cells (Th1) to effectuate their cytotoxic function. Moreover, there is increasing evidence that $CD4^+$ T cells may play a more direct role in generating efficient antitumour immunity beyond simply assisting the CTLs [3, 11].

Dendritic Cells

Dendritic cells (DCs) are professional antigen processing and presenting cells. As sentinels of the immune system, dendritic cells patrol the body seeking for endogenous and exogenous antigens to capture and present to TCRs in the context of the MHC class I or II and co-stimulatory molecules (B7, CD40, ICAM-1…). DCs form a heterogeneous population that either generate protective immunity or immune tolerance. After antigen uptake, immature DCs in the peripheral tissue undergo maturation characterised by upregulation of MHC class I and II and co-stimulatory molecules, upregulation of chemokine receptors like CCR7 and the secretion of cytokines such as IL-2. These mature DCs migrate to the secondary lymphoid organs where they present antigens to $CD4^+$ and $CD8^+$ T cells [3]. However, DCs which become activated without maturation signals will promote tolerance and immunosuppression by inducing regulatory T cells (Tregs) [1]. Nevertheless, DCs are potential targets for immunotherapeutic intervention by activating both naïve and memory T cells.

Immunosuppression

Tumour eradication and host tolerance are dictated by the type, polarity and density of infiltrating immune cells which can either reject or accept aberrant cell growth. As stated above, $CD4^+$ T cells are critical for inducing and regulating immune responses, however, tumour derived soluble factors such as transforming growth factor β (TGF-β) and IL-10 induce tolerance by promoting the expansion of $FOXP3^+$ Tregs. Induced Tregs suppress effector T cells and normally protect against auto immune disease through an array of immune suppressing factors including TGF-β, IL-10 and T lymphocyte antigen 4 (CTLA-4). Hence, Tregs appear to play a pivotal role in tumour progression and the suppression of antitumour immunity. Notwithstanding the adverse outcomes associated with the presence of Tregs in breast, hepatocellular, and gastric carcinomas, Treg densities in CRC patients seem to correlate positively with patient survival and absence of metastasis [1, 3, 8]. Hence, it is questioned if these colon and rectum residing $FOXP3^+$ cells truly represent Tregs since FOXP3 is transiently expressed by intra-tumoural T cells upon TCR stimulation, thereby suggesting that FOXP3 might poorly identify immunosuppressive T cells. Perhaps, the true prognostic relevance of Tregs lies in the balance with cytotoxic $CD8^+$ T cells [1].

Furthermore, tumour associated macrophages (TAMs) constitute a significant part of the tumour-infiltrating immune cells and are classified into heterogeneous subgroups by an array of secreted cytokines [1]. TAMs can polarise to the M1 (classical) phenotype and promote adaptive T cell immunity or they polarise to the M2 (alternative) phenotype and promote immune suppression and tumour escape. The role of TAMs in CRC is controversial since intratumoural macrophages seem to facilitate immune suppression, inflammation and angiogenesis by secreting VEGFR, IL-10, IL-16, nitric oxide and reactive species. In contrast, when TAMs are found at

the CRC invasive margins they seem to prevent tumour development (suggesting polarisation towards the M1 phenotype) and are associated with better prognosis and survival rate [10].

The diversity of CRC environments requires research to develop multiple immunotherapies. New insights into the mechanisms of immune suppression, tolerance and mutational profiles will help establish novel therapies that will circumvent these immunological barriers [1].

Cellular Treatment Modalities

Available cellular based anti-cancer immunotherapies can be roughly divided into adoptive immunotherapies that rely on administration of *ex vivo* prepared immune cells or antibodies, and active immunotherapies or vaccines that activate the host's endogenous immune system [12, 13].

Adoptive T Cell Therapy

Most adoptive cell therapies (ACTs) focus primarily on T cell therapy, due to the highly specific nature and potent killing ability of T cells [13]. In ACT, autologous T cells are collected from the tumour, draining lymph nodes or peripheral blood and are activated and expanded to large numbers *ex vivo* before being intravenously administered to the patient in an attempt to give their immune system the ability to overwhelm the remaining tumour [7]. One advantage of ACT is that *ex vivo* selection, reprogramming and activation of highly reactive T cells may overcome some tolerogenic mechanisms, which inhibit T cell activation *in vivo* [3, 13].

The primary strategies for ACT have utilised tumour infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) as starting materials. It is known that tumour-antigen-specific T cells within the tumour microenvironment are often suppressed or dysfunctional such that cancer cells overwhelm the immune response. However, T cells collected from these TILs can be restimulated *ex vivo* in a process that reverses their unresponsive state [13]. ACT was first described in 1988 by Rosenberg and colleagues in metastatic melanoma [14]. Expanded TILs re-administered to patients with metastatic melanoma promoted impressive reductions in tumour burden in early phase clinical trials [15]. Contrarily, in CRC first generation clinical trials of ACT using TILs reported only limited success [16] (Table 2.1).

Satoh and colleagues reported that two patients showed complete response and four patients showed partial response among 19 gastric or CRC patients with ACT combined with the streptococcal immunopotentiator OK-432 [17]. Another study demonstrated that adoptive TIL therapy, administered to 14 stage IV gastric and colon cancer patients after IL-2 stimulation, could induce increased or stable TCR

Table 2.1 Overview of different trials investigating the efficacy of ACT in colorectal cancer

Author	Cancer type	# patients enrolled	Treatment schedule	Toxicity	Biological response	Clinical impact
Satoh et al. [17]	Liver metastasis from gastric and colorectal cancer	24 patients	T cells from regional LN or peripheral blood cultured sonicated tumour extract antigen (SE-Ag) for 9–13 days. Transfusion through the hepatic artery after the administration of OK-432.	No		2/19 CR 4/19 PR 5 patients with resected liver metastasis still alive and RF.
Kono et al. [18]	Stage IV adenocarcinoma of the stomach and colon	14 patients 13 controls	T cells + IL-2: administered by IP bolus injection to autologous donors with malignant ascites or by IV bolus injection to autologous donors with malignant pleural effusion.	No	2/14 patients: upregulation of initially decreased TCR expression. 7/14 patients: no significant change of TCR expression. 5/14 patients: decreased expression of TCR molecules.	3/14 minor response. 4/14 no changes 7/14 PD

(continued)

Table 2.1 (continued)

Author	Cancer type	# patients enrolled	Treatment schedule	Toxicity	Biological response	Clinical impact
Gardini et al. [19]	CRC with liver metastasis	47 patients: 14 group A: immunotherapy. 14 group B: conventional chemotherapy. 19 group C: no treatment.	TIL + IL-2 treatment: T cells transfused to patients over 2–3 h. Continuous intravenous infusion of IL-2 for 5 days (West's schedule). 1-week interval, second cycle of intravenous IL-2 for 4.5 days. Maintenance therapy of 3×10^6 IU subcutaneous IL-2 for 5 consecutive days each month over a period of 6 months until disease progression.	1 case of grade 4 toxicity Grade 3 toxicity in 2 cases	Almost complete restoration of the TCR ζ and ε-chains. To a lesser degree of p56lck. A consequent increase in FasL expression.	Group A: 70 % recurrence. Group B: 55 % recurrence. No difference in DFS between groups.
Ishikawa et al. [20]	Several solid tumours including CRC	109 cancer patients: 3 post completely resected state. 106 non-operable advanced or recurrent cancers. 49 control patients.	ACT with CD3-LAK cells at 2 week interval	ND	IFN-α: unchanged. FN-γ and TNF-α: markedly increased. IL-12 (p70): decreased. IL-2: unchanged. Th2 cytokines: increased. IL-10: unchanged. Tregs: decreased.	OS significantly longer in patients with increased FN-γ and TNF-α. OS seemed longer in patients with decreased Tregs.

ACT with genetically engineered T cells						
Parkhurst et al. [21] (NCT00923806)	mCRC refractory to a minimum of four different chemotherapy regimens	3 HLAA*0201+ patients	Lymphodepleting chemotherapy regimen. $2–4 \times 10^8$ genetically modified ACT + IL2.	Grade 2 and 3 diarrhoea. Inflammatory colitis.	Serum CEA protein levels dropped by 74–99 % after ACT → transient; CEA serum levels increased at 3–4 months posttreatment	Patient 1: 17 % reduction in metastatic cancer to the lung, PD at 5 months. Patient 2: no response. Patient 3: PR at 4 months, PD at 6 months.
Morgan et al. [22]	Colon cancer metastatic to the lungs and liver, refractory to multiple standard treatments	1 patient (case report)	Nonmyeloablative lymphodepleting regimen for 7 days. Intravenous infusion of 10^{10} cells transduced with the 4D5-CD8-28BBZ ERBB2-CAR in 125 ml over 30 min.	Within 15 min after cell infusion: Respiratory distress. Dramatic pulmonary infiltrate on chest X-ray. Severe hypotension. Two cardiac arrests. Gastrointestinal bleeding. Dead: 5 days after treatment.	Marked increases in: IFN-γ GM-CSF TNF-α IL-6 IL-10 → Consistent with a cytokine storm	Dead: 5 days after treatment

(continued)

Table 2.1 (continued)

Author	Cancer type	# patients enrolled	Treatment schedule	Toxicity	Biological response	Clinical impact
ACT from sentinel lymph nodes						
Marits et al. [25]	Colon cancer with no signs of distant metastases or lymph node involvement prior to surgery	15 patients	NA (*ex vivo* study)	NA	T cell proliferation in SLNs → dose dependent and/or kinetics of antigen dependence: present in a majority of patients → weaker or absent in patients with metastatic SLNs. IFN-γ response close correlation with proliferative responses, with a few exceptions → freshly isolated lymphocytes from SLNs in CRC patients → proliferative responses against autologous tumour extracts.	NA

Karlsson et al. [4]	Disseminated or locally advanced, high-risk CRC	16 patients	On average 71 million clonally expanded autologous tumour-reactive T-lymphocytes were retransfused to each patient	No	Expansion of tumour-reactive T-lymphocytes as assessed by investigating the TCR Vβ repertoire of SLN-acquired lymphocytes before and after restimulation	Clinical response in stage IV patients (n = 9): 4 complete tumour regression. 4 SD. 1 diminished tumour burden. → Increased cumulative survival, however eventually all patients died. Clinical response in Stage III patients (n = 1): SD Clinical response in Stage II patients (n = 5): 1 patient developed liver metastasis and regression of metastases after ACT → SD. 4 SD

ACT adoptive cell transfer, *mCRC* metastatic colorectal cancer, *LN* lymph nodes, *CR* complete response, *PR* partial response, *RF* recurrence free, *IL* interleukin, *IP* intraperitoneal, *IV* intravenous, *TCR* T cell receptor, *PD* progressive disease, *IU* international units, *DFS* disease free survival, *Th2* T helper 2, *OS* overall survival, *CEA* carcinoembryonic antigen, *CAR* chimeric antigen receptors, *IFN-γ* interferon gamma, *GM-CSF* granulocyte macrophage colony-stimulating factor, *TNF-α* tumour necrosis factor alfa, *SLNs* sentinel lymph nodes

ζ-expression in some patients, which is normally downregulated with disease progression and immunosuppression [18]. Similar results were reported by Gardini and colleagues, where 14 CRC patients who underwent radical surgery for liver metastasis received reinfusion of IL-2 stimulated TILs. Although patients did not demonstrate significant clinical results, the biological findings indicated a potentially good activation of all TILs after IL-2 stimulation with preserved TCR ζ- and ε-chains [19]. Given the primarily biological responses in CRC and the absence of abundant clinical results, the use of TILs in ACT is currently limited to patients with melanoma, potentially due to a higher immunogenicity of melanoma in comparison to other cancers [13].

An alternative strategy is the use of anti-CD3 stimulated lymphokine-activated killer (CD3-LAK) cells for ACT. In a study of 109 patients with advanced cancer, including CRC, a significantly enhanced secretion ability of IFN-γ and TNF-α from peripheral blood cells (PBCs) was induced by CD3-LAK based ACT as well as a decrease in the number of Tregs in peripheral blood of patients with advanced cancer, possibly in an IFN-γ dependent manner. Furthermore, the overall survival (OS) was significantly longer in patients who had increased IFN-γ and TNF-α secretion after receiving ACT [20].

Some disadvantages of adoptive cell therapy also need to be considered such as a possible lack of immune memory, poor persistence of adoptive T cells *in vivo*, prohibitive costs and time to produce T cells (4–16 weeks), as well as risk of severe adverse effects [13]. Furthermore, the development of ACT for CRC has slowed somewhat by the lack of acceptable target antigens. However, based on growing insights into the immune system and T cell biology, there is a renewed interest to explore ACT as a novel therapeutic strategy in CRC [16]. Genetically engineered T cells expressing high avidity antigen receptors with predetermined affinity facilitate the targeting of virtually any tumour type, including CRC [13].

ACT with Genetically Engineered T Cells

A phase I trial investigated the administration of autologous T lymphocytes genetically engineered to express a murine TCR against human carcinoembryonic antigen (CEA) in 3 mCRC patients, refractory to standard treatment [21]. Although all patients experienced profound decreased serum CEA levels, and one patient had an objective clinical response, in all patients a severe transient inflammatory colitis was induced indicating the limitations of using CEA as a target for immunotherapy given its relevant expression in certain normal tissues.

Alternatively, antibody-based chimeric antigen receptors (CARs) can be used expressing a single chain variable fragment derived from a tumour antigen-recognising monoclonal antibody, fused to intracellular T cell signalling domains. CAR T cells can be used universally across all patients since they target native antigens on the surface of tumours without MHC restriction. Morgan and colleagues

investigated the administration of Her2-specific CAR T cells to one CRC patient with liver and lung metastasis refractory to multiple standard treatments. However, within 15 min after cell infusion the patient experienced respiratory distress, displayed a dramatic pulmonary infiltrate on chest X ray and despite intensive medical intervention, the patient died 5 days after treatment probably due to a cytokine storm [22].

Finally, an alternative approach was investigated to mediate the induction of multiple antigen-specific polyclonal CTLs which might be more relevant for tumours where only a few or no defined TSAs are available, like CRC. Autologous peripheral blood-derived CD8-enriched T-cells were stimulated with DCs derived from PBMCs and pulsed with autologous apoptotic tumour cells to generate CTLs with anti-tumour activity. IFN-γ secretion analysis confirmed that generation of tumour-specific CTLs is feasible from patients with CRC, and could be useful for supporting an ACT approach in CRC [23].

ACT Using Sentinel Node Acquired Lymphocytes

The sentinel node (SLN) is the first lymph node to receive lymphatic drainage from a tumour and seems to be the predominant location for activation and expansion of tumour reactive lymphocytes. Yanagawa and colleagues were the first to report the expansion of CTLs from tumour draining SLNs [24]. Subsequent studies demonstrated that SLNs serve as an enriched source of tumour reactive lymphocytes that proliferate upon stimulation with autologous tumour antigens and may be useful in future trials of ACT [25]. A pilot study of ACT using SLN acquired lymphocytes, expanded *ex vivo* against autologous tumour extracts and retransfused to 16 patients with CRC was conducted. The expanded cell population displayed the surface phenotype of memory cells and a cytokine secretion profile of Th1 cells. This study indicated that SLN acquired $CD4^+$ lymphocytes are capable of inducing tumour regression in patients with disseminated CRC, without causing serious complications [4]. Subsequently, a Phase I/II study in 71 postoperative mCRC patients, including stage I–III patients who underwent radical surgery and stage IV patients with synchronous metastases who underwent palliative surgery, demonstrated that SLN-T cell based immunotherapy was feasible and safe as an adjuvant to current standard treatment regimens for stage I-IV CRC patients with a significantly improved survival rate in stage IV patients (Zhen et al. 2015). It has been indicated that chemotherapy can modulate the tumour microenvironment to augment anti-tumour immune response. Theoretically, chemotherapy is a two edged sword; on the one hand it may decrease the proliferative capability of tumour reactive T cells since chemotherapy affects dividing cells. On the other hand, chemotherapy will lead to increased tumour cell death, hence increased antigen presentation to T cells and increased T cell reactivity [4]. Consequently, a combined approach is recommended in future randomised controlled trials [2].

Combination Strategies with ACT

Chou and colleagues [26] reported that the methylating agent 5-aza-2′-deoxicytidine (DAC) can induce expression of NY-ESO-1 and other cancer/testis (CT) antigens, that can serve as targets for ACT. CT antigens are immunogenic proteins that are expressed in germ cells, trophoblastic tissue and a wide range of cancer cells but not in normal tissue. NY-ESO-1 has made one of the fastest transitions to an immunotherapy candidate with as main characteristic its capacity to elicit spontaneous antibody and T-cell responses in a proportion of cancer patients [27]. DAC induced demethylation of these CT genes coupled with NY-ESO-1 specific CTLs showed *in vitro* activity against CRC cell lines that were HLA-matched, however, *in vivo* the induced expression of NY-ESO-1 was low and heterogeneous. These results suggest that a combination of an epigenetic modulation and immunotherapy targeted against CT antigens could provide a novel systemic treatment for CRC.

TNF-related apoptosis-inducing ligand (TRAIL) is an immune effector molecule that functions as a selective anti-tumour agent. However, tumour cells, especially metastatic tumour cells often exhibit a TRAIL-resistant phenotype. Nevertheless; therapeutic agents can sensitise tumour cells to TRAIL-initiated apoptosis through mediating TRAIL receptor expression and function. It has been shown that the combined treatment of TNF-α and IFN-γ effectively sensitised mCRC to TRAIL-induced apoptosis. Moreover, tumour specific CTL adoptive transfer immunotherapy when combined with TRAIL therapy, achieved significantly greater metastasis suppression efficacy against TRAIL resistant CRC than either therapy alone. This might be explained by the dual function of $CD8^+$ T cells which may infiltrate in the tumour and utilise TNF-α and IFN-γ and TRAIL to induce tumour cell apoptosis or when not able to infiltrate the metastatic tumour, still can secrete TNF-α and IFN-γ that might move into the tumour through peripheral blood circulation to sensitise the tumour cells. In that case, exogenous TRAIL might be applied to treat the TRAIL resistant cancer [28].

Vaccine Strategies

Cancer vaccines must effectively break immunological tolerance and induce or amplify antigen directed T cell assaults [1]. In contrast to chemotherapy or passive (adoptive) immunotherapies with antibodies or *ex vivo*-expanded T cells, therapeutic vaccines do not have a direct anti-tumour activity, but aim to reset patients' immune systems to achieve this goal [12]. The intrinsic advantage of vaccines is the feasibility of targeting multiple antigen targets, or even whole tumour cells by including multiple related epitopes of whole tumour cells as sources of cancer-related antigens [12].

Dendritic Cell Vaccines

Dendritic cell (DC) based vaccination strategies have been developed owing to the role of DCs in coordinating innate and adaptive immune responses as sentinels of the immune system. The aim of DC vaccination is to induce tumour-specific effector T cells that can reduce the tumour mass significantly and that can induce immunological memory to prevent tumour relapse [29]. This can be achieved by the use of *ex-vivo* generated DCs as carriers of cancer vaccines. In clinical vaccination trials, predominantly monocyte-derived DCs from patients are used, stimulated with differentiation stimuli (including IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF)) to differentiate monocytes towards DCs and adjuvants, mostly proinflammatory cytokines, to induce DC maturation. It is known that the immature dendritic cell is efficient in capturing and processing antigens, but inefficient when it comes to co-stimulation and activation of T-cells. On the other hand, the mature DC is efficient in T-cell activation and co-stimulation but less efficient in capturing and processing further antigens [30]. Hence, in a next step the *ex-vivo* matured DCs are loaded with the tumour-associated antigen (TAA), and then injected back into the patient where they are able to prime polyclonal T cells *in vivo* [8, 29]. This strategy is gaining momentum and is currently considered as mainstream technology for most vaccine studies [9]. In CRC, the most frequent TAAs are self-antigens expressed at low levels on normal cells and in embryonic tissue and at high levels in tumour cells. The most recognised in CRC is the carcinoembryonic antigen (CEA), which is normally expressed in fetal tissue and widely overexpressed in CRC. Other TAAs that are thought to be immunogenic in CRC are Ep-Cam HER2/neu, MUC-1, MAGE-1,2 and/or 3 and p56 [7].

To our knowledge, 26 clinical studies on DC vaccination in CRC have been reported until now [31–57]. To date, various strategies for loading DCs with CRC-associated antigens have been used, including peptide pulsing, tumour lysate pulsing, mRNA electroporation or transduction with viral vectors. No consensus has been reached on the most optimal vaccination schedule. Hence DC injections have been given intravenously, subcutaneously, intradermally and/or intranodally with concentrations ranging from 10^6 to 10^9 DCs per vaccination and administered between two and ten times in an individual patient.

Most of these early phase clinical trials documented robust tumour-specific immune responses, although the overall clinical response, defined as stabilisations or responses, whether minor, partial or complete in some patients, have been variable. Nevertheless, the vaccine proved to be safe and well tolerated [8, 9]. It seems that the method of antigen loading may not primarily dictate clinical efficacy. Lesterhuis and colleagues, compared CEA mRNA electroporation with CEA peptide loading and could not find superiority of one over the other in CRC patients [55]. However, the gradual induction of anti-tumour immunity with DC vaccines and the rapid decrease of adoptively transferred T cells may represent major limitations in complete treatment of established tumours. On the basis of these data, a

combined immunotherapy of DC vaccination following ACT was investigated [58–60]. It was shown that an immunisation of ACT followed within 1 day by a DC vaccine led to a boosting of antigen specific T cell responses and in a complete inhibition of tumour growth and prolongation of survival. This form of (synergistic) combined immunotherapy may provide a potent therapeutic strategy for cancer treatment [58].

In the future, increasing research efforts should focus on investigating combination therapies in order to increase the clinical success rate of DC vaccination. Immunopotentiators, such as Toll like receptor agonists Poly I:C, LPS, Pam3Cys and R848, optimise DC based vaccines and enhance production of type 1 T cell IL-12 [1]. Other promising candidates for synergism are antibodies that block immune inhibitory molecules (such as CTLA-4 and PD-1) or chemotherapy that induces immunogenic cell death [8].

Autologous Tumour Cell Derived Vaccines

An autologous tumour cell vaccine is a therapeutic agent produced by isolating tumour cells from an individual and processing these tumour cells into a vaccine formulation *in vitro*. The vaccine is then administered to the individual from whom the tumour cells were isolated, typically combined with an adjuvant immunostimulant such as bacillus Calmette-Guérin (BCG), Ulster strain of the Newcastle disease virus (NDV), heat shock proteins or IL-2 transfected fibroblasts. Autologous tumour cell preparations have the advantage that all antigens that are presented to the immune system are also relevant for the recognition of the tumour. However, the preparation of such personalised vaccines is time consuming, relatively costly and, therefore, clinically difficult to apply outside a clinical trial setting [61]. The first clinical study on active specific immunotherapy (ASI) using an autologous tumour cell-BCG vaccine (OncoVAX®) given intradermaly to patients with stage II and III CRC was conducted by Hoover and colleagues [62–64]. Development of anti-tumour immunity was measured by serial delayed cutaneous hypersensitivity (DCH) skin testing with autologous tumour cells compared to normal colon mucosa cells, indicating an immunisation in response to tumour-associated antigens [62]. In addition, a significant improvement in overall (OS) and disease free survival (DFS) in all eligible colon cancer patients who received ASI was suggested [64]. To our knowledge, three phase II studies and five phase III studies have been conducted in stage II-III CRC patients, using the DCH skin reaction as an indirect parameter to measure vaccine-specific immunity (Table 2.2). Two studies reported a positive correlation between DCH and improved prognosis [65, 66]. An intention to treat analysis showed that overall no significant impact on improvement of the rate of recurrence or survival could be demonstrated. However, subgroup analysis in three studies using tumour cell reinjection combined with BCG reported an effect on survival, only in stage II patients [67, 68] and only in patients with colon cancer [64]. Additionally, using NDV-infected autologous tumour cells a randomised Phase II trial reported a positive effect on OS when compared to historical controls and a

Table 2.2 Overview of clinical trials using autologous tumour cell derived vaccines

Author	Type of study	Type of cancer	Patients (n) enrolled; treated; control	Vaccination schedule	Toxicity	Immune response	Clinical impact	Concurrent therapy
Hoover et al. [64]	Randomised phase III trial (ASI/BCG or no therapy after resection)	Dukes stage B2-C3 CRC	98; 50; 48	3 intradermal vaccines in 3 weeks (10^7 tumour cells/10^7 BCG for the first 2 weeks) and 1 (10^7 tumour cells) in week 3 starting 4–5 weeks after tumour resection	No serious side effects	16/20 positive for DCH after vaccination	Overall no effect on OS/DFS. Subgroup analysis: advantage OS and DFS vaccinated colon cancer patients	Rectal cancer patients received postimmunotherapy radiation
Ockert et al. [69]	Phase II trial (ASI/NDV and ASI/BCG as control)	stage II/III CRC	57;48 (NVD) and 9 (BCG); historical controls	3 vaccines at 2 week interval starting 6–8 weeks after surgery	No serious side effects	21/31 increasing DCH reactivity. 8/31 positive DCH response during vaccination	Improved OS compared to historical controls	None

(continued)

Table 2.2 (continued)

Author	Type of study	Type of cancer	Patients (n) enrolled; treated; control	Vaccination schedule	Toxicity	Immune response	Clinical impact	Concurrent therapy
Vermorken et al. [67] Uyl-de Groot et al. [68]	Randomized phase III trial (ASI/BCG or no therapy after resection)	Stage II/III colon cancer	254; 128; 126	3 weekly vaccination starting 4 weeks after surgery with a booster vaccination at 6 months	No serious side effects,	Increasing DCH, 92 % of patients had indurations of more than 10 mm after 4th vaccine	Overall no significant impact on prognosis. Subgroup analysis: significant clinical benefit in surgically resected stage II colon cancer patients (OS, RFS, RFI)	None
Harris et al. [65]	Randomised phase III trial (ASI/BCG or no therapy after resection)	Stage II/III colon cancer	412; 205; 207	3 intradermal vaccines in 3 weeks (10^7 tumour cells/10^7 BCG for the first 2 weeks) and 1 (10^7 tumour cells) in week 3 starting 4 weeks after tumour resection	79 % of vaccinated patients local reaction at vaccination site (ulceration, drainage and crusting)	106/150 positive DCH response to the third vaccine	No significant difference in survival between treatment arms, in vaccination arm the DCH response correlated with improved prognosis	None

Baars et al. [72]	Phase II trial (ASI/BCG combined with 5FU/leucovorin)	Stage III colon cancer	104; 53; 0	3 weekly vaccination starting 4–5 weeks after surgery with a booster vaccination at 8 months, and at least 4 weeks after last cycle of chemotherapy	Grade III/IV reaction comparable to chemotherapy alone	DCH reaction after fourth vaccination still strong, despite chemotherapy in between third and fourth vaccination	Not described	5FU/leucovorin
Liang et al. [66]	Phase II trial	Stage IV gastrointestinal tumours	25; 25; 0		Not described	Not described	1 CR 5 PR 1 year survival rate was 96 %	None
	Randomised Phase III trial (ASI/NVD or no therapy after resection)	Stage I-IV CRC	567; 310; 257		Not described	>90 % positive DCH reaction 2 weeks after the immunotherapy, but negative before therapy	The magnitude of DCH was related to the prognosis. Significant difference in 5-year survival rate: 80 % in the positive response group, and 30 % in negative response group	None

(continued)

Table 2.2 (continued)

Author	Type of study	Type of cancer	Patients (n) enrolled; treated; control	Vaccination schedule	Toxicity	Immune response	Clinical impact	Concurrent therapy
Schulze et al. [70]	Randomised Phase III trial (ASI/NDV or no therapy after resection)	Complete resected liver metastasis from CRC	51; 25; 26	First vaccination 14–21 days after resection of metastasis. A complete ASI cycle involved 5 consecutive vaccinations at 2 week intervals and one boost 3 months later	No serious side effects	Not described	Overall no significant impact on survival. Subgroup analysis: significant long term benefit with respect to OS and DFS for vaccinated colon cancer patients compared to control group	None
NCT02448173	Randomised Phase III Multicenter Study	Stage II colon cancer	Will be recruiting shortly, estimated enrolment 550	Randomised 1:1 OncoVAX® plus surgery (n = 275) (intradermally following surgical resection) vs surgery alone (n = 275)	Not described yet	Not described yet	Not described yet	Surgery

ASI/BCG active specific immunotherapy/bacillus Calmette-Guérin, *CRC* colorectal cancer, *DCH* delayed cutaneous hypersensitivity, *OS* overall survival, *DFS* disease free survival, *NDV* Ulster strain of the Newcastle disease virus, *RFS* relapse free survival, *RFI* recurrence-free interval, *CR* complete response, *PR* partial response

randomised Phase III trial reported a significant effect on both OS and DFS in an colon cancer subgroup when compared to a non-vaccinated control group [69, 70]. Furthermore, clinical use of these types of vaccines has revealed no serious toxic events. While colon cancer patients seem to benefit, rectal cancer patients do not possibly due to a lack of intrinsic immunogenicity or the abolishment of the lymph nodes by pelvic irradiation before they could have full impact in the immunologic response [8].

Early clinical trials of ASI used a three-vaccination immune induction regimen after surgical resection of the primary tumour, however it was shown that the DCH response waned over 6 months after the third vaccination. Hence, the need for a booster vaccine at 6 months was suggested and Vermorken and colleagues completed a Phase III trial showing that this strategy reduced the risk of recurrence by 61 % in patients with Stage II colon cancer, recurrence-free survival was significantly longer and there was a trend towards improved OS [67].

At the time this ASI trial was conducted, awareness on the biologic heterogeneity of colon cancer, including the microsatellite status, and its possible clinical implications was still limited. Therefore, the association between response to ASI treatment and microsatellite status in colon cancer was retrospectively investigated on the original patient group of the above described phase III study. No impact of the autologous tumour vaccine was detected in patients with microsatellite instable (MSI) tumours; they did well irrespective of treatment arm and tumour stage. Hence, it could be argued that these patients could do without further immunotherapy after surgical excision of the primary tumour. Consequently, it was concluded that the clinical benefit, measured as recurrence-free survival, from adjuvant ASI treatment was restricted to colon cancer patients with MSS Dukes B tumours [71].

All these clinical trials indicated that high quality vaccines are needed in order to obtain a positive effect and that treatment is most effective in patients with minimal residual disease (stage II).

Despite these results with autologous tumour cell-derived vaccines, a paucity in clinical ASI trials was introduced probably due to lack of pharmaceutical support for tailor-made vaccines and the problems to optimise the vaccines based on limited immunological data [61]. Moreover, FDA requirements made a confirmatory OncoVax® trial needed with a sterile vaccine. Such a confirmatory trial in stage III colon cancer patients (NCT02448173) has been set up and will most probably be activated in the last quarter of 2016. This international trial plans to enrol 550 patients from over 40 sites, (both in the U.S. and in Western Europe). Patients will be randomised 1:1 to either receive surgery alone or OncoVAX® given intradermally following surgical resection. The primary outcome measure of the trial is DFS at 5 years, while secondary measures are OS and recurrence-free interval. The study is estimated to collect its final data on primary outcomes in July 2021 and to be completed in July 2023. Future trials should also focus on patients with minimal subclinical disease and should take MSI into account.

Furthermore, possible combination strategies should be explored. In preclinical models, ASI combined with chemotherapy were shown to have a synergistic anti-tumour effect, possibly due to depleting Tregs and enhancing CTL responses. Additionally, this combined approach of chemotherapy or irradiation might kill the bulk of cancer cells while immunotherapy might keep residual cancer stem cells and

differentiated cancer cells in check, thereby abrogating the replenishing pool of CRC cells. Moreover, chemotherapeutically killed tumour cells will disperse large amounts of intracellular antigens to an awaiting immune system [3, 72]. To our knowledge only one non-randomised multicenter Phase I/II study of ASI combined with adjuvant 5-fluorouracil (5-FU) and leucovorin, administered between the third and the fourth vaccination, in patients with stage III colon cancer (NCT00016133) has been performed. This study showed that the ASI-induced immune response is only minimally impaired by consecutive 5FU/Leucovorin and that the combined treatment does not cause unexpected toxicity. Nevertheless, other types of chemotherapy might have a more pronounced negative effect on anti-tumour immunity, so future combinations of immune therapy and chemotherapy will have to be carefully tested before being applied on large scale in a clinical setting [72].

Conclusion and Future Perspectives

The limitations of surgery and adjuvant chemo and/or radiation therapy in treating CRC patients necessitate the development of alternative treatment approaches including immunotherapy. Despite evidence of antigen-specific responses in the absence of serious adverse events, indicating a clear role for T cell based immunity in the final outcome of CRC, there is hardly any evidence generated to demonstrate the clinical impact of these immunological strategies. Furthermore, it is also essential to keep in mind that traditional RECIST criteria may not fully capture the clinical benefit of immunotherapeutic strategies and that immune related (ir)PFS and OS, based in the immune-related response criteria (irRC) are important. Furthermore, it is unlikely that immunotherapeutic strategies will be able to induce the desired clinical responses on their own, but will need to be combined with delivery systems and other modalities, including adjuvants, blockers of immunosuppressive mechanisms and conventional therapies that target regulatory mechanisms in order to overcome immunological tolerance and promote tumour regression.

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Chapter 3
Therapeutic Cancer Vaccines

Chris Heery, Anteneh Tesfaye, Benjamin Weinberg, and John Marshall

Introduction

Immune therapy for cancer has evolved significantly in the past several decades. With the recent dramatic successes of novel immune modulating agents in certain cancers, the scientific community has redoubled efforts to expand the impact of these treatments for more patients. Cancer vaccines have been developed and tested in gastrointestinal (GI) cancers for more than two decades, mostly with little success. As a result, there has been little interest or investment in this field of research. However, now that opportunities for combination immune therapies have become more attractive, having an understanding of the past work in cancer vaccines is critical (Fig. 3.1). In this chapter, we will review the most important and representative results of cancer vaccine research in GI cancers. The fundamentals of this work will not only serve future GI cancer research, but also other tumour types.

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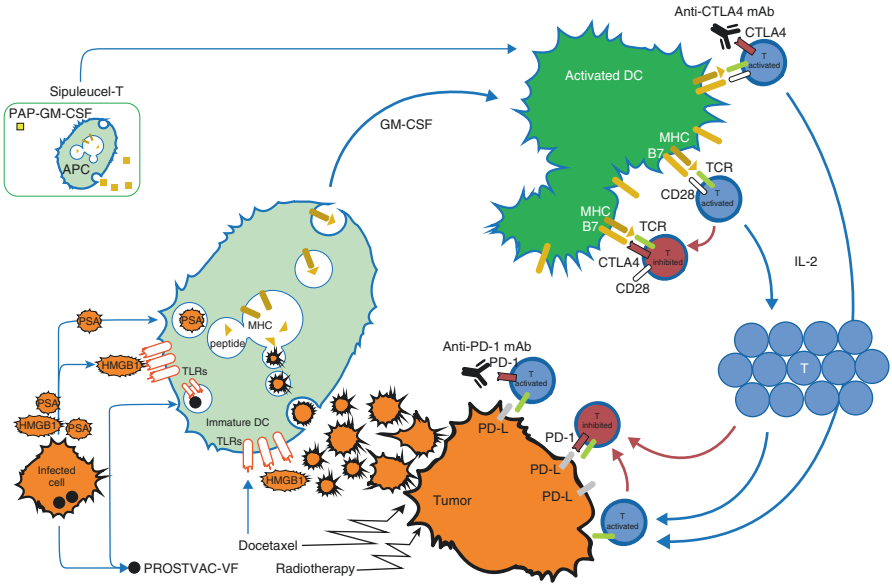

Fig. 3.1 Cancer vaccination and induction of T cell activation

Vaccine Platforms and Vectors

Common Mechanisms of Action

This section will discuss various therapeutic cancer vaccine constructs, including peptide, DNA, whole tumour cell, vector-based, and *ex vivo* processed dendritic cell (DC) vaccines. All vaccines require dendritic cell activation with presentation of the target antigen via major histocompatibility (MHC) complexes (signal 1) and a costimulatory signal (signal 2) (Fig. 3.2) [1]. While this final step of activated DCs inducing T cell activation is common, each vaccine platform has a unique method of DC stimulation. For instance, a peptide vaccine is composed of a target peptide, which represents the target antigen overexpressed on the tumour cell via MHC class 1 and another chemical, called an adjuvant, which has the role of stimulating DC maturation toward an activated state. On the other hand, vector based platforms may trigger DC activation via the presence of the foreign vector, as in the case of a yeast-based vaccine platform. In each case, the vaccine design must include a method of delivering the target antigen for DC uptake and presentation while also containing a component of the innate immune response that will induce a "danger signal" [2] and drive the DC to activate the adaptive immune response [3, 4]. Selection of the target antigen, the concentration and dwell time of the vaccine, along with the concentration of administered adjuvant are all critical to driving an effector T cell phenotype necessary for anti-tumour activity [5].

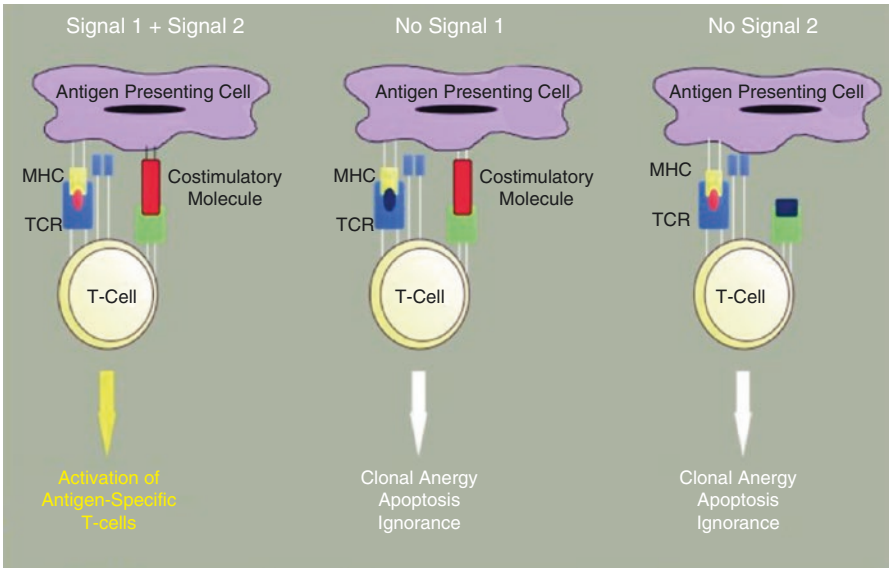

Fig. 3.2 T cell dependence on co-stimulation

Peptide Vaccines (Table 3.1)

Mutant RAS

RAS oncogenes are associated with a variety of malignancies and are mutated in many GI cancers. RAS proteins function as GTPases that playing critical roles in signal transduction pathways, serving as an on/off switches and promoting cell proliferation when switched on [6]. Mutated *RAS* genes—*KRAS*, *HRAS*, and *NRAS*—code for altered RAS proteins, which lead to constitutive activation of the downstream signalling pathways [7]. *RAS* mutation status conveys vital clinical information, guiding therapeutic selection in metastatic colorectal cancer (CRC); In particular, cetuximab and panitumumab have decreased efficacy in tumours with *RAS* mutations due to circumvention of EGFR inhibition [8, 9]. These mutations are extremely common in pancreatic ductal adenocarcinoma (approximately 90 % of tumours harbour a *KRAS* mutation, the majority of which are in codon 12, with others in codons 13 and 61) [10, 11]. Given that *RAS* mutations carry a pivotal role in oncogenesis, *RAS* vaccines have been developed to selectively target GI cancers.

One such *RAS* vaccine is GI-4000, which has been studied in colorectal, pancreatic, and non-small cell lung cancers (NSCLC) [12]. GI-4000 is comprised of four different strains of heat-inactivated *Saccharomyces cerevisiae* yeast expressing the 7 most common *RAS* mutations; each strain has a fusion protein of three different *RAS* mutations (GI-4014: Q61L, G12V, and Q61R; GI-4015: Q61L, G12C, and Q61R; GI-4016: Q61L, G12D, and Q61R; and GI-4020: Q61L, G12R, and Q61H)

Table 3.1 Peptide vaccine clinical trials

Vaccine	Cancer type	Trial design	n	Treatment arms	mOS
Mutant RAS					
GI-4000 [13]	Pancreatic	Adjuvant, phase II	176	3 weekly doses of GI-4000 vs. placebo, followed by 6 cycles adjuvant gemcitabine	17.5 vs. 14.8 months
CTN-95002 CTN-98010 [171]	Pancreatic	Adjuvant, phase I/II	23	6 administrations on weeks 1, 2, 3, 4, 6, and 10	27.5 months
CEA					
CEAVAC (3H1) + TriAb (CALGB89903) [24]	Colorectal	Resected metastatic, adjuvant, phase II	56	4 administrations every 2 weeks for 2 months, then monthly for 2 years, and then every 2 months for 3 years	Not reached (2 year OS: 94%)
CEAVAC + TriAb [23]	Colorectal	Metastatic, phase III	630	5-FU/leucovorin plus CEAVac vs. placebo	21.3 vs. 18.5 months (in patients receiving ≥8 cycles)
β-hCG					
CTP37-DT [43]	Colorectal	Advanced, phase II	77	3 administrations on days 0, 28, and 70	10.5 vs. 5.5 months (high- vs. low-responders)
Avicine (conjugated to DT) [44]	Pancreatic	Advanced, phase II	55	Gemcitabine plus Avicine vs. gemcitabine alone	6.6 vs. 4.7 months
Gastrin					
G17DT [58]	Pancreatic	Advanced, phase II	30	3 administrations on weeks 0, 2, and 6	6.2 months (all), 7.2 vs. 4.0 months (high- vs. low-responders)
G17DT [60]	Gastric	Untreated unresected or metastatic, phase II	103	Cisplatin, 5-FU, and G17DT	9.0 months
G17DT [61]	Pancreatic	Advanced, phase II	154	G17DT vs. placebo	5.0 vs. 2.7 months

G17DT [62]	Colorectal	Metastatic, progressed on irinotecan, phase II	161	G17DT plus irinotecan	9.0 vs. 5.6 months (immune responders vs. non-responders)
CD55					
105 AD7 [67]	Colorectal	Advanced, phase II	162	105 AD7 vs. placebo	4.1 vs. 6.1 months
CD17-1A					
CD17-1A [78]	Colorectal	Metastatic, phase IB/II	20	CD17-1A plus GM-CSF plus IL-2	8.0 months
Edrecolomab [80]	Colorectal	Adjuvant (stage III), phase III	2761	5-FU/leucovorin plus edrecolomab vs. 5-FU/ leucovorin alone vs. edrecolomab alone	Not reached (3 year OS: 74.7 % vs. 76.1 % vs. 70.1 %)
Telomerase					
GV1001 [82]	Pancreatic	Unresectable, Phase I/II	48	Low-, intermediate-, and high-dose groups	4.0 vs. 8.7 vs. 5.1 months
GV1001 (TeloVac) [84]	Pancreatic	Advanced, Phase III	1062	Gemcitabine and capecitabine, gemcitabine and capecitabine followed by GV1001, or concurrent	7.9 vs. 6.9 vs 8.4 months
WT1					
WT1 [85]	Pancreatic	Advanced, phase I	32	Gemcitabine plus WT1 vaccine	10.9 vs. 3.9 months (immune responders vs. non-responders)
WT1 [86]	Pancreatic or biliary	Advanced, phase I	25	Gemcitabine plus WT1 vaccine	9.6 months (biliary) and 8.6 months (pancreatic)

[13]. In an adjuvant phase II trial, 176 patients with *RAS* mutant pancreatic cancer status-post resection were randomised 1:1 to receive 3 weekly doses of GI-4000 or placebo followed by six cycles of gemcitabine (1000 mg/m^2 IV on days 1, 8, and 15 every 21 days) with monthly GI-4000 or placebo administered during off-weeks. Patients were stratified by resection status (R0 or R1).

Among 39 patients who had R1 resections, those who received GI-4000 had a median overall survival (mOS) of 524 days versus 444 days in patients who received placebo [13]. In addition, patients treated with GI-4000 had a higher mutation-specific T cell response (determined by enzyme-linked immunosorbent spot (ELISPOT) assay [serum interferon γ analysis, 46.7 % vs. 8.3 %, P = 0.032]). Accordingly, GI-4000 treated immune responders had the greatest survival advantage (mOS = 596 days vs. 444 days with placebo).

Among patients who had R0 resections, those with G12R mutations had better mOS irrespective of receiving GI-4000 or placebo (335 days longer compared with other mutations), and these patients also benefited from GI-4000 over placebo (mOS was 568 days longer for patients treated with GI-4000) [14]. This finding may be attributed to a significant decrease in inhibitor T regulatory cells (Tregs; 26.2 % of patients treated with GI-4000 vs. 8.8 % treated with placebo had at least a 2-fold decrease in Tregs, P = 0.048). Given these mixed results, phase III studies of GI-4000 have not been performed.

A retrospective analysis looked at 20 evaluable patients with resected pancreatic adenocarcinoma treated with 2 Norwegian *KRAS* peptide vaccines on 2 prior adjuvant phase I/II trials (CTN-95002, 4 different peptides of *KRAS* 5-21 corresponding to the patient's *KRAS* mutation, and CTN-98010, a mixture of 7 peptides of *KRAS* 5-21). Seventeen of the 20 patients had an immunologic response to the vaccine, based on positive delayed-type hypersensitivity (DTH) test or presence of *KRAS*-specific T cells in the peripheral blood after vaccination. The mOS for all patients was 27.5 months, the 5-year survival rate was 22 % (29 % in immune responders), and 20 % of patients were alive at 10 years. Although this was a retrospective analysis involving only a small number of patients, the study still manages to demonstrate the potential for a durable immune responses to *RAS* peptide vaccines.

CEA (See Also Section "Vector-Based Vaccines")

Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion, which is normally produced during fetal development. It is also a tumour-associated antigen that is expressed on over 90 % of colorectal and pancreatic adenocarcinomas, 70 % of NSCLC, and 50 % of breast cancers [15–17]. Given its frequent presence on the surface of tumour cells, there have been many attempts to incorporate CEA into vaccines. CEA by itself is poorly immunogenic due to immune tolerance, although cytotoxic T-lymphocytes (CTLs) recognise CEA epitopes that bind to

major histocompatibility complexes (MHC) A2, A3, and A24 [18]. Further modification of the HLA-A2 CEA CAP-1 epitope to CAP1-6D has been shown to increase its immunogenicity, enhancing sensitisation of CTLs by 100–1000 fold [19, 20].

Foon et al. [21] first attempted to use an anti-idiotype antibody that is the internal image of CEA (3H1, the precursor to CEAVac, given subcutaneously at a dose of 1, 2, or 4 mg every 2 weeks for 4 injections, and then monthly thereafter) in a phase Ib trial of 23 patients with metastatic CEA-positive CRC. Seventeen patients developed antibodies against the vaccine, indicating an immune response, and the presence of this response correlated with progression free survival (PFS) and overall survival (OS), although disappointingly, there were no objective clinical responses. These findings led to a single-arm phase II trial of CEAVac at the 2 mg dose in 32 patients with resected and incompletely resected CRC (14 patients were also treated concurrently with 5-fluorouracil (5-FU) chemotherapy regimens) [22]. All 32 patients generated high-titre antibody responses (based on increased IgG to CEA), and these were not affected by co-administration with 5-FU. A phase III trial of 630 patients with stage IV CRC randomised patients receiving 5-FU/leucovorin to CEAVac versus. placebo. Whereas over 75 % of patients in the CEAVac arm developed anti-CEA antibodies, there was no difference in mOS between the treatment arms, although there was a significant difference in patients who received at least 8 cycles of treatment (21.3 vs. 18.5 months with placebo, $P = 0.04$) [23].

In the phase II Cancer and Leukaemia Group B (CALBG) 89903 study, 52 patients with resected metastatic colorectal cancer (mCRC) to the liver received 4 biweekly treatments of anti-idiotype monoclonal antibodies against CEA (CEAVac) combined with human milk fat globule (TriAb), followed by monthly treatments for 2 years and then every other month for 3 years. Although the treatment was well tolerated with only 10 % of patients experiencing a grade 3 adverse event (AE), the recurrence-free survival rate at 2 years was 39 %, which was not an improvement on the expected rate of 40 % following hepatic resection alone [24].

Following suggestions that tumour antigen peptide emulsification in Montanide adjuvant combined with granulocyte-macrophage colony-stimulating factor (GM-CSF) could stimulate DC differentiation and improve DC recruitment, a phase I study of a modified CEA peptide (CAP1-6D)/montanide/GM-CSF-vaccine was conducted. All patients included in the study had pancreatic adenocarcinoma expressing CEA and HLA-A2, which had been previously treated. Nineteen patients were randomised to receive 3 different doses of the vaccine every 2 weeks at doses of 10 μg, 100 μg, or 1000 μg. The CTL response (measured by ELISPOT testing) was dose-dependent, with 248 spots per 10 [4] CD8 cells following a 1000 μg dose, compared with 37 spots following a 10 μg dose ($P = 0.037$) [25].

Although this study demonstrated a strong immune response to the CAP1-6D/montanide/GM-CSF-vaccine, along with limited toxicity, it has not been further studied.

MUC-1

Mucin 1 (MUC-1) is a transmembrane protein with anti-adhesion properties that may promote metastasis and is expressed on many adenocarcinomas [26]. Thus, MUC-1 is of interest as a tumour-associated antigen for use in therapeutic vaccines. In an early phase I trial, 63 patients (including 24 with pancreatic cancer and 30 with CRC) were treated with a synthetic mucin peptide mixed with the bacillus calmette-guérin (BCG) vaccine [27]. Seven of 22 patients tested had a two to four-fold increase in mucin-specific CTL, but there were no objective tumour responses and only three patients had stable disease. Other early studies used a fusion protein with MUC-1 conjugated to mannan. Karanikas et al. [28], gave four to eight subcutaneous injections of MUC-1/mannan fusion protein to 25 patients with advanced cancers—16 with colorectal, one with gastric, and 8 with breast cancers—on weeks 1, 2, 3, 4, 7, 9, 11, and 13 at escalating doses (10–500 μg per injection). Thirteen of the 25 patients had a detectable antibody response by ELISA, and CTL responses were seen in 2 out of 10 evaluable patients. In subsequent phase I trials, an intraperitoneal route of administration tended to amplify immune response, but the use of cyclophosphamide to increase cellular immunity was found to be of no benefit [29].

Kimura et al. [30] published the results of a phase I/II immunoprevention study, in which 39 patients with premalignant colonic adenomas were given a MUC-1 peptide vaccine with a toll-like receptor (TLR)3 agonist (polyinosinicpolycytidylic acid stabilised with poly-L-lysine and carboxymethylcellulose, poly-ICLC) on weeks 0, 2, and 10. Seventeen of the 39 vaccinated patients achieved an immune response, manifested by high anti-MUC-1 immunoglobulin G (IgG) levels with lasting immune memory—12 of 16 patients who responded at week 12 also responded to a booster injection at week 52. Those patients who did have an immune response tended to have higher levels of myeloid-derived suppressor cells (MDSCs) prior to vaccination. This prevention strategy is being further evaluated in an ongoing randomised, double-blind, placebo-controlled phase II trial (NCT02134925).

SART3

Squamous cell carcinoma antigen recognised by T cells 3 (SART3) is a tumour-rejection antigen that is expressed in over 70% of CRCs. SART3 possesses two antigenic epitopes ($SART3_{109\text{-}118}$ and $SART3_{315\text{-}323}$), and can induce CTLs in HLA-A2 and HLA-A24 positive patients [31–34]. In a phase I study, Miyagi et al. [35], administered a vaccine containing the two SART3 antigenic peptides mixed with incomplete Freund's adjuvant to 12 patients with advanced CRC (all were refractory to 5-FU-based chemotherapy except for 1 patient who was treatment naïve; all patients were confirmed to be HLA-A24 positive). At 5 weeks, 9 patients had stable

disease and 2 had progressive disease (one was not evaluable); a CTL response was seen in 7 out of 11 evaluable patients. Tit was found that the CTL response did not correlate with improved outcomes, and no specific IgG or immunoglobulin E (IgE) responses to the peptides were detected. No further clinical trials using SART3 peptide vaccines have been performed.

β-*hCG*

Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of α and β subunits. β-hCG is used as a marker for pregnancy, although it is also elevated in trophoblastic and non-trophoblastic malignancies [36, 37]. In patients with CRC, wide ranges of serum hCG levels (2–41 %) and tumour hCG expression (17–52 %) have been reported, and both have been shown to have a negative prognostic significance [38, 39]. Importantly, β-hCG is not expressed on normal colonic mucosa or benign colonic lesions [40–42]. In a randomised phase II trial, Moulton et al. [43], studied CTP37-DT, a synthetic β-hCG peptide vaccine conjugated to diphtheria toxoid (DT), in 77 patients with advanced CRC, randomised into low-dose and high-dose groups. The vaccine was administered on days 0, 28, and 70. It was found that 73 % of patients produced anti-hCG antibodies, and those with an antibody titre above the median value had improved survival compared with those with a titre below the median (44.9 vs. 23.6 weeks, P = 0.0002). There were no statistically significant differences between the low- and high-dose groups in terms of antibody response, survival, and toxicity.

Another phase II study randomised 55 patients with treatment-naïve advanced pancreatic cancer to receive gemcitabine or gemcitabine plus Avicine, a vaccine composed of two synthetic β-hCG peptides conjugated to DT. All immunised patients produced anti-β-hCG antibodies and mOS was improved in this treatment cohort, compared with those receiving gemcitabine alone (6.6 vs. 4.7 months; 1-year survival 30 % vs. 14 %) [44]. Despite this result, no further investigations of peptide-based β-hCG vaccines have been published.

Survivin

Survivin is a member of the inhibitor of apoptosis family of proteins, and functions by inhibiting caspase activation and preventing programmed cell death [45, 46]. Survivin is expressed in fetal tissue and many tumours, but is absent in normal, fully differentiated cells, making it a potential target for cancer vaccines [47, 48].

Hirohashi et al. [49], identified survivin-2B80-88, an HLA-A24-restricted peptide recognised by CD8+ CTLs. Survivin-2B80-88 was subsequently studied in combination with incomplete Freund's adjuvant (IFA) and type-1 interferon α (IFN) for treatment of advanced CRC [50, 51] and pancreatic cancer [52]. In the CRC

study, two groups of patients were treated. The first group of five patients were treated with peptide and IFA only. The second group of eight patients were treated with peptide, IFA, and IFN. One patient in the first group had stable disease, while the other four in this group had progressive disease. Out of the eight patients in the second group, four had stable disease, while the other four had progressive disease [51]. Patients in the IFN group also had an increase in peptide-specific CTL clones by tetramer staining, indicating an enhancement in immunogenicity. The pancreatic cancer study similarly treated patients with peptide, IFA, and IFN, and four out of six patients treated with this combination displayed stable disease. The same four patients also had evidence of an immunologic response by tetramer staining and ELISPOT assay [52].

Finally, a phase I study by Lennerz and colleagues [53] used EMD640744, a combination of 5 HLA class I-binding survivin peptides, and administered this with the adjuvant Montanide™ ISA 51 VG to 49 cancer patients, including 10 patients with CRC. Considering all 49 patients, the best overall response was stable disease (seen in 28 % of patients), and 63 % of patients had peptide-specific CTL immunologic responses by multimer staining and ELISPOT testing [53].

While survivin-based peptide vaccines have clearly demonstrated an ability to elicit peptide-specific immune responses (especially with the addition of IFN), their use has not progressed beyond early phase clinical trials given their modest clinical responses.

G17DT

G17DT is an immunoconjugate of gastrin-17 linked to DT. It is administered intramuscularly, inducing antibody formation against gastrin-17 and cell-associated precursor gastrin molecules [54]. Gastrin functions as a growth peptide in both colorectal [54, 55] and pancreatic cancers [56, 57]. Brett et al. [58], studied the vaccine in 30 patients with advanced pancreatic cancer as part of a phase II study. Tweinty vaccine-treated patients (approximately 67 %) achieved an antibody response, and those exposed to higher dose level had a higher response rate (82.4 % in the group receiving 250 μg G17DT vs. 46.2 % in the 100 μg group, P = 0.018). mOS was significantly longer in antibody responders compared with non-responders (217 days vs. 121 days, P = 0.0023).

Another phase II study of G17DT in 52 patients with stage I-IV gastric adenocarcinoma also found that antibody responses were dose-dependent, with 11 out of 12 stage I-III patients, and 8 out of 14 stage IV patients treated at 250 μg achieving a response [59]. Detailed clinical response data were not published from this study.

Ajani et al. [60], studied the addition of cisplatin (100 mg/m^2 IV on day 1 of each 28 day cycle) and 5-FU (1000 mg/m^2/day continuous IV infusion on days 1–5) to G17DT in 103 patients with untreated metastatic or unresectable gastric or gastro-esophageal adenocarcinoma as part of an international phase II study. Ninety six patients were evaluable (seven patients were overdosed on 5-FU and were not

analysed), and only 79 were evaluable for response (24 patients were excluded; one patient did not receive therapy according to protocol, and 23 patients did not undergo follow-up assessment). Of these patients, there was a 50% best overall response rate (ORR), but the confirmed ORR was only 30%. In the intent-to-treat (ITT) population, best ORR was 42% and confirmed ORR was 25%. mOS was 9.0 months and was significantly longer in the 90% of patients who had a positive antigastrin antibody titre by ELISA than the 10% who did not (10.3 months vs. 3.8 months; $P<0.0001$) [60]. Grade 3–4 AE were notable for neutropenia (24%), leukopenia (9%), anaemia (12%), febrile neutropenia (7%), thrombocytopenia (5%), nausea (20%), vomiting (20%), stomatitis (19%), diarrhoea (5%), myositis (13%), fatigue (12%), anorexia (12%), dehydration (11%), peripheral neuropathy (9%), and syncope (5%). Survival statistics were comparable with published data on 5-FU/cisplatin regimens, although there was a possible added benefit for immune responders in this trial.

In another phase II study, Gilliam and colleagues [61] randomised 154 patients with advanced pancreatic cancer who were unwilling or unable to undergo chemotherapy to receive G17DT or placebo. mOS was longer in patients who received G17DT (151 days vs. 82 days, $P=0.03$), and those patients who developed antibody responses (73.8% of the G17DT arm) also had longer mOS than those who did not respond, or those taking placebo (176 days vs. 63 days vs. 83 days, respectively; log-rank test, $P=0.003$). However, in the ITT population, mOS was not statistically improved following G17DT administration (150 days vs. 83 days with placebo, $P=0.054$). To summarise, although well tolerated, and with apparently improved survival in immune responders, G17DT effected only a trend toward significantly improved mOS in this population overall.

Finally, a phase II study by Rocha-Lima et al. [62], treated 161 patients with mCRC who had progressed on an irinotecan-containing regimen with G17DT plus irinotecan. In the ITT population, there were very few clinical responses (3% had partial responses (PRs), 32% had stable disease (SD)). Sixty-two percentage of patients had an immune response, and these patients had improved mOS compared with non-responders (9.0 vs. 5.6 months, $P<0.001$) [62].

G17DT showed only modest antitumour efficacy in early phase clinical trials and has thus not been further pursued.

CD55

CD55, decay accelerating factor is a complement regulatory protein overexpressed by cancer cells to prevent them from complement-mediated destruction. 105 AD7 is a human anti-idiotype monoclonal antibody that mimics CD55 and stimulates CD4 and CD8 antitumour responses in patients with appropriate haplotypes (HLA/A1,3,24 and HLA/DR1,3,7) [63]. Interestingly, CD55 is expressed on 70–80% of CRCs [64, 65], and a small neoadjuvant study treated 35 patients with resectable CRC with 105 AD7 before undergoing surgery. Approximately 83% of patients expressing a permissive haplotype had antitumour responses, whereas only 12% of

patients without these haplotypes had a response. Sixty-five percentage of patients were disease-free at a median follow-up time of 4 years [66]. Maxwell-Armstrong and colleagues [67] randomised 162 patients with advanced CRC to 105 AD7 or placebo. Only 50 % of patients received the 3 planned doses of vaccine, and mOS was not prolonged with 105 AD7 (124 days vs. 184 days with placebo, P = 0.38). Finally, Ullenhag et al. [68], randomised 67 patients with resectable CRC 2:1 to neoadjuvant/adjuvant 105 AD7 (with or without BCG) or no treatment. Fifty-three percentage of immunised patients had an immune response on ELISPOT or proliferation assays, but survival was not assessed in this study. Therefore, despite demonstration of immunogenicity, 105 AD7 has not yet been shown to have a survival benefit in CRC.

CD17-1A

CD17-1A (edrecolomab) is an anti-idiotype murine monoclonal antibody that targets the tumour-associated glycoprotein GA733-2 [69]. Early clinical studies using CD17-1A monotherapy in advanced gastrointestinal cancers, and CD17-1A in combination with 5-FU, adriamycin, and mitomycin (FAM) in patients with advanced pancreatic cancer demonstrated that CD17-1A is well tolerated [70, 71]. Two other studies combined CD17-1A with interferon gamma in mCRC and advanced pancreatic cancer but failed to show a clinical benefit or a clear correlation between antibody development and improved survival [72, 73]. A larger phase II trial of CD17-1A monotherapy in advanced pancreatic cancer patients also failed to demonstrate efficacy [74].

Despite these findings, CD17-1A was tested in a large adjuvant CRC trial. A total of 189 patients with fully resected Dukes' stage C CRC were randomised to adjuvant CD17-1A or observation. The overall death rate was decreased by 32 % in the CD17-1A arm compared with no treatment (log-rank P = 0.01), and the recurrence rate was decreased by 23 % (log-rank P = 0.07) [75, 76]. Also, the incidence of distant metastases was significantly reduced in the CD17-1A arm (log-rank P = 0.004) but local recurrences were not (log-rank P = 0.83) [76]. This study demonstrated that CD17-1A might be of clinical benefit in the adjuvant setting.

The addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) to augment antibody-dependent cellular cytotoxicity (ADCC) conferred clinical benefit in a study of 20 patients with mCRC treated with CD17-1A: 2 patients achieved a complete response (CR), one achieved a partial response (PR), and 3 experienced stable disease (SD) for more than 3 months [77]. In another study of 20 patients with mCRC, this protocol was extended to include the addition of IL-2 to CD17-1A and GM-CSF. One patient on this treatment experienced a PR and 2 had SD [78]. Thus, IL-2 did not appear to improve the clinical efficacy of this regimen.

Finally, CD17-1A was studied in a phase III trial as adjuvant therapy in stage II colon cancer [79]. A total of 377 patients were randomised 1:1 to CD17-1A or observation, and the study was terminated early due to discontinuation of drug

supply. Neither mOS nor disease-free survival (DFS) were significantly different between the study groups [79]. Similarly, a study of CD17-1A as adjuvant therapy in 2761 patients with stage III colon cancer randomised patients 1:1:1 to receive 5-FU/leucovorin with CD17-1A, 5-FU/leucovorin alone, or CD17-1A alone. There was no difference in 3-year OS between combination treatment and chemotherapy alone (74.7 % vs. 76.1 %; P = 0.53). Additionally, DFS was significantly reduced following CD17-1A treatment alone compared with chemotherapy alone (53.0 % vs. 65.5 %; P < 0.0001) [80]. Although well tolerated, the addition of CD17-1A did not show any clinical benefit.

To summarise, targeting CD17-1A has yet to show promise in large clinical trials despite early signals of potential benefit in the adjuvant setting for CRC.

GV1001

Telomerase, expressed by 85–90 % of human cancer cells while restricted in normal cells, functions to preserve telomeres at the end of chromosomes, thus promoting immortalisation. GV1001 is a telomerase peptide that therefore specifically targets malignant cells [81, 82]. In a phase I/II study of 48 patients with unresectable pancreatic cancer, 24 of 38 evaluable patients had immune responses, and these immune responders had significantly improved mOS (216 days vs. 88 days, P = 0.0001) [82]. Patients in the intermediate dose group had the longest mOS (260 days) compared with the low dose (119 days, P = 0.006) and high dose groups (153 days, P = 0.05).

A phase II trial of a combination of GV1001, GM-CSF, and cyclophosphamide in 40 patients with advanced hepatocellular carcinoma (HCC) did not demonstrate any objective tumour responses, and only 46 % of patients had SD on this regimen [83].

Finally, a large phase III trial (the TeloVac trial) focused on the treatment of locally advanced or metastatic pancreatic cancer patients with gemcitabine and capecitabine, with or without GV1001/GM-CSF. This trial failed to show an overall survival benefit following the addition of GV1001 to standard chemotherapy [84]. Patients (n = 1062) were randomised 1:1:1 to receive chemotherapy alone, sequential chemotherapy and GV1001/GM-CSF, or concurrent chemotherapy and GV1001/GM-CSF. mOS was not significantly different in the chemotherapy group when compared with the sequential group (7.9 months vs. 6.9 months, HR 1.19, 98.25 % CI 0.97–1.48, P = 0.05) or the concurrent group (8.4 months, HR 1.05, 98.25 % CI 0.85–1.29, P = 0.64) [84].

WT1

Wilms tumour gene (WT1) peptide-based cancer vaccines have been studied in pancreatic and biliary tract cancers. In a phase I study, 32 patients with advanced pancreatic cancer received a WT1 vaccine in combination with gemcitabine [85].

Within the 29 patients who completed 2 cycles of treatment, notable grade 3–4 AEs included neutropenia (44.8 %), leukocytopenia (27.6 %), lymphopenia (27.6 %), anaemia (6.9 %), and non-neutropenic biliary infection (24.1 %). There was one episode of cerebrovascular ischemia, a dose-limiting toxicity. Otherwise, AEs were comparable to those observed in patients receiving gemcitabine alone. Fifty-eight percentage of patients on study developed a delayed-type hypersensitivity (DTH) reaction, which was indicative of an immune response, and responders had improved mOS (10.9 months vs. 3.9 months, log-rank P = 0.003) [85]. A similar phase I study of WT1 peptide vaccine plus gemcitabine in 25 patients with advanced pancreatic or biliary tract cancers was disappointing. Although 59 % of patients had an immune response, this unfortunately did not translate into clinical benefit (mOS was 288 days for biliary cancer patients and 259 days for pancreatic cancer patients) [86]. WT1 vaccines have been extensively studied in a number of cancers, but at this point there are no published phase II or III data for WT1 vaccines in gastrointestinal cancers.

DNA Vaccines

Plasmid DNA Targeting CEA

Plasmid DNA vaccines targeting CEA have been investigated with limited success. In a phase I trial, Staff et al. treated patients with adjuvant CRC with a plasmid DNA vaccine construct containing a modified CEA gene fused to a T helper epitope of tetanus toxoid (CEA 66 DNA) [87]. GM-CSF was also administered, as was cyclophosphamide (to deplete regulatory T-cells). Ten patients received this regimen and only 2 had recurrent disease. The regimen was well tolerated with no evidence of grade 3 or 4 AEs.

Conry and colleagues studied a DNA vaccine encoding both CEA and hepatitis B surface antigen in 17 patients with mCRC [88]. Unfortunately only five patients had SD and none of the patients developed CEA-specific antibodies.

CEA DNA vaccines have not been further explored in clinical trials.

Whole Tumour Cell Vaccines

GVAX/CRS-207

GVAX is a lethally-irradiated allogeneic GM-CSF-secreting whole cell pancreatic tumour vaccine that has been studied alone and in combination with CRS-207, a live-attenuated *Listeria*-based vaccine expressing mesothelin (a cell surface tumour-associated antigen expressed on the majority of pancreatic cancer cells and thought to be implicated in cell adhesion and metastasis) [89, 90].

In a phase I study, Jaffe et al. first demonstrated safety and immune activation in 14 pancreatic cancer patients treated with GVAX in the adjuvant setting, delivered before and after chemoradiation [91]. A single-arm phase II study of 60 patients in the same setting showed a median DFS of 17.3 months (95 % CI 14.6–22.8 months) and mOS of 24.8 months (95 % CI 21.2–31.6 months) [92]. Treatment was well tolerated; the only grade 3–4 AE was eosinophilia, which was observed in two patients.

Cyclophosphamide was added 1 day before GVAX treatments in order to inhibit regulatory T-cells and enhance the anti-tumour immune response. Laheru et al. [93], enrolled 30 patients treated with GVAX alone, and another 20 patients treated with pre-vaccine cyclophosphamide. Although not randomised, patients who received GVAX alone had a mOS of only 69 days, as opposed to 130 days for patients who received cyclophosphamide prior to GVAX administration. Encouragingly, treatment was also relatively well tolerated. To investigate the addition of CRS-207, Le and colleagues [94] randomised 93 patients with pre-treated metastatic pancreatic cancer 2:1 to receive a combination of cyclophosphamide and GVAX or cyclophosphamide, GVAX, and CRS-207. mOS was improved in the CRS-207 arm in both the full analysis (6.1 months vs. 3.9 months; $P = 0.0343$) and per-protocol analysis (9.7 months vs. 4.6 months; $P = 0.0335$). The treatment was well tolerated; the only grade 4 AE in the CRS-207 arm were lymphopenia (two patients). This was the first study to establish a survival advantage using immunotherapy in pancreatic cancer.

Next, investigators attempted to augment this immune response using checkpoint inhibitors. The anti-cytotoxic T lymphocyte-associated antigen-4 (anti-CTLA-4) antibody—ipilimumab—was combined with GVAX in a phase Ib study of 30 patients with previously treated advanced pancreatic cancer [95]. Patients were randomised 1:1 to receive 10 mg/kg IV ipilimumab alone or in combination with GVAX, administered every 3 weeks for 4 doses followed by maintenance dosing every 12 weeks. mOS was improved in the combination arm, but unfortunately, this finding was not statistically significant (5.7 months vs. 3.6 months; $P = 0.072$) [95]. AEs were comparable between the study groups (grade 3–4 events included one episode of colitis in each arm and one episode of pneumonitis in the combination arm).

An ongoing phase II trial (STELLAR) is investigating the addition of nivolumab, a humanised IgG4 anti-programmed cell death protein 1 (anti-PD-1) antibody, to GVAX/CRS-207 and cyclophosphamide in patients with previously treated metastatic pancreatic cancer (NCT02243371). An estimated 108 patients will be randomised 1:1 to receive cyclophosphamide and GVAX/CRS-207 with or without nivolumab (3 mg/kg IV every 2 weeks), we await the results of this trial with interest. [96]

OncoVAX

OncoVAX is a vaccine comprised of autologous tumour cells with the immunomodulating adjuvant BCG. A phase III trial of OncoVAX in stage II and III colon cancer patients in the adjuvant setting randomised 254 patients 1:1 to receive OncoVAX or no further treatment [97]. In the ITT population, there was a trend

toward OS benefit in stage II patients treated with OncoVAX (5-year OS: 82.5 % vs. 72.7 %; HR 0.544, 95 % CI 0.276–1.071; log-rank P = 0.074) but not among stage III patients (63.6 % vs. 70 %; HR 1.168, 95 % CI 0.522–2.469; log-rank P = 0.685) [97]. Given the lack of statistically significant survival benefit, OncoVAX has not been further studied in the stage II and III colon cancer setting.

Algenpantucel-L

Algenpantucel-L is an irradiated whole-cell allogeneic pancreatic cancer vaccine composed of two human pancreatic adenocarcinoma cell lines (HAPa-1 and HAPa-2) with a retrovirally inserted murine α(1,3)-galactosyltransferase (αGT) gene [98]. Expression of the αGT enzyme causes hyperacute rejection with complement and antibody-dependent cytotoxicity [98–102]. In a single-arm phase II study, 73 patients with resected pancreatic cancer received adjuvant algenpantucel-L, gemcitabine, and chemoradiation with infusional 5-FU. Median DFS was 14.1 months, and the treatment was well tolerated with no grade 4 adverse events attributed to algenpantucel-L. This survival outcome compared favourably to historical controls (11.4 month median disease-free survival in the RTOG-9704 trial; adjuvant 5-FU-based chemoradiation plus gemcitabine) [103]. The phase III PILLAR trial of algenpantucel-L in locally advanced pancreatic cancer has completed enrollment of 302 patients, randomised 1:1 to FOLFIRINOX plus algenpantucel-L or FOLFIRINOX alone; however, results have not yet been published (NCT01836432).

Vector-Based Vaccines

A vector-based vaccine approach can take various approaches to DC activation. These agents have in common the expression of, or encoding of, the target antigens. The use of a foreign particle provides a method to initiate the innate immune response, which can then stimulate the adaptive immune response against the specific target, as discussed in specific cases below. There are advantages and disadvantages to each vector, which have to be carefully considered during development of a vaccine and decisions on how it will be used clinically. Specifically, if the vector requires infectious capability to be efficacious, host-neutralising immunity against the vector can be a major limitation. Similarly, underlying hypersensitivity to a vector may also be a limitation.

Yeast Vectors

A yeast platform provides an example of a vector that does not initiate DC infection to trigger activation. Instead, this strategy exploits the underlying innate immunity against yeast particles to drive DC and then T cell activation. The transgene for the target antigen is transfected into yeast; the yeast cells are then grown in culture until

they express the target antigen, after which they are heat-killed. This results in a non-infectious delivery mechanism for the target antigen that will be engulfed by DCs and trigger signalling through TLRs in a pathogen-associated molecular pattern (PAMP) [104]. The activated DCs then produce type I cytokines, including IL-12 and TNF-α, and induce T cell activation against the antigens they are presenting, which include the tumour-associated antigen (TAA) target of the vaccine. In preclinical models, yeast-based vaccines have been seen to induce activated T cells [105] that are capable of killing tumour cells expressing the TAA *in vitro*, which was found to slow tumour growth and improve OS in murine models [106].

Multiple clinical trials of yeast-based vaccines demonstrated the safety of the platform and the ability to generate specific T cell responses against a variety of target antigens, including KRAS (GI-4000), CEA (GI-6207), and brachyury [107–109], a transcription factor involved in the epithelial to mesenchymal transition in many adenocarcinomas [110–112]. The RAS, CEA, and brachyury targets are all potentially applicable in GI malignancies. In a phase 1 clinical trial of the yeast-brachyury vaccine, 17 of 31 evaluable patients developed brachyury-specific CD4 and/or CD8 T cell responses post-vaccination, and the proportion of responders increased at the higher dose levels (5 of 6, 83%, at the highest dose level) [113]. These immune responses were observed in the context of minimal AEs, most commonly injection site reactions; no dose-limiting toxicities (DLTs) were observed. Clinical efficacy of these agents, as with most vaccine platforms, is an area of ongoing investigation.

Viral Vectors

A commonly employed platform for therapeutic cancer vaccines is a genetically modified virus. These viral vectors typically have been genetically modified to reduce the infection risk and immunogenicity of the viral particle to allow for multiple safe administrations, and increase the likelihood of a targeted T cell response against the target antigen. Modified viral vectors are commonly altered to include the transgene for the target protein, resulting in translation of that transgene into expression of the target protein within the infected cell. In most cases, the target of infection is a subcutaneous DC. Once infected, the DC will translate the full-length protein and then present it via MHC. Some viral vectors also include mechanisms to help activate the DC, triggering migration to the draining lymph node and presentation of the target antigen in combination with costimulatory molecules, providing signal 1 and signal 2 to drive T cell activation against the target antigen [114].

Poxviral Vectors

The poxviral platform has a long history in development as therapeutic cancer vaccines. Initial studies were performed using recombinant vaccinia (rV) across a variety of target antigens. rV-CEA was the first vaccinia vector tested in humans,

including patients with GI malignancies. Evaluation of the rV platform demonstrated that host neutralising immunity of the vaccinia vector limited its applicability for booster dosing [115]. Hodge et al. later demonstrated that priming with vaccinia followed by booster dosing with replication-incompetent poxviral vectors, including canarypox and fowlpox, allowed for administration of multiple booster doses, resulting in greater T cell activation and persistence in preclinical models [116]. This prime and boost strategy proved to be effective in a clinical trial conducted by Marshall and colleagues using rV-CEA followed by avipox-CEA boosts [117]. Present strategies include further refinement of the vaccinia priming dose to minimise safety risk. To that end, a modified version of vaccinia, called MVA (modified vaccinia Ankara) has become the priming dose of choice in studies of poxviral vaccines [118].

Breaking Tolerance via Costimulation

The majority of recent immunotherapy successes have come from the recognition and targeting of inter-cellular cross talk between immune cells, both stimulation and inhibition of the immune cascades. The poxviral platform is unique in its flexibility to include the transgenes for costimulatory molecules capable of driving a more optimal T cell response. The three co-stimulatory molecules, called TRICOM, are B7.1, intracellular adhesion molecule (ICAM-1), and lymphocyte function-associated antigen (LFA-3). Preclinical and clinical studies have demonstrated the effects of the inclusion of TRICOM in this platform with various tumour antigens [116, 117, 119–121]. The platform includes a vaccine that targets CEA and MUC-1 (previously called PANVAC, now called CV-301, Bavarian Nordic) [121, 122], which may be an ideal vaccine to consider in GI cancers. This is because GI cancers commonly overexpress both of these tumour-associated antigens. We, and others have demonstrated the safety and immune stimulation of these viral constructs, supporting ongoing larger clinical trials [123]. An additional target, brachyury, has been linked to prognosis in colorectal [124, 125] and hepatocellular carcinoma [126], and is the target of an ongoing phase 1 study using this platform with initially reported good safety profile and immune response data [127].

Adenovrial Vectors

Another viral platform employs an adenovirus genetically modified to cause replication incompetence and limit host-neutralising immunity against the vector [128]. This vector has been evaluated in preclinical [129, 130] and clinical studies [131], and has been demonstrated to be safe and capable of inducing T cell specific responses both in models and in humans. Notably, CEA-specific responses were generated even in patients with known anti-adenoviral immunity using the

CEA-targeting adenoviral vaccine (called Ad-CEA) [131]. This platform is safe, immunogenic, and relatively easy to manufacture. Further clinical evaluation is ongoing to determine efficacy in gastrointestinal tumours.

Ex vivo Dendritic Cell Vaccines

DCs are critical to initiating the immune response and much work has gone into understanding how to harvest, train, and re-administer antigen pulsed DCs as cancer therapy. Morse et al. have performed a series of elegant clinical translational experiments demonstrating the feasibility of this approach, including a phase 2 randomised trial comparing the DC-based vaccine approach with the viral vector approach described above. First, their team performed a series of phase 1 studies of active immunotherapy with CEA peptide (CAP-1)-pulsed, autologous human cultured dendritic cells and then with dendritic cells modified with fowlpox encoding CEA and costimulatory molecules in patients with metastatic malignancies expressing CEA [132, 133]. Having demonstrated safety and encouraging efficacy, the group then led a national randomised phase II study of immunisation with dendritic cells modified with poxvectors encoding CEA and MUC-1, compared with the same poxvectors plus GM-CSF for resected mCRC. The outcomes for both patient groups were better than an unvaccinated historical control, and were comparable with each other. This suggested that the more cumbersome and expensive technique of DC based immune therapy was not superior to the viral based treatment. As a result further DC work was abandoned [134]. Significant work using adoptive T cell therapy remains but is outside the scope of this chapter.

Agonist Epitopes

As previously mentioned in this chapter, one strategy to increase the effectiveness of a therapeutic cancer vaccine is to include mechanisms within the vector that will enhance costimulation. In the yeast platform, for instance, the foreign antigens serve to activate DCs through TLR signalling. In viral vectors, encoding the transgenes for costimulatory molecules is a novel approach capable of driving T cell activation more efficiently [120]. The poxviral vectors encoding TRICOM for example, which are expressed by the infected DCs, provide the second signal for T-cell activation in conjunction with the MHC-presented antigen. Therapeutic cancer vaccines are different from vaccines developed for prevention of infectious diseases because they are required to overcome self-tolerance. The addition of costimulatory molecules within a vector platform appear to improve the likelihood of overcoming tolerance and generating activated T cells capable of killing tumour cells [116].

Pathogen-Associated Molecular Patterns (PAMPs)

The innate immune system, in addition to the acquired immune system, has been the focus of recent scrutiny in cancer immunotherapy. Inflammation has been linked with the various phases of tumour development, from initiation to progression and perhaps resolution. Human malignancies have been demonstrated to have sterile chronic inflammation, similar to the inflammation that follows microbial infections, once the infective agent has been cleared [135]. The risk of neoplastic transformation resulting from chronic inflammatory states have been well documented as in the case of inflammatory bowel disease leading to the development of adenocarcinoma of the colon [136]. Many chronic infections have also been linked to multiple malignancies. On the flip side, inflammatory changes and immune cell infiltration of the tumours have been shown to correlate with effectiveness of immunotherapy in the resolution of metastatic carcinomas [137, 138].

Pattern recognition receptors (PRR) are activated by certain pathogenic molecules with specific molecular patterns, often called pathogen-associated molecular patterns (PAMPs). Such receptors that bind to PAMPs include, but are not limited to, TLRs, NOD-like receptors, RIG-like receptors, and C-type lectin receptors. The widely recognised TLRs bind to a wide variety of PAMPs and stimulate the innate immune system. Widely recognised PAMPs include lipopolysaccharide (LPS), a molecule common to Gram-negative bacteria; peptidoglycan and muramyl dipeptide; viral RNA; β-glucans; cytosolic bacteria; and viral DNA [139, 140]. The general immune response to PAMPs includes the upregulation of MHC and costimulatory molecules, and the production of inflammatory cytokines, resulting in the recognition and clearance of the pathogen associated with the PAMP. One major downstream effect of the binding of PAMPs to PRR is the activation of NF-kB, which is a transcription factor associated with septic and aseptic inflammation and promotion of oncogenesis [137]. The earliest insight for the role of PAMPs in cancer treatment dates back to 1891 when Coley injected live cultures of *Staphylococcus pyogenes* into a surgically unresectable sarcoma, resulting in an infection and complete resolution of the tumour [141]. It is unclear where the distinction between the tumour suppressive effects of the immune system and the inflammatory microenvironment that facilitates tumour growth lies, as there is a significant overlap of inflammatory mediators. PAMP-mediated uncontrolled TLR signalling in cancer provides a microenvironment that enables tumour cells to proliferate, while at the same time enhancing the immune response against it [142].

The interaction of PAMPs with their respective PRRs and other molecules is believed to potentiate the innate immune response to danger-associated molecular patterns (DAMPs) and the specific immune response to tumour-associated antigens [141, 143].

Damage-Associated Molecular Patterns (DAMPs)

The process of tissue growth and regeneration in the human body involves replacement of continually dying cells with new ones. Apoptotic cell death often leads to the secretion of transforming growth factor (TGF)- β, interleukin (IL)-10, and prostaglandin E2; all of which dampen inflammatory response [144–146]. On the contrary, cell death that occurs after pathogen exposure often triggers an inflammatory reaction that will help in the containment of the inciting agent and the repair of the resulting tissue injury.

The release of pro-inflammatory molecules by dying cells has been shown to activate dendritic cells, which in turn promote T cell response to antigens released by the dying cells. Such pro-inflammatory molecules include uric acid, free extracellular DNA and RNA, spliceosome-associated protein 130 (SAP130), high mobility group box 1 (HMGB1), high mobility group nucleosome binding domain 1 (HMGN1), H_2O_2, extracellular ATP, heat shock protein (hsp), and many more. As these molecules are released in response to tissue damage, they are often referred to as damage associated molecular patterns (DAMPs). A multitude of DAMP receptors have been identified, but not all have been shown to mediate activation of DCs or be an adjuvant to other immunogenic molecular patterns [147].

TLR engagement is a central mechanism by which immune cells are constantly activated, and it plays a central role in a host of autoimmune and inflammatory disorders. The TLR bind to PAMPs, DAMPs, or complexes of the two, and cause variable downstream effects, in which there is significant overlap [148].

Some DAMPs induce changes in DCs that are similar to those observed in response to PAMPs, including the up-regulation of MHC and costimulatory molecules, and production of inflammatory cytokines that promote antigen acquisition and presentation. The activation of TLR receptors 7, 8 and 9 by self-DNA and RNA has been shown to activate dendritic cells (DC) [147, 149].

Several reports have emphasised that the mode of cell death can profoundly influence the subsequent availability of antigens for cross-priming in that induction of autophagy prior to cell death is more potent in facilitating antigen delivery to DCs [150, 151]. Additionally, caspase-mediated cleavage of cellular proteins during apoptosis has been shown to generate neoantigens that act as efficient substrates for DC presentation, and may lead to priming of reactive CD8+ T cells [152]. The recognition of DAMPs by the innate cell population is a key part of the cancer immune response. One of the major DAMP-driving host antitumour immune responses is tumour-derived DNA, sensed by the stimulator of interferon gene (STING) pathway and driving type I IFN production [153]. The release of DAMPs from cellular injury resulting from radiation, chemotherapy, or biological therapy may augment the presentation of tumour antigens by DCs, and therefore antitumour immunity [141].

Understanding the contribution of DAMPs and PAMPs to cancer immunology helps in the design of better clinical trials that may increase success of immunotherapy

in gastrointestinal malignancies [154, 155]. We are not aware of any specific cancer treatment modality that targets these molecular patterns (PAMPs and DAMPs) currently undergoing clinical testing.

Vaccination Combined with Other Immunotherapies

Immunotherapy has become an area of interest in the broader oncology research community due to the activity of therapies that bind to and block the signalling of immune checkpoints, including CTLA-4 and PD-1/L1. These agents have demonstrated the ability to induce tumour shrinkage and improve OS in melanoma [156–159] and lung cancer [157, 160], with further evidence of clinical benefit in a variety of other solid tumours. However, with the exception of tumours with microsatellite instability, checkpoint inhibitors have shown minimal activity in GI cancers [161]. It has been hypothesised that this lack of response may be due to a relatively small number of mutations in GI tumours, which limits the potential number of antigens that would be seen as foreign. Other hypotheses include the ability of GI tumours to create a disruptive tumour microenvironment for T cell penetration and killing [162]. In either case, it may be possible to overcome these barriers by using therapeutic cancer vaccines that are capable of inducing specific T cell activation against overexpressed tumour-associated epitopes such as CEA, MUC-1, and others. Other agents may also be capable of affecting the tumour in a way that allows better T cell activation and killing, including inhibition of negative regulatory molecules such as transforming growth factor-beta (TGF-beta), interleukin-8 (IL-8), and Indoleamine 2,3-dioxygenase (IDO), amongst others. Ongoing clinical trials with these agents will determine the safety of their use, and subsequent trials in combination with vaccines and checkpoint inhibitors will answer the question of whether combination therapy in GI cancers can be effective.

Vaccination Combined with Standard Therapy

While some inhibitory factors within the tumour microenvironment may need to be addressed with novel interventions, standard therapies may offer another potential avenue to overcome these issues (Fig. 3.3). Some of the most commonly used agents in GI cancer have been demonstrated to have immunogenic effects *in vitro* and in animal models [163–169]. Oxaliplatin and radiation are known to be excellent inducers of a process dubbed "Immunogenic Cell Death," in which tumour cells die in a fashion that causes DC activation and therefore T cell activation against the antigens released from within [168, 169]. Radiation and many chemotherapy agents, including taxanes, 5-FU, and platinums are capable of inducing "Immunogenic Modulation," a process in which tumour cells that are not killed by cytotoxic agents are phenotypically altered making them more amenable to T cell mediated killing [166, 167]. Gemcitabine has been shown to have immunomodulatory effects on the tumour microenvironment, while inducing specific T cell activation in pancreatic

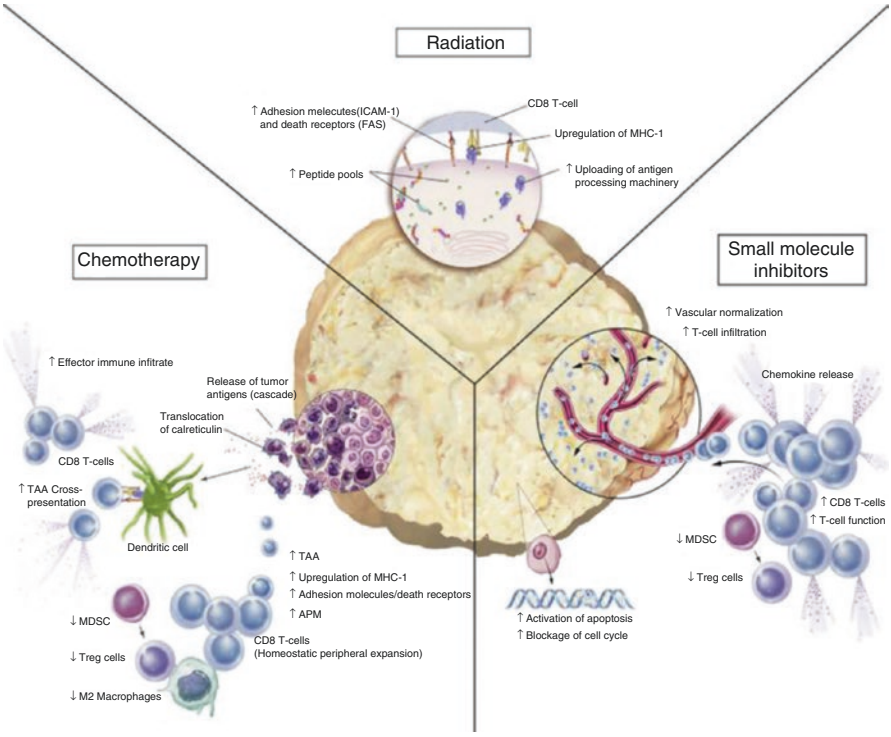

Fig. 3.3 Vaccine combination with standard therapy (Taken from Hodge et al. [170])

cancer [163–165]. Due to the complexity of the immune response, careful modelling of standard agents in combination with novel immunotherapeutics is required to yield maximal benefit for patients prior to initiating clinical trials.

Summary

Great advances in immune therapy have been made in the past decade and we anticipate more to come. Through the combination of immune stimulatory agents such as the vaccines, reviewed in this chapter, with novel immune modulating agents such as checkpoint inhibitors and other anti-cancer agents, we anticipate further benefit for more GI cancer patients in our future.

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Chapter 4
Antibody Drug and Radionuclide Conjugates for GI Cancers

Beverly A. Teicher

In the mid-1980's, after it was found that patients rapidly developed neutralising antibodies to tumour-directed 'therapeutic mouse antibodies', the concept of using these mouse antibodies to deliver cytotoxic therapeutics emerged. However, success was limited. Anti-mouse antibodies developed even with a limited number of infusions of the antibody-drug conjugates, and often the cytotoxic agents were released in the blood stream producing serious toxicities. The field of antibody-conjugates was quiet while technology for humanisation of mouse antibodies and the means of producing fully human antibodies evolved, and linker chemistry was developed, which allowed stable transport of antibody-conjugates to the target cells and release of the cytotoxic agent inside the target cells (Fig. 4.1) [1–3]. The first antibody-drug conjugate to reach Food and Drug Administration (FDA) approval in 2000 was gemtuzumab ozogamicin (Mylotarg), which targeted CD33 on the surface of acute myeloid leukaemia cells and delivered the potent cytotoxic agent, calicheamicin, inside the leukaemia cells. In 2010, gemtuzumab ozogamicin was withdrawn from the market after failing to produce sufficient efficacy in follow-up clinical trials [4]. Two antibody-drug conjugates (ADCs), trastuzumab emtansine (T-DM1; Kadcyla) and brentuximab vedotin (SGN-35; Adcetris) reached FDA approval in 2014 and 2011 for treatment of metastatic breast cancer and refractory Hodgkin lymphoma and systemic anaplastic large cell lymphoma, respectively [5, 6]. Approximately 50 ADCs have reached clinical trial to date, with more than 40 in trials currently and nearly 20 in, or having completed Phase 2 clinical trials (Table 4.1). The ADCs under investigation target haematological and solid tumours [7, 8]. The current chapter focuses on those that may be useful in the treatment of GI cancers.

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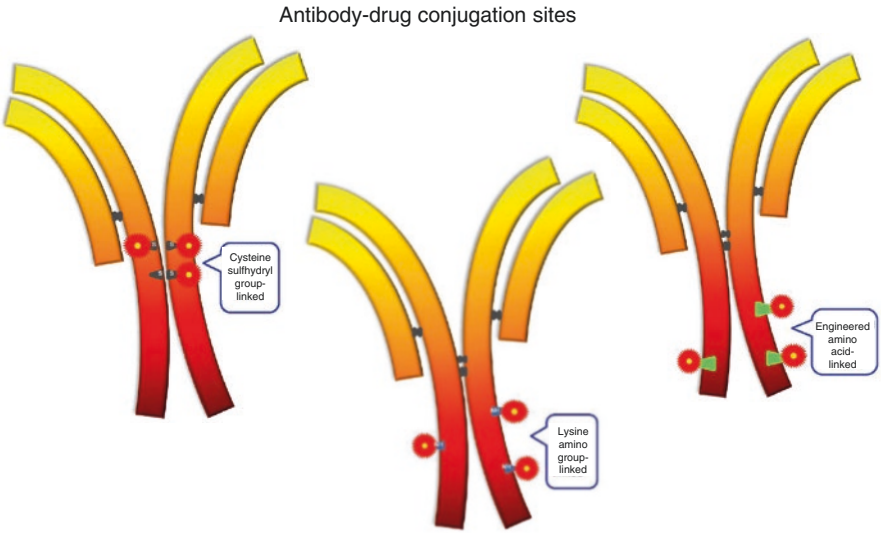

Fig. 4.1 Structure of antibody-drug conjugates. Highly potent small molecule drugs are frequently linked to antibodies via a short linker molecule covalently bound to the protein by breaking the disulphide bonds between the heavy chains of the antibody; or by binding to accessible epsilon amino groups of lysines; or via unnatural amino acids genetically engineered into specific sites of the antibody protein

Currently, there are three 'naked' antibodies that have reached FDA approval for treatment of colorectal cancer; anti-EGFR antibodies, cetuximab and panitimumab, and anti-VEGF, bevacizumab. 'Naked' antibodies targeting EpCAM, DR5, IGFR, HER2, Lewis-Y as well other cell surface targeting antibodies are in clinical trial along with anti-CTLA-4 and anti-PD-1 antibodies to stimulate immune system response to the cancer [9]. Several studies have extensively characterised gene expression and cell surface antigen expression of colorectal cancers providing additional potential antibody targets in these diseases [10–12]. Cetuximab, trastuzumab and bevacizumab have been tested in oesophageal and gastric cancer clinical trials [13]. The addition of cetuximab to chemotherapy did not alter the outcome compared to chemotherapy alone. Trastuzumab plus chemotherapy resulted in a more positive outcome than chemotherapy alone and bevacizumab plus chemotherapy improved overall response rate and progression free survival but not overall survival compared with chemotherapy alone. Trastuzumab in combination with platinum and 5-fluorouracil is approved for first-line therapy in patients with HER2 positive gastric cancer and ramucirumab, anti-VEGFR2, is approved for treatment of patients with metastatic gastric cancer [14, 15]. Hepatocellular cancer is rising world-wide and treatment remains difficult [16]. Recently, glypican-3 (GPC3) was identified as a potential antibody target for liver cancer. Currently, GPC3 CAR T cells are in early clinical trial in hepatocellular carcinoma [17, 18]. Pancreatic cancer remains one of the greatest therapeutic challenges [19]. Several antibody therapeutics including anti-mesothelin, anti-HER2, anti-EGFR and antibody combinations have been

Table 4.1 ADCs in clinical development, previously in clinical development and nearing clinical development

Conjugate name	Target	Status	Company
Gemtuzumab ozogamicin, Mylotarg	CD33	Withdrawn	Pfizer
Trastuzumab emtansine, Kadcyla	HER2	Approved	Roche-Genentech
Brentuximab vedotin, Adcetris	CD30	Approved	Seattle Genetics-Takeda
Inotuzumab ozogamicin; CMC-544	CD22	Phase 2	Pfizer
Glembatumumab vedotin; CDX-011	GPNMB	Phase 2	Celldex Therapeutics
Coltuximab ravtansine; huB4-DM4	CD19	Phase 2	Immunogen
Lorvotuzumab mertansine, IMGN901	CD56 (NCAM)	Phase 2	ImmunoGen
IMGN529	CD37	Phase 2	ImmunoGen
PSMA ADC 2301; MLN-2704	PSMA	Phase 2	Progenics and Takeda
Pinatuzumab vedotin; RG7593	CD22	Phase 2	Roche
Mirvetuximab sorvatansine; IMGN853	FOLR1	Phase 2	ImmunoGen
Denintuzumab talirine;SGN-19A	CD19	Phase 2	Seattle Genetics
IMMU-132	Trop-2	Phase 2	Immunomedics
Labetuzumab-SN-38; IMMU-130	CEACAM5	Phase 2	Immunomedics
Indatuximab ravtansine; BT-062	CD138 (syndecan-1)	Phase 1/2	Biotest
Polatuzumab vedotin; RG7596	CD79b	Phase 1/2	Roche
Milatuzumab-DOX; hLL1-DOX	CD74	Phase 1/2	Immunomedics
MLN0264	Guanylyl cyclase	Phase 1/2	Millenium-Takeda
BAY 94-9343 & BMS-986148	Mesothelin	Phase 1/2	Bayer Pharma & BMS
HuMax-TF	Tissue Factor	Phase 1/2	Genmab
Vadastuximab talirine; SGN-CD33A	CD33	Phase 1/2	Seattle Genetics
SAR566658, huDS6-DM4	CA6 (Muc1)	Phase 1	Sanofi
SGN-CD70A	CD70	Phase 1	Seattle Genetics
AMG-595	EGFRvIII	Phase 1	Amgen
AMG-172	CD70	Phase 1	Amgen

(continued)

Table 4.1 (continued)

Conjugate name	Target	Status	Company
AGS-15ME	SLITRK6	Phase 1	Agensys/Astellas
AGS-16C3F	ENPP3	Phase 1	Agensys/Astellas
Vandortuzumab vedotin; RG7450	STEAP1	Phase 1	Roche
GSK2857916; J6M0-mcMMAF	BCMA(B-cell maturation antigen)	Phase 1	GSK
Lifastuzumab vedotin; RG7599	SLC34A2 (NaPi2β)	Phase 1	Roche
LY3076226	FGFR3	Phase 1	Eli Lilly and Company
SC16LD6.5	Dll3	Phase 1	Stem CentRx
SGN-LIV1A	LIV1 (ZIP6)	Phase 1	Seattle Genetics
SAR408701	CEA CAM5 (CD66e)	Phase 1	Sanofi
PCA062	P-cadherin	Phase 1	Novartis
RG7841	Ly6E (lymphocyte antigen 6 complex, locus E)	Phase 1	Roche
AGS-5ME	SLC44A4	Phase 1	Agensys/ Astellas
Enfortumab vedotin; AGS-22ME	Nectin-4	Phase 1	Astellas
RG7986		Phase 1	Roche
DS-8201	HER2	Phase 1	Daiichi Sankyo
IMGN-242	CanAg	discontinued	ImmunoGen
IMGN-388	Alpha v-integrin	discontinued	ImmunGen
RG7458; RG7882	MUC16/CA125	discontinued	Roche
BAY79-4620	CAIX	discontinued	Bayer
MEDI-547	EphA2	Phase 1 terminated	MedImmune
Bivatuzumab mertansine; BIWI1	CD44v6	Phase 1 terminated	Boehringer Ingelheim
PF-06263507	5 T4	Phase 1 terminated	Pfizer
IMGN-289	EGFR	Phase 1 terminated	ImmunoGen
IMGN-779	CD33	preclinical	ImmunoGen/Sanofi

tested in clinical trial in pancreatic cancer without success [20, 21]. Preclinically, an anti-EpCAM-amanitin antibody-drug conjugate was very effective in treatment of human pancreatic cancer xenografts [22].

TACSTD2/TROP2 and IMMU-132

Trophoblast antigen 2 (Trop2) is the transmembrane glycoprotein encoded by the *TACSTD2* gene. Trop2 is a type 1 transmembrane protein with a large N-terminal extracellular domain with N-linked glycosylation, a transmembrane domain and a

cytoplasmic tail and is involved in intracellular calcium signal transduction. Trop2 is a GA733 protein family which includes GA733-1 (Trop2) and GA722-2 (EpCAM). Several signalling pathways have been implicated in the function of Trop2; however, its exact role in the cell remains under investigation [23–25]. To make Trop2 a good antibody drug conjugate target, it is not essential to fully understand the function of Trop2 in cells, however, it is essential for Trop2 to be abundantly expressed by the targeted malignant diseases, that it be minimally or not expressed by critical normal tissues and finally, that the Trop2:antibody drug conjugate complex be internalised into target cells upon binding of the antibody to its epitope.

Stepan et al. [26] evaluated the transcription and translation of Trop2 in human normal and tumour tissues by RT-PCR and immunohistochemistry, respectively, and identified that Trop2 is overexpressed in some carcinomas relative to the corresponding normal tissue. Crucially, however, Trop2 is highly expressed at both the transcript and protein levels by several essential normal tissues. Trop2 is expressed by carcinomas of the breast, cervix, colon, oesophagus, lung, ovary, pancreas, prostate, stomach, thyroid, urinary bladder, and uterus. Immunohistochemical staining showed a strong plasma membrane pattern or a mixed membrane and cytoplasmic pattern indicating that Trop2 may be an appropriate target for an ADC. While there was strong expression on ovarian, colon, and thyroid carcinoma specimens, there was little to no expression in the corresponding normal tissue. However, there was also strong immunohistochemical staining by normal tissues including prostate, cervix, lung, breast, uterus, kidney, skin, pancreas and liver, salivary gland, and oesophagus. The Trop2 immunohistochemical staining in carcinoma of the breast, cervix, oesophagus, lung, pancreas, prostate, and uterus was similar in intensity to the corresponding normal tissue. Trop2 is overexpressed in colon and colorectal cancer [27]. Trop2 expression leads to decreased survival in colon cancer. High expression can indicate poor prognosis in colon cancer. Trop2 is a significant predictor of poorer patient survival and relates to the chance of disease recurrence and liver metastasis in colon cancer. Expression in left-sided colon cancer is much higher than in right-sided colon cancer and may, thus, be a potential independent prognostic factor [28]. Trop2 expression is highly expressed in colorectal tumours and is associated with an unfavourable outcome [29].

Trop2 expression was detected on oesophageal carcinomas. Protein levels were much higher in oesophageal squamous cell carcinoma lines than in normal tissues and are notably higher in mild hyperplasia of oesophageal mucosae. Trop2 was overexpressed in gastric cancer. Trop2 was an independent prognostic marker for disease recurrence in the intestinal type gastric cancer. In gastric cancer in Chinese populations, Trop2 gene expression was higher in gastric cancer than in adjacent normal tissue [28]. In these patients, high Trop2 expression was associated with poor survival. In intestinal-type gastric cancer, Trop2 expression was correlated with shorter disease-free survival. In a gallbladder cancer immunohistochemical study, Trop2 expression was significantly correlated with histologic grade, tumour stage, lymph node metastasis and loss of the epithelial marker E-cadherin [30]. Overall, Trop2 overexpression was predictive of overall survival and poor disease-free survival in lymph node positive patients.

Trop2 was overexpressed in pancreatic cancer. High Trop2 expression was correlated with the development and malignancy of pancreatic cancer. Trop2 was associated with poor prognosis and could be a novel prognostic biomarker [31]. Given the Trop2 protein expression pattern, it may be important to select patients most likely to benefit from a Trop2 ADC using a diagnostic test to identify patients with very high Trop2 expressing tumours. The GI cancer patients eligible for treatment with a Trop2 ADC will most likely have had surgery to remove the primary tumour and may have had prior chemotherapy and/or radiation therapy. It would be important to confirm Trop2 expression in metastatic disease.

Therapeutics Targeting Trop2

hRS7 is a humanised monoclonal anti-Trop2 antibody that was developed from the RS7-3G11 murine monoclonal antibody. The murine monoclonal antibodyRS7-3G11 is an IgG_1 with pancarcinoma reactivity. RS7-3G11 was raised against a crude membrane preparation derived from a surgically removed human primary lung squamous-cell carcinoma [32, 33]. Early studies demonstrated the efficiency of tumour targeting and the efficacy of RS7-3G11 in nude mice bearing human MDA-MB-468 breast carcinoma. The tumour:non-tumour ratios of RS7-3G11 were 1.9–2.1 times higher than those for Ag8 (the control antibody) on day 14, except in the heart. Radioimmunotherapy of nude mice bearing MDA-MB-468 100 mm^3 xenografts using 250 pCi of ^{131}I-labelled RS7-3G11 resulted in the disappearance of tumours in 6 of 10 animals at 2 weeks postinjection [34]. Humanised RS7 (hRS7) was developed by standard procedures. The genes encoding Vk and VH sequences of murine RS7 were cloned by RT-PCR and the sequences were determined. A chimeric RS7 (cRS7) IgG containing human light and heavy chain constant region domains was generated and shown to have comparable binding specificity and affinity as murine RS7. The V genes of humanised CDR-grafted RS7 antibody were then engineered by a combination of long DNA oligonucleotide synthesis and PCR. hRS7 was expressed in Sp2/0-Ag14 cells. A high hRS7-producing clone was developed [35].

Numerous hRS7 drug and radionuclide conjugates have been prepared. Primary ovarian tumours with high Trop2 expression, both serous or clear cell histology, are highly susceptible to hRS7-mediated antibody-dependent cellular cytotoxicity (ADCC) in the presence of effector cells in cell culture. Although clinically, these tumour cells are resistant to multiple standard cytotoxic therapies, in culture they were sensitive to lysis by natural killer cells when exposed to hRS7. Negligible cytotoxicity against chemotherapy-resistant ovarian cancers was seen in the absence of hRS7 or in the presence of a rituximab control [36]. Two SN-38 derivatives, CL2-SN-38 and CL2A-SN-38, were conjugated to hRS7. The hRS7 conjugates of the two SN-38 derivatives were equivalent in drug substitution (about 6 SN-38 molecules per antibody molecule), cell binding ($K_d = 1.2$ nmol/L), cytotoxicity ($IC_{50} = 2.2$ nM), and serum stability in vitro ($t_{1/2} = 20$ h). Antitumour effects were produced by

hRS7-SN-38 at nontoxic doses in nude mice bearing Calu-3 non-small cell lung carcinoma, Capan-1 pancreatic carcinoma, BxPC-3 pancreatic carcinoma, and COLO 205 colon carcinoma when compared to non-targeting control ADCs. Mice tolerated a dose of 2 × 12 mg/kg SN-38 equivalents with short-lived elevations in ALT and AST liver enzymes [37].

hRS7 conjugated with the active metabolite of irinotecan, SN-38 was designated sacituzumab govitecan (IMMU-132). In IMMU-132 a moderately stable carbonate bond is used to couple SN-38 to the linker-antibody. A pharmacokinetic study of IMMU-132 in mice indicated a mean residence time of 15.4 h. IMMU-132 treatment of mice bearing human gastric cancer xenografts (17.5 mg/kg; twice weekly × 4 weeks) resulted in antitumour effects compared to that of mice treated with a non-specific control. Clinically relevant dosing schemes of IMMU-132 administered either every other week, weekly, or twice weekly in mice bearing human pancreatic or gastric cancer xenografts demonstrated similar, antitumour effects in both models [38, 39]. The delivery of SN-38 to Trop-2–expressing tumours and to several normal tissues was assessed in nude mice bearing human Capan-1 pancreatic cancer and NCI-N87 gastric cancer xenografts after a single injection of irinotecan (40 mg/kg /mouse) or IMMU-132 with an average of 7.6 molecules of SN-38/per antibody molecule. In serum, the mice cleared >98 % irinotecan within 5 min; peak levels of SN-38 and SN-38G (glucuronidated SN-38) were detected in equal amounts at this time, and were below the limit of detection at 6–8 h post administration. IMMU-132 was detected in the serum over 3 days, and at each interval, >95 % of SN-38 was bound to the antibody. Intact IMMU-132 cleared with a half-life of 14 h. The IgG protein portion of the conjugate cleared with a half-life of 67.1 h. Area under the curve analysis indicated that IMMU-132 delivers 20-fold to as much as 136-fold more SN-38 to tumours than irinotecan, with tumour:blood ratios favouring IMMU-132 by 20- to 40-fold [40, 41].

A Phase I/II clinical trial (ClinicalTrials.gov, NCT01631552) was initiated in patients with diverse epithelial cancers, administering IMMU-132 by intravenous infusion on days 1 and 8 of 21-day treatment cycles. Treatment was continued based on tolerance or until disease progression. Phase I was a dose escalation study; a total of 23 assessable patients were given 8, 10, 12, or 18 mg/kg of IMMU-132 and dose-limiting neutropenia was found to occur at 18 mg/kg. For Phase II, enrollment was expanded to multiple cycles of 8 and 10 mg/kg. Safety data from 123 patients given 8–10 mg/kg showed multiple adverse events, including grade 3 neutropenia (National Cancer Institute's Common Terminology Criteria for Adverse Events, NCI CTCAE), febrile neutropenia, and diarrhoea. Other G3 toxicities included anaemia, fatigue, leucopenia, vomiting, and asthenia. Encouragingly, no patients developed antibodies to the ADC. Among assessable patients that is, patients who completed the therapeutic regimen, 15 patients with a median of 3 prior therapies, 2 had partial responses and 7 (46 %) has stable disease for a median of 5.0 months. All 3 gastric carcinoma pts had stable disease. Of 14 pancreatic cancer patients with a median of 2 prior therapies, 7 (50 %) had stable disease for a median of 3.4 months. In 26 colorectal cancer patients with a median of 4 prior therapies, there was 1 partial response, 14 (54 %) stable disease. Repeated cycles of IMMU-132 monotherapy

were well tolerated. Objective responses in oesophageal cancer and extended stabilisation in colorectal cancers were encouraging (NCT01631552; [42–46]). In the same clinical trial, 58 patients with metastatic triple negative breast cancer with a median of 6 prior lines of therapy, were treated with IMMU-132. The tumour response occurred in 30 % of 56 metastatic triple negative breast cancer patients, including 2 with complete response, and a clinical benefit ratio of 46 % (57 % with >4 months), with 20 patients continuing treatment after 1st assessment. The median progression free survival was 7.0 months in 40 patients treated at the 10 mg/kg dose, at 50 % maturity. Immunohistochemistry in archival specimens currently showed 97 % Trop-2 positivity among 34 specimens evaluated (79 % had 2+/3+ staining [47, 48].

Next steps include determining whether IMMU-132 can be incorporated in combination therapy regimens. Preclinically, IMMU-132 was combined with microtubule inhibitors (paclitaxel or eribulin mesylate) or a poly (adenosine diphosphoribose) polymerase (PARP) inhibitor (olaparib) in mice bearing human triple negative breast cancer xenografts. Mice bearing human MDA-MB-468 or HCC1806 triple negative breast cancer xenografts were treated with paclitaxel (15 mg/kg weekly × 5 weeks) and IMMU-132 at either 10 mg/kg or 12.5 mg/kg on days 1, 8, 22, and 29 or with olaparib (50 mg/kg, qdx5d, × 4 weeks; 33 % of human dose equalling 800 mg daily) and IMMU-132 (10 mg/kg, twice weekly × 4 weeks). Mice bearing HCC1806 tumours were treated for 2 cycles with IMMU-132 (12.5 mg/kg) and 0.5 mg/kg of eribulin mesylate weekly for 2 weeks on a 21-day cycle. Mice with MDA-MB-468 tumours treated with IMMU-132 and paclitaxel exhibited >11-fold tumour shrinkage, in comparison to 1.4-fold shrinkage in the IMMU-132 alone group or 11.4-fold increase in tumour size in mice treated with paclitaxel alone. In the HCC1806 bearing mice, the combination treatment regimen improved survival to 38 days from 17.5 to 17.0 days for paclitaxel and IMMU-132 alone, respectively. Mice treated with the combination of IMMU-132 plus eribulin mesylate had a median survival of 23 days compared to eribulin (18 days) or IMMU-132 (14 days) as single agents. Combining IMMU-132 therapy with olaparib was superior to single agent therapy in mice bearing MDA-MB-468 tumours [49]. IMMU-132 is completing Phase II clinical trial and preparing to enter Phase III. A Phase III pivotal trial of IMMU-132 in triple negative breast cancer is planned in calendar year 2016.

CEACAM5/CEACAM5 and IMMU-130

Human carcinoembryonic antigen (CEA) now designated CEACAM5 (CD66e) is a highly glycosylated glycosylphosphatidylinositol(GP)-anchored protein that is over-expressed by cancers of the ga tract including pancreas, liver, gallbladder and colorectal cancer [50]. CEACAM5 was initially thought to be a tumour-specific antigen and as such was one of the early immunotherapy targets, however, it has since been found in many normal tissues. CEACAM5 tends to localise in the plasma

membrane in micro-domains or lipid rafts and functions as an inter-cellular adhesion molecule via anti-parallel binding of CEACAM5 molecules on the extracellular surface of adjacent cells. CEACAM5 expression on epithelial cells may directly influence tumour development by CEACAM5–CEACAM5 bridges between tumour cells or between tumour and stromal cells. The related protein CEACAM6 (NCA-90) is also highly expressed in many solid tumours [51, 52]. The transforming growth factor beta (TGF-β) signalling pathway has been implicated in the stimulation of CEACAM5 secretion by TGF-β-sensitive colon cells. Data indicate that CEACAM5 is a target gene for Smad3-mediated TGF-β signalling [53]. The CEACAM5 glycoprotein is detectable in circulation of about 85 % of colon cancer patients [54]. Blocking the binding of CEACAM5 to CEACAM1 on purified human natural killer (NK) cells via a CEACAM5-specific humanised monoclonal antibody, PR1A3, that recognises the membrane-bound CEA, produced extensive killing of human colon carcinoma cells. Cell lysis occurred through antibody-dependent cellular cytotoxicity (ADCC). The anti-CEACAM5 monoclonal antibody, CC4 exhibited similar properties [55]. CEACAM5 plays an active role in colorectal cancer liver metastasis development. Pretreatment of mice with soluble CEACAM5 favoured enhanced development of experimental liver metastatic nodules in nude mice, even when using weakly metastatic colorectal cancer cells negative for CEACAM5 expression.

The camptothecin derivative irinotecan is a well-used anticancer drug for the treatment of colorectal cancer, however, it's use is limited by associated toxicities. Early ADCs directed toward CEACAM5 were designed to improve efficacy and reduce the gastrointestinal toxicity of camptothecin by covalently linking the active form of irinotecan, SN-38, to an anti-CEACAM5 antibody, labetuzumab (hMN-14), for targeted chemotherapy. In a lung metastatic model of GW-39 human colon carcinoma in nude mice, treatment with two labetuzumab-SN-38 conjugates extended median survival time versus controls. In subcutaneously implanted LS174T xenografts, labetuzumab-SN-38 conjugates produced tumour growth control and increased median survival time versus controls [56]. The selected labetuzumab-SN-38 conjugate designated IMMU-130, was evaluated in three Phase I clinical trials of heavily pretreated patients with metastatic colorectal cancer. The conjugate was manufactured with a near-homogeneous drug substitution of 7–8 SN-38 molecules/antibody molecule and with a linker that released 50 % of the drug in 20 h. A tolerability study in rabbits showed a safety margin, with a no-observed-adverse-effect level (NOAEL) corresponding to a cumulative human-equivalent protein dose of 40–60 mg/kg. The preclinical findings appeared to be corroborated in two phase I clinical trials, with tolerability and evidence of antitumour activity, including objective responses [57]. In the on-going Phase II clinical trial in patients with metastatic colorectal cancer, IMMU-130 is being administered in 3 week cycles either once weekly or twice weekly for the first 2 weeks followed by 1 week of rest [58, 59]. A novel anti-CEACAM5 maytansinoid-antibody-drug conjugate for the treatment of colorectal, lung and gastric tumours is under development [60].

EGFR

EGFR is a cell surface receptor tyrosine kinase which can be activated by several ligands including epidermal growth factor (EGF) and EGF family members such as TGF-b, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, GP30 and vaccinia virus growth factor. The EGFR triggered pathway is involved in control of cell growth and differentiation. EGFR isoform 2/truncated isoform may act as an antagonist. Several anti-EGFR-drug conjugates using the antibody C225 (IMC-C225, Erbitux) linked to varied taxanes or doxorubicin have been described [61–63]. In early studies, antibody C225 was coupled to a poly(L-glutamic acid)-co-polyethylene glycol block copolymer linked to doxorubicin [64]. The resulting C225-Dox conjugate bound to human carcinoma A431 cells which endogenously express high EGFR. The conjugate was internalised and was potently cytotoxic toward the A431 cells. Paclitaxel was conjugated to the anti-EGFR C225 through an amide linkage. The conjugate designated PTXC225 was assessed in cell culture and in vivo in nude mice bearing A431 xenograft tumours [65]. Although the antibody localised to the tumour site in the mice, there was no difference in the efficacy between the antibody-drug conjugate and treatment with paclitaxel as the small molecule.

Recently, IMGN289, a novel ADC consisting of the humanised anti-EGFR antibody, J2898A, covalently linked to the maytansinoid, DM1 was reported. J2898A was comparable in potency to cetuximab in vitro against a panel of EGFR-dependent tumour cell lines and in vivo against two head and neck tumour xenograft models. In cultures of human primary keratinocytes, J289A was less cytotoxic than cetuximab and did not affect TNFα-induced cytokine production. In culture, IMGN289 was more potent against all HNSCC cell lines than cetuximab. Cetuximab and gefitinib resistant cell lines were sensitive to IMGN289 [66]. A safety study in cynomolgus monkeys demonstrated that IMGN289 was well tolerated and exhibited a similar toxicity profile to that of trastuzumab emtansine (T-DM1) [67]. IMGN289 was evaluated in EGFR-positive SCCHN xenograft models with EGFR expression comparable to clinical disease. Immunodeficient mice bearing established xenografts were treated with a single intravenous injection of IMGN289 at 1, 2.5 or 5.0 mg/kg. In the FaDu colon carinoma xenograft model, IMGN289 was active with a minimally efficacious dose of 1 mg/kg, highly active at 2.5, and at 5 mg/kg produced 5/6 partial regressions and 2/6 complete regressions in the mice. IMGN289 was active in the HSC-2 xenograft model with tumour regression at 5 mg/kg with 6/6 partial regressions and 4/6 complete regressions [68].

ABT-414 is an ADC comprised of an anti-EGFR antibody (ABT-806) conjugated to the monomethylauristatin F. ABT-414 binds a unique EGFR epitope which is largely inaccessible when EGFR is expressed at physiological levels but is accessible in tumours that express EGFRde2-7 (EGFR variant III (EGFRvIII) and in tumours with wild type amplified EGFR or excessive wild type EGFR activation; therefore, ABT-414 effects on normal tissues may be limited. ABT-414 is effective in both mutant and wild type EGFR-positive human tumour xenografts. Patients

with recurrent glioblastoma multiforme (Glioblastoma multiforme, GBM) have few treatment options and a very poor prognosis. GBM tumours often exhibit aberrant EGFR proliferative signalling. ABT-414 was active in preclinical GBM tumour models harbouring either wild type EGFR or EGFRvIII. Preliminary safety data demonstrated a unique toxicity pattern related to MMAF-induced corneal epithelial microcysts. Preliminary responses in 3/9 pts with temozolomide refractory GBM, included 1 complete response [69]. ABT-414 doses were escalated using a modified continual reassessment method. Primary objectives were safety, maximum tolerated dose and ABT-414 recommended phase 2 dose. Accrual included 18 patients treated in 4 dose groups (0.5, 1.0, 1.25, 1.5 mg/kg) and 28 patients treated at 1.25 mg/kg. Treatment-emergent adverse events occurring in ≥ 25 % of patients were blurred vision, fatigue, foreign body sensation in the eyes, photophobia, nausea, constipation, and liver enzyme increase. Dose-limiting toxicities were corneal deposits and liver enzyme increase. The ABT-414 recommended phase 2 dose was 1.25 mg/kg. Confirmed responses were durable, ranging from 5 to 16 months [70]. Interestingly, EGFR amplification was found in all patients with confirmed responses.

Another phase 1/II trial evaluated the safety, pharmacokinetics and efficacy of ABT-414 in patients with solid tumours. In general, by immunohistochemistry, >70 % of squamous lung and oesophageal cancers and >90 % of oral cavity tumours have moderate to strong staining for EGFR [71]. Fifty-three patients received ABT-414. Most common treatment-emergent adverse events were blurred vision (49 %, due to transient microcystic keratopathy), fatigue (42 %), nausea (42 %), and dry eyes (36 %). In the EGFR-amplified cohort, 1 partial response was observed. Stable disease occurred in 11 of 53 patients. The ABT-414 recommended phase 2 dose administered once every 3 weeks was 3 mg/kg. The preliminary efficacy observed in patients with EGFR-amplified tumours suggests antitumour activity [72]. Further clinical investigation of ABT-414 is on-going.

HER2/CD340

T-DM1 is an ADC whose mechanism of action includes all of the effects of trastuzumab plus the effects of the conjugated maytansine derivative. Initially, T-DM1 binds HER2, then the HER2/T-DM1 complex undergoes internalisation, followed by lysosomal degradation resulting in intracellular release of DM1, which prevents microtubule polymerisation. T-DM1 retains mechanisms of action of trastuzumab, including disruption of the HER3/PI3K/AKT signalling pathway and Fcγ receptor–mediated engagement of immune effector cells, which leads to antibody-dependent cellular cytotoxicity [73, 74]. All clinical trials of T-DM1 used HER2 protein over-expression and/or HER2 gene amplification as an inclusion criteria. Ongoing clinical trials are evaluating T-DM1 treatment in other solid tumours that either over-express HER2 protein and/or amplify the HER2 oncogene (e.g., gastric cancer) or carry HER2 mutations (e.g., NCI-MATCH trial). T-DM1 phase I and phase II trials showed objective responses in patients with HER2+ metastatic breast

cancer with an acceptable toxicity profile, leading to the design of a pivotal phase III randomised trial (EMILIA). In this study patients with HER2+ metastatic breast cancer were assigned to T-DM1 or to capecitabine in combination with lapatinib. One inclusion criterion was prior trastuzumab- and taxane-based chemotherapy treatment. T-DM1 therapy improved response rate, time to progression and overall survival rate compared to the capecitabine plus lapatinib and was better tolerated than capecitabine plus lapatinib [75].

In xenograft models, T-DM1 and pertuzumab result in improved tumour inhibition compared with either agent alone [76]. T-DM1 and pertuzumab were combined at full doses with no unexpected toxicities. In previously treated patients T-DM1 + pertuzumab had similar activity to that observed with single-agent T-DM1 and for pertuzumab plus trastuzumab [77–80]. In treatment naive patients, the objective response rate (ORR) was 57 % and the median progression free survival (PFS) was 7.7 months [81]. The order of treatment may be important as in preclinical models where pretreatment with pertuzumab appeared to blunt the efficacy of T-DM1 [82]. Additional trials explore the potential to combine T-DM1 with a variety of chemotherapy agents including paclitaxel, docetaxel, and capecitabine among others. Alternatively, T-DM1 was substituted for the taxane/trastuzumab portion of adjuvant therapy high-risk patients in the ongoing KAITLIN trial. Inhibition of HER2 activity along with oestrogen receptor inhibition has been a successful treatment strategy [83, 84]. The ADAPT HER2+/HR+ trial tested T DM1 + endocrine therapy (tamoxifen, aromatase inhibitors), which resulted in a greater median fractional decrease in proliferation (Ki67) after 3 weeks of therapy [85]. SYD985 is another HER2-targeting ADC combining trastuzumab and a duocarmycin payload with a cleavable linker. Even in cell lines with low HER2 expression, SYD985 had activity [86]. Thus, the target population of breast and gastric cancer patients that may respond to HER2-targeted ADCs could include FISH-negative / IHC low HER 2+ patients.

The response of human NCI-N87 HER2+ gastric cancer cells to exposure to T-DM1 was studied in cell culture in the presence/absence of pertuzumab. Binding of T-DM1 to cell surface HER2 was increased in the presence of pertuzumab. Simultaneous exposure to T-DM1 and pertuzumab led to a reduction of phosphorylated EGFR in EGF or heregulin-stimulated cells, and the pERK or pAkt pathways. The results suggested that the combination of T-DM1 with pertuzumab may benefit patients with HER2+ gastric cancer [87].

The breast cancer Phase III MARIANNE trial randomly assigned 1,095 patients with metastatic HER2+ breast cancer to one of three treatment arms; T-DM1 plus pertuzumab, T-DM1 plus placebo, or a taxane along with trastuzumab [88]. At 35 months median follow-up, the T-DM1 regimens had non-inferior progression free survival, compared with a taxane plus trastuzumab. A randomised Phase II/III clinical trial (GATSBY) of T-DM1 versus a taxane in patients with previously treated HER2+ locally advanced, metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma was conducted. Patients were randomised to T-DM1 3.6 mg/kg every 3 weeks, T-DM1 2.4 mg/kg weekly, or to a taxane. At the point of clinical cutoff, 415 patients had been accrued. Results revealed that T-DM1 treatment did

not show an efficacy benefit over taxane use [89]. A combination study of T-DM1 and capecitabine in patients with HER2+ metastatic breast cancer and patients with HER2+ locally advanced or metastatic gastric cancer is currently underway (NCT01702558). In another active clinical trial patients with unresectable HER2 overexpressing gastric or GEJ cancers will be treated with T-DM1 (NCT02318901).

The premise of cancer precision medicine is that a therapeutic will be effective in any tumour expressing the target, regardless of the disease. To test this hypothesis, a phase II "basket" trial with T-DM1 is on-going in patients with HER2 amplified or HER2 over-expressed advanced lung, bladder, endometrial, gastric cancers, amongst others. All patients will receive T-DM1 every 21 days until disease progression or unacceptable toxicity, with a primary endpoint of ORR. HER2 amplification assessment by next-generation sequencing correlates well with amplification by *in-situ* hybridisation. Exploratory analysis will examine the concordance among HER2 gene amplification and/or mutation, and protein over-expression [90]. The application of T-DM1 in combination with CTLA-4 and/or PD-1 blockade is being explored [91]. Many new HER2-targeted ADCs are being developed using unique antibodies with newer toxins and linkers [92–98].

MUC1/Mucin 1

The application of antibodies as carriers for therapeutically active radionuclides has a history similar to ADCs, with agents evolving from mouse antibodies and suboptimal chelators to the present products with human or humanised antibodies and stable chelators [99, 100]. There are currently two FDA approved radioimmunotherapy drugs: ^{131}I tositumomab (Bexxar) and ^{90}Y ibritumomab tiuxetan (Zevalin), both for the treatment of lymphoma. Progress in the treatment of solid tumours with radionuclide-conjugated antibodies has been slower [101]. Radionuclide-conjugated antibodies are under investigation in a variety of solid tumours, GI malignancies, pancreatic cancer and hepatocellular carcinoma are farthest along.

Pancreatic cancer is among the most deadly diseases and was named in the Recalcitrant Cancer act as a disease needing further study. In 2015 within the USA, the estimated number of new cases of pancreatic cancer was 49,000 and the number of deaths from pancreatic cancer was 40,500 [102]. The high disease mortality is often attributed to late diagnosis, further work is needed to increase early detection and as yet, no specific pancreatic tumour markers have been identified [103].

Chemotherapy is the primary treatment modality for patients with locally advanced or metastatic pancreatic cancer and gemcitabine has long been the standard of care. Gemcitabine demonstrated activity in patients with pancreatic cancer [104–106]. A phase III trial of gemcitabine versus 5-fluorouracil as first-line therapy in patients with advanced or metastatic adenocarcinoma of the pancreas reported an improvement in survival among patients treated with gemcitabine (1-year survival was 18 % with gemcitabine compared with 2 % with 5-fluorouracil) [105]. Recently, a multicentre, international phase III trial with metastatic pancreatic adenocarcinoma

patients, who had not previously received chemotherapy for metastatic disease, has been undertaken. Patients were randomised to receive gemcitabine and nab-paclitaxel weekly for 3 of 4 weeks or gemcitabine alone [107]. The median overall survival was 8.5 months in the nab-paclitaxel plus gemcitabine group compared with 6.7 months in the gemcitabine alone group. Thus, nab-paclitaxel plus gemcitabine is now a treatment option for patients with advanced pancreatic cancer.

PAM4 is a monoclonal antibody with selectivity for pancreatic ductal adenocarcinoma and other pancreatic cancers. A PAM4-based serum immunoassay detected 71 % of early pancreatic cancer and 91 % of advanced pancreatic cancer. However, 20 % of chronic pancreatitis was positive for circulating PAM4 antigen [108]. PAM4 antibody selectivity is critical to application in serum-based and immunohistochemical pancreatic cancer detection. The PAM4 antibody had a greater selectivity for pancreatic cancer than did 4 other antibodies (MUC1, MUC4, CEACAN5/6 and CA19-9) and may be suitable as a diagnostic for this extremely difficult disease [108]. A humanised PAM4-radionuclide conjugate, ^{90}Y-hPAM4 (90Y-clivatuzumab tetraxeta), has been developed. The ^{90}Y-hPAM4 plus hRS7-SN-38 targeting Trop2 combination produced higher survival and greater tumour-free response in animals (90 %) than radiation therapy alone in animals with pancreatic cancer xenografts [38]. A phase I study evaluated a single dose of ^{90}Y-clivatuzumab tetraxetan (^{90}Y-labeled hPAM4) in patients with advanced pancreatic cancer. Patients first received ^{111}In-hPAM4 for imaging and serum sampling before ^{90}Y-hPAM4. Twenty patients received ^{90}Y doses of 15, 20, and 25 mCi/m2. Three patients at the highest dose had dose-limiting toxicity with grade 4 cytopenia for >7 days, thus establishing 20 mCi/m^2 as the maximal tolerated ^{90}Y dose. Most patients progressed rapidly and with CA19-9 increasing within 1 month of therapy, but 7 remained progression-free by for 1.5–5.6 months, including 3 transient partial responses [109]. A subsequent ^{90}Y-hPAM4 trial studied fractionated radioimmunotherapy combined with low dose gemcitabine in metastatic pancreatic cancer. Previously untreated patients received gemcitabine (200 mg/m^2) weekly for 4 weeks with ^{90}Y-hPAM4 given weekly in weeks 2, 3, and 4. In part 1 of the study, patients received escalating weekly ^{90}Y doses of 6.5 mCi/m^2, 9.0 mCi/m^2, 12.0 mCi/m^2, and 15.0 mCi/m^2. In part 2, patients received weekly doses of 9.0 mCi/m^2 or 12.0 mCi/m^2. The maximum tolerated ^{90}Y-hPAM4 dose was 9.0 mCi/m^2 weekly for 3 weeks. Six of 38 patients had partial responses, and 16 patients had disease stabilisation [110]. A Phase Ib clinical trial of fractionated radioimmunotherapy was undertaken administering ^{90}Y-hPAM4 with or without radiosensitising doses of gemcitabine. Patients were treated with ^{90}Y-clivatuzumab tetraxetan, weekly 6.5 mCi/m^2 doses × 3, plus gemcitabine, weekly 200 mg/m^2 doses × 4 starting 1 week earlier or with ^{90}Y-clivatuzumab tetraxetan alone, weekly 6.5 mCi/m^2 doses × 3, repeating cycles after 4-week delays. Cytopaenia was the only significant toxicity. Two patients receiving the combination regimen had partial responses by RECIST criteria. Kaplan-Meier overall survival for the combination regimen was 7.9 versus 3.4 months. Three of 27 patients receiving the combination regimen survived >1 year. A Phase III trial of this combination is now underway in metastatic pancreatic cancer [111].

Conclusion

Gastrointestinal cancers are a therapeutic challenge. Antibody drug and antibody radionuclide conjugates directed toward cell surface molecular targets in this varied family of diseases are in early clinical trial. With the continued clinical success of these agents in gastrointestinal cancers, additional promising drugs can be brought to bear against these deadly cancers.

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Chapter 5
Antibodies that Inhibit Specific Cellular Pathways in Gastric Cancer

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Monoclonal antibodies have been developed to target specific therapeutic targets overexpressed on cancer cells. The mechanisms of action of monoclonal antibodies include the inhibition of ligand/receptor binding, the down-regulations of oncoproteins, and the stimulation of immune system effector mechanisms, such as, complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). Here, we describe interesting therapeutic targets in gastric cancer and studied antibodies.

Introduction

The biology of gastric cancer has been revealed through technologic advances. Deng et al. performed a comprehensive genomic analysis of 233 gastric cancer and 98 matched non-malignant gastric samples and identified 22 recurrent genetic alterations (13 amplifications and 9 deletions) [1]. They reported that 5 distinct gastric cancer subgroups could be defined by specific genetic alterations, namely, amplifications of *HER2*, *FGFR*, *KRAS*, *EGFR*, or *MET*. Dulak et al. also reported similar results using high-density genomic profiling arrays, and detected amplification of *HER2*, *FGFFR1*, *FGFR2*, *EGFR*, or *MET* in 37% of gastric/oesophageal tumours [2]. Presumably, novel targeted agents could be specifically developed against these genes.

A recent TCGA (The Cancer Genome Atlas) study that involved comprehensive molecular profiling of gastric cancers identified four different molecular subtypes; Epstein-Barr virus (EBV) associated tumours, microsatellite unstable tumours

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(MSI), genomically stable tumours (GS), and tumours with chromosomal instability (CIN) [3].

EBV-associated gastric cancers account for 5–10 % of all gastric cancers. This subtype is characterised by intense lymphoid stroma histologically and epigenetic alterations. In particular, DNA methylation of the promoter regions of tumour suppressor genes, are frequent, probably due to host reactions to viral infections. This cancer subgroup has a strong signature of interleukin-12 signalling events, which reflect abundant immune cell infiltration. The TCGA study also identified critical associations with signal pathways, such as, PI3K/AKT and JAK2, and elevated *PD-L1* and *PD-L2* expressions in EBV-associated gastric cancers.

MSI is a genetic alteration characterised by the deactivation of DNA mismatch repair (MMR) genes. MSI gastric cancers usually have an intestinal histology, and are associated with good prognoses and low recurrence rates. These cancers often exhibit an aberrant epigenetic pattern and in this subtype *MLH1* methylation is a key biomarker. EGFR-MAPK and PI3K pathways are frequently activated in MSI gastric cancers, which have higher mutation burdens than other subtypes, and higher mutation rates of genes, such as, *PIK3CA*, *ERBB3*, *ERBB2*, and *EGFR*. Interestingly, however, amplifications of these genes are not observed. *KRAS* and *BRAF* mutation rates are extremely low in gastric cancer, and *KRAS* mutation is most frequently observed in MSI gastric cancer.

GS subtype is characterised by the absence of extensive somatic copy-number aberrations and enhancement of the diffuse histological subtype. *RHOA* and *CDH1* mutations and *CLDN18-ARHGAP6* or *-ARHGAP26* fusions are frequently observed in this subtype, and these genetic alterations underlie a poor cohesive morphology, resistance to anoikis, and epithelial-mesenchymal transition of carcinoma cells.

CIN gastric cancer is characterised by an intestinal histology and frequent *p53* mutation (71 % in the TCGA study). In the CIN subtype, many molecules, such as, HER2, EGFR, c-MET, and FGFR2, have been recently been identified as treatment targets. Phosphorylation of EGFR (pY1068) is significantly elevated in the CIN subtype, which is consistent with the amplification of *EGFR* in this subtype. For *HER2*, most cases showing amplification are of the CIN subtype followed by the EBV associated subtype. *MET* amplification is observed in around 6 % in gastric cancers, predominantly in the CIN subtype. *KRAS/NRAS* amplification is also mainly observed in CIN subtype. This subtype also shows elevated *p53* expression, which is consistent with frequently observed *p53* mutation and aneuploidy. Recurrent amplification of the gene encoding ligand VEGFA is also observed, and frequent amplifications of cell cycle mediators (*CCNE1*, *CCND1* and *CDK6*) are detected in the CIN subtype.

HER Family as a Therapeutic Target in Gastric Cancer

The human epidermal growth factor receptor (HER) family of tyrosine kinases consists of HER1 (EGFR, ERBB1), HER2 (HER2/neu, ERBB2), HER3 (ERBB3) and HER4 (ERBB4). HER receptors are composed of an extracellular domain (ECD), a

transmembrane domain, and an intracellular tyrosine kinase domain. Ligand binding to its ECD triggers conformational changes of receptors and exposes dimerisation domain(s) used to bind to another receptor. Following dimerisation, the kinase domain of one dimer allosterically activates its neighbour and becomes trans-autophosphorylated. This phosphorylation of kinase domain leads to the activation of downstream signalling pathways that ultimately stimulate tumour cell proliferation and survival [4].

EGFR

EGFR is a transmembrane glycoprotein of 170 kDa, coded by the C-*ERB1* proto-oncogene situated in the 7q22 chromosome, and its known ligands are EGF, TGF alpha, amphiregulin, heparin-binding EGF, betacelulin, epiregulin, and NRG2-alpha. EGFR overexpression occurs in 27–55 % of oesophagogastric adenocarcinomas, and its presence correlates with poor prognosis [5].

Cetuximab, panitumumab, and nimotuzumab are EGFR-targeting antibodies. Cetuximab is a recombinant human-mouse chimeric IgG1 monoclonal antibody, and its binding to EGFR induces receptor dimerisation, internalisation, and receptor down-regulation. These processes lead to cell-cycle arrest via the upregulation of cyclin dependent kinase inhibitor p27, the potentiation of apoptosis (correlated with the induction of Bax), and the activation of caspase [6]. Other effects include the reduction in VEGF production by tumours, which reduces tumour microvessel density, and the inhibition of invasion and metastasis through modulation of matrix metalloproteinases [7]. Panitumumab is a totally human, high affinity IgG2 monoclonal antibody against human EGFR, and thus, its immunogenicity is minimal or non-existent and its use avoids the problem of generating human murine antibodies and therefore minimises the risk of hypersensitivity reactions. However, panitumumab is an IgG2 subtype and may not act on ADCC, because only IgG1 antibodies are able to induce ADCC [8]. Nimotuzumab is a humanised IgG1 monoclonal antibody against human EGFR [9]. Good tolerance and interesting activity were observed in initial Phase I studies [10, 11]. As compared with cetuximab, nimotuzumab has a longer half-life, a higher dose-effect rate, and less severe dermatological toxicity. Notably, no skin toxicity or hypersensitivity reactions were reported in these trials [12].

The EXPAND trial was a randomised, phase III study of capecitabine and cisplatin with or without cetuximab [13]. A total of 904 chemotherapy-naïve patients with gastric or gastroesophageal junction (GEJ) cancer were enrolled. No patient selection based on any biomarker, including EGFR status, was performed. The primary endpoint was progression-free survival (PFS). The addition of cetuximab to capecitabine/cisplatin was found to provide no additional benefit over chemotherapy alone. PFS was 4.4 months in the cetuximab arm and 5.6 months in the chemotherapy alone arm ((HR) 1.09, P = 0.32). Overall survival (OS) was not improved by cetuximab (9.4 months vs 10.7 months, HR1.00, P = 0.95), and overall response and

disease control rates were similar in the two arms. Common Terminology Criteria for Adverse Events (Common Terminology Criteria for Adverse Events, CTCAE) Grade 3 and 4 skin reaction (13 % vs 0 %), acne-like rash (11 % vs 0 %) and mucositis (4 % vs 2 %) were more frequently observed in the cetuximab arm.

The REAL3 trial was another randomised, phase III study of panitumumab [14]. A total of 553 patients were enrolled regardless of EGFR status, and randomised to EOC chemotherapy (epirubicin, oxaliplatin, capecitabine) with or without panitumumab. In the panitumumab arm, the dose of EOC chemotherapy was reduced because excessive toxicity was observed in a previous phase I study [15]. The primary endpoint was OS. OS in the panitumumab arm was poorer (8.8 months vs 11.3 months, HR 1.37, P = 0.0013), and grade 3/4 diarrhoea (17 % vs 11 %), rash (11 %, vs 1 %), mucositis (5 % vs 0 %), and hypomagnesemia (5 % vs 0 %) were more common in the panitumumab arm. At the time of writing, EGFR antibodies were not recommended in gastric or GEJ cancer.

In a randomised phase II study of nimotuzumab, 83 patients that progressed after previous 5-FU based therapy were randomly assigned to irinotecan or irinotecan plus nimotuzumab [16]. OS and PFS were no different in the two arms. However, overall survival of patients exhibiting EGFR overexpression (2+ or 3+) were 11.9 months in the nimotuzumab arm and 7.6 months in the irinotecan monotherapy arm. Based on this finding, a phase III study of nimotuzumab and irinotecan as second-line treatments in EGFR overexpressed gastric or GEJ cancer was initiated (ClinicalTrials.gov Identifier: NCT01813253).

HER2/HER3

Of the four HER family receptors, HER2 has the most potent catalytic kinase activity. No ligand is known to bind to HER2, which is always in the open conformation and easily dimerises with other HER family receptors. In contrast to HER2, the kinase domain of HER3 does not possess catalytic activity. Ligand-activated HER3 preferentially binds to HER2, and activates HER2 signalling. In fact, HER2/HER3 dimer is most potent signalling dimer [17, 18].

HER2 is not infrequently overexpressed or amplified in gastric cancer. In the past, *HER2* overexpression or amplification was reported in 20–25 % of gastric cancers, but more recent studies have revised this figure to 11–16 % of gastric cancers [19–21].

Trastuzumab is a fully humanised monoclonal antibody that binds to the ECD of HER2 receptor, and thus prevents receptor dimerisation, the activation of HER2-related signalling, and the induction of ADCC [22].

The ToGA trial was a randomised multicenter phase III study in HER2-positive gastric cancer. A total of 584 HER2-positive patients were randomised to chemotherapy (5-FU/cisplatin or capecitabine/cisplatin) with or without trastuzumab. OS (the primary endpoint) was found to be significantly prolonged (HR 0.74 [95%CI: 0.60–0.91], P = 0.0046), and all other efficacy endpoints, including objective

response rate (ORR) and PFS, were improved. Furthermore, the benefit of trastuzumab was substantially greater in patients with HER2 3+ or HER2 2+/FISH (+) (HR 0.65 [95 %CI: 0.51–0.83]), and median survival of these patients was 16.9 months. This was the first successful immunotherapy trial in gastric cancer.

Although trastuzumab-based first-line treatment is the standard for HER2-positive gastric cancer, not all patients benefit, and reported ORRs are variable (about 32–68 %) [23, 24]. In a recent report, it was suggested that degree of *HER2* gene amplification is predictive of responsiveness to trastuzumab-based therapy in gastric cancer; patients with a *HER2/CEP17* ratio of >4.7 were found to have more favourable clinical outcomes [25]. In another study, it was suggested that a cutoff value for *HER2/CEP17* ratio of 3.69 be considered for the selection of patients with HER2 immunohistochemistry (IHC) ≤ 2+ to receive trastuzumab; this value is higher than the conventional consensus cutoff of 2.0 [26]. However, in patients with IHC 3+, information obtained by *HER2* gene amplification might not influence clinical decisions regarding trastuzumab-based treatment. On the other hand, in patients with IHC ≤ 2+, further information regarding *HER2* gene amplification status could provide clinicians with additional guidance for prospectively selecting patients that might benefit from trastuzumab.

Pertuzumab binds to the dimerisation domain (ECD II) of HER2, and this leads to the blocking of ligand-induced HER2 heterodimerisation. The HER2 binding site for pertuzumab is not the same as that used for trastuzumab. *In vitro*, pertuzumab was more effective than trastuzumab at blocking HER1/HER2 and HER3/HER2 dimerisations [27]. Pertuzumab mediates ADCC in a manner similar to trastuzumab [28]. In combination, trastuzumab and pertuzumab were reported to synergistically inhibit tumour growth *in vitro* and *in vivo* [28, 29]. Preclinical studies in a human HER2-positive gastric cancer xenograft model showed enhanced anti-tumour activity for pertuzumab and trastuzumab in combination versus either antibody alone; this anti-cancer activity was attributed to the potentiation of cell growth inhibition, apoptotic activity, cell killing by ADCC, and antiangiogenic activity [30].

The JOSHUA study was conducted to investigate the pharmacokinetics (PK) of pertuzumab in combination with trastuzumab/capecitabine/cisplatin and to determine the pertuzumab dose that produces a steady-state trough serum concentration (Cmin) of >20 μg/ml in at least 90 % of HER2-positive gastric cancer patients [31]. Thirty patients were randomised to receive an initial pertuzumab dose of 840 mg for cycle 1, followed by a dose of 420 mg for cycles 2–6 (Arm A) or pertuzumab 840 mg for cycles 1–6 (Arm B). Mean pertuzumab Cmin for the 840/420 mg dose (40 μg/ml) was 37 % lower than that observed for the same dose in the breast cancer CLEOPATRA study [32]. In contrast, mean pertuzumab Cmin for the 840 mg every 3 weeks dose (62.7 μg/ml) was similar to that observed for the 840/420 mg dose in the CLEOPATRA study. Based on the JOSHUA study, a pertuzumab dose of 840 mg every 3 weeks is expected to provide greater treatment benefit than the 840/420 mg dose in patients with HER2-positive gastric cancer.

The JACOB study phase III trial of trastuzumab/capecitabine/cisplatin with or without pertuzumab in HER2-positive gastric and GEJ cancer was ongoing at the time of writing (ClinicalTrials.gov Identifier: NCT01774786). The dose of

pertuzumab being used was 840 mg every 3 weeks (based on the findings of the JOSHUA study). In this study, HER2-positivity was defined as HER2 IHC3+ or IHC2+/FISH+, the primary endpoint was OS and secondary endpoints were PFS, ORR, duration of response, clinical benefit rate and safety. Up to 780 patients were enrolled in the study.

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate (ADC) of trastuzumab linked to DM1 (a microtubule polymerisation blocker). After the internalisation of HER2 receptor, T-DM1 complex releases DM1-containing moieties from T-DM1, which inhibits cell division and leads to cell death. The GATSBY phase II/III study was undertaken to evaluate the efficacy and safety of trastuzumab emtansine versus standard taxane treatment in HER2-positive second-line gastric or GEJ cancer patients (ClinicalTrials.gov Identifier: NCT01641939). The primary endpoint was OS. Disappointingly, T-DM1 failed to improve OS as compared with taxane chemotherapy [33]. OS for T-DM1 was 7.9 months and that of taxane was 8.6 months (HR 1.15, p = 0.86).

HGF-cMET as a Therapeutic Target in Gastric Cancer

In gastric cancer, *MET* is overexpressed in 21.5 % of IHC 2+ patients and in 2.3 % of IHC 3+ patients, and 3.4 % of patients exhibit *MET* gene amplification [34]. Patients with *MET* overexpression were found to have the poorest prognosis. Hepatocyte growth factor (HGF) is the only ligand of MET receptor, and elevated HCF serum concentrations have been shown to be associated with disease stage and reduced after primary tumour resection [35, 36].

Rilotumumab is a fully human anti-HGF neutralising IgG2 antibody, and was tested in a randomised phase II study [37]. A total of 121 unresectable or metastatic gastric or GEJ adenocarcinoma patients were randomised to ECX (epirubicin/cisplatin/capecitabine) + placebo, ECX + rilotumumab 15 mg/kg, or ECX+ rilotumumab 7.5 mg/kg arms. PFS (the primary endpoint) was 5.7 months (HR 0.60 vs placebo, P = 0.016) in combined rilotumumab arms, and 4.2 months in the placebo arm. Grade 3/4 neutropenia (44 % vs 28 %), venous thromboembolism (20 % vs 10 %), and any grade of peripheral oedema (27 % vs 8 %) were more frequently observed in the rilotumumab arm. According to analysis based on tumour *MET* expression levels, OS was much shorter in the MET-positive subgroup than in the MET-negative subgroup of the placebo arm (5.7 months vs 11.5 months). Interestingly, in the MET-positive subgroup, OS was improved by the addition of rilotumumab (10.6 months vs 5.7 months), and in the MET-negative subgroup, OS was similar in the rilotumumab and placebo arms (11.1 months vs 11.5 months). Based on this finding, the RILOMET-1 phase III study of rilotumumab was conducted [38]. In this study, only HER2-negative, MET-positive gastric, or GEJ cancer patients were enrolled. Patients were randomised to ECX with or without rilotumumab and overall survivals were compared. A total of 609 patients were enrolled, but the study was stopped prematurely because of a death imbalance. OS was even

poorer in the rilotumumab arm than in the placebo arm (9.6 months vs 11.5 months, HR 1.37, P = 0.016). No patient subgroups seemed to benefit from rilotumumab, including those with higher percentages of cells exhibiting ≥1+ IHC *MET* expression. The most common adverse events observed in rilotumumab arm were peripheral oedema, hypoalbuminemia, deep vein thrombosis, and hypocalcaemia.

Onartuzumab is a monovalent (one-armed) humanised monoclonal antibody that binds to the Sema domain in the extracellular part of MET to block HGF binding. The novel monovalent design of onartuzumab, which was achieved using the "knobs-into-holes" method to minimise formation of the bivalent antibody configuration [39], and thus, prevents MET dimerisation, which leads to subsequent pathway activation that occurs for some bivalent anti-c-MET monoclonal antibodies [40]. METGastric was a phase III study of onartuzumab in combination with mFOLFOX6 in patients with metastatic HER2-negative and MET-positive gastric or GEJ cancer [41]. The study was designed for up to 800 patients and powered to demonstrate an improvement in OS from 9 months to 12.3 months (Intention to treat, ITT population; HR 0.73) and 9 months to 18 months (MET 2+/3+ population; HR 0.49). However, enrollment was stopped prematurely due to the negative final results of a phase 2 trial on mFOLFOX6 + onartuzumab [42]. A total of 562 patients were enrolled, and 39 % exhibited MET 2+/3+ expression. In the ITT population, OS was similar in the onartuzumab and placebo arms (11.0 months vs 11.3 months, HR 0.82, p = 0.244). In MET2+/3+ population, OS was also similar in onartuzumab and placebo arms (11.0 months vs 9.7 months, HR 0.64, P = 0.062). PFS and ORR were not improved by adding onartuzumab in the ITT or MET2+/3+ populations.

FGFR as a Therapeutic Target in Gastric Cancer

In gastric cancer, 4.2 % of Korean patients and 7.4 % of UK patients harbour *FGFR2* amplification, and intratumoural heterogeneity is observed in 24 % of *FGFR2* amplified cases [43]. About 20 % of patients show *FGFR2* polysomy, and *FGFR2* amplification and polysomy have been reported to be associated with poor OS in Korean (1.83 years vs 6.17 years, P = 0.0073) and UK (0.45 years vs 1.9 years, P < 0.0001) cohorts.

FPA144 is a humanised monoclonal antibody for FGFR2b, and is the result of a clinical development to provide a targeted immune therapy for tumours overexpressing *FGFR2b*. FPA144 binds specifically to FGFR2b and prevents the bindings of certain fibroblast growth factors that promote tumour growth. In addition, it was engineered to drive the immune-based killing of tumour cells by ADCC through the recruitment of natural killer cells [44]. In the Phase 1 trial of FPA144, patients with solid tumours were initially enrolled, and at the time of writing selected gastric cancer patients exhibiting *FGFR2* gene-amplified or FGFR2b protein overexpressing tumours were being recruited (ClinicalTrials.gov Identifier: NCT02318329).

VEGF/VEGFR as a Therapeutic Target in Gastric Cancer

The VEGF-VEGFR pathway is activated in gastric cancer, and much evidence indicates activation of this pathway indicates a poor prognosis [45, 46].

The originally named VEGF has been renamed VEGF-A to distinguish it from other family members, which include VEGF-B, VEGF-C, and VEGF-D and placental growth factor (PLGF). These growth factors have been demonstrated to differ in terms of their expression patterns, receptor specificities, and biological functions [47]. Furthermore, several distinct variants of VEGF-A have been identified (VEGF121, VEGF145, VEGF148, VEGF165, VEGF183, VEGF189, and VEGF206), which also have different receptor specificities and functions [48]. Downstream signalling of VEGF in cancer cells is mediated through receptor tyrosine kinases, such as, VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 and KDR), and VEGFR3 (also known as FLT4) [49]. The majority of these receptors are expressed by endothelial cells and many tumour types, and interestingly, the expression patterns of these receptors in tumours are correlated with some clinical parameters [50].

Traditionally, anti-angiogenesis treatment is believed to act mainly through endothelial fenestrations by reducing vascular sprouting and vessel patency and suppressing blood flow. In could be considered that antiangiogenic surveillance involves the negative regulation of endothelium by the immune system during tumour growth. Lymphocyte subsets produce specific angiogenic regulators; for example, VEGF is highly expressed by regulatory T cells (Treg), while the antiangiogenic protein IFN-γ is secreted by CD8+, CD4+, and natural killer (NK) cells [51, 52]. Thus, T cell–secreted VEGF and IFN-γ play opposing roles in the regulations of tumour angiogenesis and tumour growth [53, 54]. Antiangiogenic surveillance is now appreciated to be a unique antitumour aspect of traditional immunosurveillance [55–58].

Bevacizumab is a recombinant humanised IgG1 monoclonal antibody against VEGF-A, and was tested in the AVAGAST phase III study [59]. A total of 774 treatment naïve gastric cancer patients were randomised to capecitabine/cisplatin with or without bevacizumab arms. The primary endpoint was OS. PFS (6.7 vs 5.3 months, HR 0.80, P = 0.0037) and ORR (46.0 % vs 37.4 %, P = 0.0315) were improved by adding bevacizumab. However, OS was not prolonged (12.1 months vs 10.1 months, HR 0.87, P = .1002). Interestingly, the benefit afforded by bevacizumab appeared to differ in geographic regions. The most common grade 3–5 adverse events were neutropenia (35 %, bevacizumab vs 37 %, placebo), anaemia (10 % v 14 %), and decreased appetite (8 % v 11 %). The AVATAR trial (Chinese phase III study) also failed to show that the addition of bevacizumab improved of OS (HR 1.11, P = 0.5567) [60].

Ramucirumab is a fully human IgG1 monoclonal antibody against VEGFR2. Ramucirumab leaves the VEGFR1 alone, which behaves as a decoy receptor and enhances the VEGFR2 inhibitory effect of ramucirumab. VEGFR2 is expressed on macrophages as well as endothelial cells, and the inhibition of macrophage activity by ramucirumab reduces tumour immune infiltration along with cytokine and chemokine release, thereby decreasing tumour growth and proliferation. Ramucirumab

was tested in a second-line setting in gastric cancer. The REGARD trial was a double-blind, placebo-controlled, phase III study in gastric or GEJ cancer patients that had previously received fuoropyrimidine or platinum-based chemotherapy [61]. A total of 355 Eastern Cooperative Oncology Group (Eastern Cooperative Oncology Group, ECOG) performance status 0 or 1 patients were enrolled and randomised to ramucirumab or placebo in a 2:1 ratio. The primary end-point was OS. The study population was composed of 76 % Caucasians and 15 % Asians. OS was found to be extended by ramucirumab (5.2 months vs 3.8 months, HR 0.77, p = 0.047), and the absolute OS observed in the ramucirumab arm was comparable to those obtained by cytotoxic chemotherapy in a 2nd-line setting for gastric cancer. PFS was also improved from 1.3 months to 2.1 months (HR 0.483, P < 0.0001). Response rates were similar in the two arms (3 % vs 3 %), but the disease control rate was significantly better in the ramucirumab arm (49 % vs 23 %). Hypertension was more frequently observed in the ramucirumab arm (all grades 16 % vs 8 %), but bleeding (13 % vs 11 %), arterial thromboembolism (2 % vs 0 %), venous thromboembolism (4 % vs 7 %), proteinuria (3 % vs 3 %) and fistula formation (<1 % vs <1 %) rates were similar in the two arms.

The RAINBOW trial was another phase III study of ramucirumab in a 2nd-line setting conducted on gastric or GEJ adenocarcinoma patients [62]. A total of 665 patients with disease progression during or within 4 months of the last dose of first-line platinum and fluoropyrimidine doublet with or without anthracycline were enrolled and randomised to ramucirumab plus paclitaxel or placebo plus paclitaxel in a 1:1 ratio. Asian patients composed 33 % of the ramucirumab arm and 36 % of the placebo arm. Primary outcome was OS. OS was significantly higher in the ramucirumab plus paclitaxel group than in the placebo and paclitaxel group (9.6 months vs 7.4 months, respectively; HR 0.807, p = 0.017). PFS was also improved by ramucirumab (4.4 months vs 2.9 months; HR 0.635, p < 0.0001), as was response rate (28 % vs 16 %, p = 0.0001) in the ramucirumab arm. However, the incidence of grade 3 or 4 adverse events was higher in the ramucirumab plus paclitaxel group, and included grade 3 or 4 neutropenia (41 % vs 19 %), leucopenia (18 % vs 6 %), grade 3 hypertension (14 % vs 2 %), abdominal pain (6 % vs 3 %), and fatigue (12 % vs 5 %).

At the time of writing clinical trials on the use of ramucirumab in a 1st-line gastric cancer setting were ongoing to determine the efficacy of ramucirumab combined with standard chemotherapy. (ClinicalTrials.gov Identifier: NCT02314117, NCT02539225)

Conclusion

To date, trastuzumab and ramucirumab are the only antibodies that provide gastric cancer patients a survival benefit. Other antibodies that target interesting pathways in gastric cancer, such as, anti-EGFR, anti-MET, and anti-HGF antibodies, have failed to provide benefits. Nevertheless, these antibodies require further investigation to determine whether appropriate patient selection and specific partner drug strategies confer benefits.

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Chapter 6
PD1 and PD-L1 Immune Checkpoint Inhibitors in Gastrointestinal Cancer

Eirini Pectasides and David McDermott

Introduction

Gastrointestinal cancers are a major cause of morbidity and mortality worldwide. Cancers of the oesophagus, stomach, pancreas, gallbladder, liver, colon and rectum account for 29 % of new cancer cases and 37 % of cancer deaths [1]. The majority of these cancers are diagnosed at an advanced stage and outcomes remain poor. Systemic therapy for gastrointestinal tumours primarily relies on cytotoxic chemotherapy, with very few molecularly targeted agents incorporated in the treatment of these diseases. Recent advances have included the addition of trastuzumab, a monoclonal antibody against HER2, to cytotoxic chemotherapy in advanced *HER2* amplified gastroesophageal adenocarcinoma, the addition of the monoclonal antibodies against EGFR, cetuximab or panitumumab, in advanced *KRAS* wild-type colorectal cancer, and the angiogenesis inhibitors bevacizumab, ramucirumab and sorafenib in advanced colorectal, gastric cancer and hepatocellular carcinoma, respectively [2–8]. Although these therapies have improved clinical outcomes, their benefit is modest due to the development of acquired resistance. Therefore, more effective therapies leading to durable responses are needed for the treatment of gastrointestinal cancers.

In recent years, immunotherapy has led to marked advances in the management of melanoma, renal cell carcinoma, and non-small cell lung cancer. In these malignancies, new approaches based on monoclonal antibodies that block immune checkpoints, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), have produced meaningful improvements in survival and manageable toxicity. Immune checkpoint blockade has been an area of active

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investigation in several other malignancies, including gastrointestinal cancers. Herein, we discuss the preclinical data supporting the use of PD-1 pathway inhibitors in gastrointestinal malignancies and review the results of the clinical trials exploring the clinical activity of these agents. In addition, we discuss the efforts to identify predictive biomarkers of response and to develop rational therapeutic combinations that will enhance the efficacy of immune checkpoint blockade.

The Role of PD-1 Pathway in Cancer

Most human cancers are characterised by an inflammatory microenvironment. Tumours harbour several genetic alterations that can be recognised by the host immune system as foreign, triggering an immune response. Specifically, cytotoxic T cells are activated in response to tumour antigen stimulation and infiltrate the tumour microenvironment, with the goal of eliminating cancer cells. Under physiologic conditions, the immune response is fine-tuned by counterbalancing stimulatory and inhibitory signals. This balance is critical in order to eradicate infectious pathogens or malignant cells, while at the same time minimising persistent damage from excess inflammation in the normal tissue [9]. In many human cancers, tumour cells overexpress the ligands to immune checkpoints, which can shut down an effective immune response.

In contrast to CTLA-4, which regulates the early activation of T cells in the lymphatic tissue, PD-1 is important in the peripheral tissues and tumours, where it downregulates the activity of effector T cells and therefore limits the immune response [10]. PD-1 is expressed on activated T cells and overexpressed on exhausted T cells. Engagement of PD-1 by its major ligands, programmed death ligand-1 (PD-L1 [B7-H1 or CD274]) and programmed death ligand-2 (PD-L2 [B7-DC or CD273]), leads to inhibition of downstream kinases, decreased cytokine production, diminished T cell activity and eventually apoptosis of the T cells [9]. Therefore, inhibition of the PD-1 pathway by blocking the interaction between PD-1 and its ligands reverses T cell exhaustion, restores immune surveillance and results in tumour regression, as has been observed in several cancer types [11–14].

Immune Features of Gastrointestinal Cancers

In order to determine how to best harness the immune system to fight gastrointestinal cancers, an improved characterisation of the immune microenvironment of these tumours is essential. The analysis of the different immune components will enhance our understanding of the effect of host immune response on tumour growth, invasion and metastasis. In recent years, there have been several efforts to investigate the immune cell populations in gastrointestinal cancers and correlate them with clinicopathological characteristics and clinical outcomes.

Oesophageal Cancer

The two major histologic subtypes of oesophageal cancers are squamous cell carcinoma and adenocarcinoma. Oesophageal squamous cell carcinoma primarily occurs in the upper oesophagus and is associated with tobacco and alcohol exposure, while adenocarcinoma is mostly seen in the lower oesophagus and develops in the setting of intestinal metaplasia due to gastric reflux. However, a common feature of both histologic subtypes is the presence of carcinogen-induced chronic inflammation. Infiltration of tumours by lymphocytes is common and is associated with improved overall survival in both squamous cell carcinoma and adenocarcinoma [15–17]. Specifically, an inverse relationship was observed between tumour-infiltrating CD8+ T cells and tumour grade, stage and lymph node metastasis. In addition, the presence of tumour-infiltrating lymphocytes (TILs) was found to be an independent prognostic factor of prolonged progression-free (PFS) and overall survival (OS) [16]. In addition to CD8+ T-cells, other immune cell populations likely play a significant role in tumour progression in this disease. Infiltration of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the tumour microenvironment was correlated with poor outcomes in patients with oesophageal cancer [18, 19], whereas infiltrating natural killer (NK) cells were associated with a prolonged overall survival [20].

A small number of studies have reported on the expression of PD-1 and its ligands in oesophageal cancer. One such study used gene expression to investigate the clinical significance of PD-L1 and PD-L2 in 41 cases of oesophageal squamous cell carcinoma. In this series, 43.9 % of patients had PD-L1 or PD-L2-positive tumours, determined by real-time quantitative PCR. PD-L1 and PD-L2 positivity was found to be a poor prognostic factor, in both univariate and multivariate analysis [21]. In contrast to squamous cell carcinoma where PD-L1 and PD-L2 expression are equally prevalent, PD-L2 expression seems to be the predominant feature of oesophageal adenocarcinoma. In a large series of 354 oesophageal adenocarcinomas, 51.7 % of the tumours showed moderate-strong epithelial expression of PD-L2, by immunohistochemistry. PD-L1-positive tumour cells were observed in only 1.7 % of cases, whereas PD-L1-positive inflammatory cells (mostly macrophages) were seen in 18 % of cases. PD-1-positive TILs were identified in 58.9 % of tumours, and both PD-L1 and PD-L2-positive tumours had a higher number of PD-1-positive TILs, compared to PD-L1 or PD-L2-negative tumours. Evaluation of clinical correlates in relation to PD-1 and PD-L1/2 expression revealed that tumours with PD-L2 expression were more likely to be early-stage, low-grade and have evidence of intestinal metaplasia (Barrett's oesophagus). In contrast, tumours with PD-L1 expression had no evidence of Barrett's oesophagus. Furthermore, PD-1 positivity in TILs was associated with advance stage of disease, high tumour grade and increased mortality in univariate but not multivariate analysis. PD-L2-positive tumours had a trend towards improved survival, whereas there was no association between PD-L1 expression and clinical outcomes [22]. As the role of PD-L2 in immune evasion is less clear, future studies will need to investigate the functional implications of this phenomenon in oesophageal adenocarcinoma.

Gastric Cancer

Similar to oesophageal cancer, infiltration of gastric tumours by immune cells is a common phenomenon. Increased numbers of TILs were associated with improved outcomes [23], whereas high density of MDSCs was shown to be an adverse prognostic factor in gastric cancer [24]. Interestingly, the role of Tregs is controversial; several studies have shown that high proportion of Tregs is associated with advanced stage and poor survival [18, 25, 26], while others have found a correlation with prolonged survival [24, 27]. The latter finding is surprising but may be a result of the ability of Tregs to control tissue inflammation and therefore suppress tumour progression. Another common feature of gastric cancer is the expression of PD-L1 on tumour cells. In several case series of gastric adenocarcinoma, PD-L1 expression was observed in 42–63 % of tumours and was associated with advanced clinicopathological stage, lymph node mestastasis and increased mortality [28–30].

Recently, comprehensive molecular characterisation of gastric cancer by The Cancer Genome Atlas (TCGA) Network furthered our understanding of the biology of these tumours and led to the identification of four subclasses that were associated with Epstein-Barr virus (EBV), microsatellite instability (MSI), chromosomal instability (CIN), and genomic stability (GS) [31]. This comprehensive analysis also revealed important information about the immunogenicity of two of these subgroups, the EBV-associated tumours and the MSI tumours. The EBV-associated tumours (approximately 15 % of gastric cancers) harbour recurrent amplifications at the 9p24.1 locus, which contains the genes *CD274 (PD-L1)* and *PDCD1LG2 (PD-L2)*. Further transcriptional analysis showed upregulation of *PD-L1* and *PD-L2* gene expression, supporting the evaluation of immune checkpoint blockade in this subtype of gastric cancer. The MSI tumours (21 % of gastric cancers) are characterised by hypermethylation at the *MLH1* promoter. This DNA mismatch-repair deficiency leads to the accumulation of somatic mutations, which results in the abundance of neoantigens that can be recognised by the host immune system. As recent studies in melanoma and non-small cell lung cancer have shown, high mutation rates lead to increased neoantigen burden and are associated with a higher likelihood of response to CTLA-4 and PD-1 inhibition [32, 33]. Therefore, there is a clear rationale for the use of checkpoint inhibition in MSI tumours. The CIN tumours, which account for over 50 % of gastric tumours, are primarily characterised by focal genomic amplifications and deletions, which in many cases are related to *Helicobacter pylori*-induced damage [34]. In preclinical studies, gastric epithelial cells upregulated PD-L1 expression in response to *Helicobacter pylori* exposure. In addition, PD-L1 expression was higher in gastric biopsies from *Helicobacter pylori*-infected subjects compared to non-infected subjects [35].

Colorectal Cancer

Cancers of the colon and rectum are densely infiltrated by inflammatory cells. This immune microenvironment plays an important role in the development and progression of colorectal cancer. Several studies have demonstrated the antitumoural effects

of infiltrating cytotoxic CD8+ T cells. Galon and colleagues analysed the tumour-infiltrating immune cells in a large cohort of stage I to III colorectal cancer samples by immunohistochemistry and gene expression [36]. Tumours from patients without recurrence had higher density of immune cell (CD3+, CD8+, memory CD45RO+ T cells), than did those from patients whose tumours had recurred. Furthermore, immune cells in the centre and the invasive margin of the tumour were analysed. High density of immune cells in both regions predicted for prolonged disease-free and overall survival. A strong immune reaction in both regions was associated with a favourable prognosis regardless of the local extent of the tumour and of lymph node invasion, whereas a weak immune reaction in both the centre of the tumour and the invasive margins correlated with a poor prognosis even in cases with minimal tumour invasion. In addition, patients with low densities of CD3+ cells and CD45RO+ memory T cells in both regions had a poor prognosis, similar to patients with metastatic disease. Additional studies also confirmed the inverse relationship between high densities of cytotoxic CD8+ and memory CD45RO+ T cells and local tumour invasion. Furthermore, they validated the value of CD8+ and CD45RO+ T cell infiltration as a favourable prognostic factor [37, 38].

In contrast to other solid tumours, most studies in colorectal cancer have shown that tumour-infiltrating FOXP3+ Tregs have a protective role against disease progression [39–42]. Salama and colleagues demonstrated that high density of tumour-infiltrating FOXP3+ Tregs was associated with improved survival in patients with stage II and III colorectal cancer [39]. In addition, Correale and colleagues analysed tumour samples from patients with advanced colorectal cancer undergoing chemo- or chemo-immunotherapy. Patients with increased Treg infiltration in their tumours had a longer PFS and OS [40]. While most studies suggest a beneficial role of Tregs in colorectal cancer, some studies did not find a significant correlation between Treg infiltration and outcomes [43, 44]. However, no studies have demonstrated an adverse role of Tregs in colorectal cancer, as has been observed in other malignancies. A possible explanation for this finding is that colorectal cancers develop in a septic environment, where gastrointestinal bacteria have proinflammatory and pro-angiogenic effects that enhance tumour growth through activation of transcription factors, such as NF-kB and STAT3. Thus, by suppressing bacteria-induced inflammation, tumour-infiltrating Tregs may have an anti-tumourigenic effect [45].

A subtype of colorectal cancer that is characterised by dense immune cell infiltration is the microsatellite unstable or mismatch repair deficient (dMMR) subtype. Microsatellite instability, which is observed in approximately 15 % of colorectal cancers, leads to the accumulation of somatic mutations. The majority of these tumours are sporadic and result from inactivation of the DNA mismatch repair system, similar to MSI gastric cancer. Approximately 20 % of dMMR tumours are a consequence of the Lynch syndrome due to inherited mutations of genes involved in the DNA mismatch repair pathway (*MSH2*, *MLH1*, *MSH6*, *PMS2*) [46]. Several studies have shown a strong association between microsatellite instability and the presence of TILs [47–49]. It is postulated that this inflammatory response is the result of immune recognition of neoantigens due to the accumulation of mutations in this subset of colorectal cancer [50]. As dMMR tumours have a low metastatic potential and carry a better prognosis [51], it has been suggested that the stronger

immune response may explain the more favourable outcomes. Guidoboni and colleagues studied the relationship between clinical outcomes and the immune microenvironment in dMMR and MMR-proficient (pMMR) colorectal cancers [52]. Indeed, this study not only confirmed that dMMR tumours have denser cytotoxic CD8+ T cell infiltrates than pMMR tumours, but also showed that within the dMMR group, tumours with a higher number of CD8+ T cells had an even better prognosis. In addition to cytotoxic CD8+ T cells, dMMR tumours are also characterised by higher density of intraepithelial Tregs [53, 54], which may also contribute to better outcomes. To counterbalance this active immune microenvironment, dMMR tumours upregulate the expression of immune checkpoints, including PD-1, PD-L1, CTLA-4, LAG-3 and IDO [54]. As inhibitors against these checkpoints are currently in clinical practice or under development, these findings provide the rationale for their testing in dMMR colorectal cancer.

Pancreatic Cancer

Pancreatic cancer has a unique tumour microenvironment, compared to other gastrointestinal malignancies. Tumour stroma occupies the majority of the tumour mass, with non-neoplastic cells outnumbering cancer cells. One of the most prominent characteristics of pancreatic cancer is its dense desmoplastic stromal reaction, which promotes tumour growth and metastasis, while simultaneously hindering drug delivery [55]. These abundant fibroblasts promote immune evasion, both by serving as a physical barrier to immune cells and producing immunosuppressive cytokines [56, 57]. In contrast to other solid tumours, cytotoxic CD8+ T cells were found in small numbers in the tumour microenvironment and were decreased compared to normal pancreatic tissue and non-malignant chronic pancreatitis [58–60]. Interestingly, Tregs and MDSCs were more prevalent in pancreatic ductal adenocarcinoma [59–61]. The presence of infiltrating cytotoxic CD8+ T cells was associated with a better overall survival [58, 59], while high numbers of Tregs and MDSCs in the tumour microenviroment or the circulation correlated with advanced stage and poor survival [19, 59, 62].

Several studies have investigated the expression of co-inhibitory molecules in the tumour microenvironment of pancreatic ductal adenocarcinoma. PD-L1 expression was upregulated in tumour tissue compared to normal pancreas, with approximately 50 % of pancreatic tumours being PD-L1 positive [63, 64]. Expression of PD-L1 has been associated with advanced tumour stage, high-grade tumours and poor overall survival [63–67]. In addition to PD-L1, overexpression of other co-inhibitory proteins, including CTLA-4, LAG-3, IDO, in the pancreatic cancer cells and the immune microenvironment correlated with advanced stage and poor prognosis [66, 68].

The most prevalent genomic feature of pancreatic ductal adenocarcinoma is the presence of *KRAS* mutations, most commonly *KRAS G12D* [69]. KRAS mediates interactions between tumour cells and the surrounding stroma. In genetically engineered mouse models of pancreatic cancer harbouring KRAS mutations, tumours

cells were shown to secrete cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF), which promoted infiltration of MDSCs and inhibited the accumulation of CD8+ cytotoxic T cells [70, 71]. Abrogation of GM-CSF in these models inhibited the growth of pancreatic cancer by suppressing MDSCs and enhancing CD8+ T cell-driven antitumour immunity. Furthermore, in surgical resection specimens from patients with pancreatic cancer, tumour cells prominently expressed GM-CSF. These findings implicate oncogenic KRAS in restraining the immune response through production of GM-CSF.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) develops in a background of chronic inflammation. Common risk factors for HCC include hepatitis B (HBV) infection, hepatitis C (HCV) infection, alcoholic cirrhosis and non-alcoholic steatohepatitis (NASH) [72, 73]. With the exception of select patients with chronic HBV infection who can develop HCC in the absence of cirrhosis, the vast majority of HCC cases arise in the setting of liver fibrosis and cirrhosis. To prevent excessive tissue injury due to the chronic inflammatory response to viral pathogens or toxins, the liver maintains a strong immunosuppressive milieu. Another contributing factor to this tolerogenic microenvironment is the constant exposure of the liver to gut microbiota and their products through the portal vein circulation. Dendritic cells in the liver become refractory to continuous stimulation by bacteria-derived antigens, known as endotoxin tolerance [74, 75]. Furthermore, chronic HBV and HCV infection lead to exhaustion of cytotoxic CD8+ T cells expressing a variety of co-inhibitory receptors, such as PD-1, CTLA-4, TIM-3 [76, 77]. In addition, the frequency of Tregs in patients with persistent HBV and HCV infection strongly correlated with the viral load, suggesting that these cells prevent viral clearance and may play an important role in the progression to HCC [78].

As premalignant lesions progress to HCC, immune suppression remains evident. In a series of 163 consecutive HCC surgical cases, only 11 (6.7 %) displayed diffuse inflammatory cell infiltration. This immune infiltrate was primarily comprised of T lymphocytes, with a predominance of CD8+ T cells. Interestingly, patients whose tumours had a marked lymphocyte infiltration had a lower 5-year recurrence rate and a higher 5-year overall survival rate, compared to patients with no inflammatory cell infiltration (9.1 % vs. 47.7 %, and 100 % vs. 65.1 %, respectively) [79]. Furthermore, in murine models of HCC, tumours induced profound T cell tolerance by down-regulation of the T cell receptor, which resulted in lack of antigen recognition, decreased interferon-γ (IFN-γ) production and impaired cytotoxicity [80]. In contrast, circulating and intratumoural Tregs were significantly increased in patients with HCC and suppressed CD8+ T cell cytotoxicity by inhibiting the production and release of granzyme A and B and perforin [81]. High frequency of Tregs in both the circulation and the tumours correlated with poor disease-free and overall survival [81, 82]. In addition to Tregs, a significant increase in MDSCs was also observed in patients with HCC, and correlated with tumour progression and stage

[83]. MDSCs were shown to impair NK cell cytotoxicity and induce Tregs, which led to further suppression of the host immune response [84, 85]. In patients who underwent curative radiofrequency ablation therapy, the frequency of MDSCs after treatment was inversely correlated with recurrence-free survival [83].

The PD-1 pathway plays an important role in immune evasion in both chronic hepatitis and HCC. In patients with chronic HBV infection, the percentage of PD-1-expressing, circulating CD8+ and CD4+ T cells increases with disease progression to liver cirrhosis and HCC [86]. Furthermore, upregulation of circulating PD-1/PD-L1 was associated with tumour progression in patients with HBV-associated HCC and was predictive of poor recurrence-free survival post cryoablation [87]. In another series of surgically resected HCC, increased expression of PD-1 and PD-L1 was inversely correlated with disease-free survival after surgery. PD-L1 upregulation in hepatoma cells was shown to promote apoptosis of CD8+ T cells, while PD-L1 blockade restored CD8+ T cell proliferation and enhanced IFN-γ production [88].

Additional co-inhibitory receptors, including CTLA-4, TIM-3, LAG-3, are expressed in the tumour microenvironment of HCC and promote tumour progression by suppressing the immune response. In patients with hepatitis-induced HCC, CD14+ regulatory dendritic cells expressing CTLA-4 inhibited T cell cytotoxicity by producing the immunosuppressive molecules IL-10 and IDO [89]. In addition, TIM-3 expression was significantly increased in macrophages from patients with HCC and correlated with poor survival. Transforming growth factor-β (TGF-β) stimulated TIM-3 expression and facilitated the alternative activation of macrophages, which in turn promoted tumour growth via the NF-kB/IL-6 axis [90]. Moreover, LAG-3 expression was found to be upregulated in tumour-infiltrating CD8+ T cells in HBV-associated HCC and resulted in T cell exhaustion [91].

Clinical Trials of PD-1 and PD-L1 Inhibition in Gastrointestinal Cancers

The unprecedented success of immune checkpoint inhibition in melanoma and other solid tumours has spurred interest in testing these agents in gastrointestinal cancers. Several early phase trials of PD-1 and PD-L1 inhibitors have included patients with gastrointestinal malignancies and showed promising results. Based on these results, larger trials are currently being pursued.

Oesophageal Cancer

Treatment options for unresectable or metastatic oesophageal cancer are limited. Two recent studies investigating the safety and efficacy of PD-1 blockade in refractory advanced oesophageal cancer have shown encouraging results.

Pembrolizumab, a highly selective humanised monoclonal antibody against PD-1, was evaluated in the multicohort, phase Ib KEYNOTE-028 trial [92]. The oesophageal cohort of this study included patients with advanced or metastatic oesophageal squamous cell carcinoma or adenocarcinoma, who had progressed on standard therapy and whose tumours were PD-L1 positive (defined as PD-L1 immunohistochemical staining in the stroma or in ≥1 % of tumour cells). Pembrolizumab was given at a dose of 10 mg/kg every 2 weeks for up to 2 years or until confirmed progression or unacceptable toxicity. Of the 90 patients with oesophageal cancer who were screened, 37 (41 %) had PD-L1+ tumours. Of the 23 patients enrolled in the study, 77 % had squamous cell carcinoma, 18 % had adenocarcinoma and 5 % had mucoepidermoid carcinoma. The vast majority of patients (87 %) had received at least two prior therapies for metastatic disease. After a median follow-up duration of 7.1 months, pembrolizumab demonstrated an objective response rate (ORR) of 30.4 %, while 13 % of patients had disease stabilisation. Of 17 patients with squamous cell carcinoma, 5 (29.4 %) achieved an objective response, while the ORR in patients with oesophageal adenocarcinoma was 40 % (2 of 5 patients). The 6-month and 12-month PFS were 30.4 % and 21.7 %, respectively. Interestingly, the median duration of response was 40 weeks, ranging from 24.1 to 46.1 weeks (Table 6.1). In an effort to identify a biomarker of response to pembrolizumab, a 6-gene signature associated with IFN-γ immune response was selected (IDO1, CXCL10, CXCL9, HLA-DRA, STAT1, IFN-γ). Patients whose tumours had a higher signature score had a higher ORR to PD-1 blockade (43 % for the high signature score group vs. 11 % for the low signature score group).

Nivolumab, a fully human monoclonal IgG4 antibody specific for PD-1, was tested in a phase II trial for patients with advanced oesophageal cancer [93]. In this study, patients with advanced oesophageal squamous cell carcinoma who had failed standard therapies were treated with nivolumab 3 mg/kg every 2 weeks. Of 65 patients, 44 (66.7 %) had at least 3 prior lines of therapy. In this heavily pretreated cohort, 11 (17.2 %) of 64 evaluable patients experienced an objective response [1 complete response (CR) and 10 partial responses (PR)], while 25.0 % of patients had stable disease. The median PFS was 1.5 months and the median OS was 10.8 months. As has been observed with PD-1 inhibition in other solid tumours, patients who had CR or PR had durable responses (Table 6.1).

Table 6.1 Completed trials of PD-1/PD-L1 inhibitors in oesophageal cancer

Phase	Regimen	Patient population	Results
I	Pembrolizumab (anti-PD-1 antibody)	Advanced solid tumours, including oesophageal cancer	ORR: 30.4 % (29.4 % in squamous cell carcinoma vs. 40 % in adenocarcinoma); 12-month PFS: 21.7 %
II	Nivolumab (anti-PD-1 antibody)	Advanced oesophageal squamous cell carcinoma	ORR: 17.2 %; median PFS: 1.5 months; median OS:10.8 months

ORR objective response rate, *PFS* progression-free survival, *OS* overall survival

Based on these promising results, two large, randomised phase III trials of PD-1 blockade in oesophageal cancer have been initiated. The first study is evaluating the efficacy of pembrolizumab versus standard chemotherapy in patients with advanced oesophageal cancer who have progressed after first-line therapy (NCT02564263). The second study is designed to compare nivolumab with taxane chemotherapy in patients with unresectable advanced or recurrent oesophageal cancer in the second-line setting (NCT02569242). Several studies are also investigating the efficacy of PD-L1 inhibition in this patient population. In addition to PD-1 monotherapy, combinations of PD-1 and PD-L1 inhibitors with chemotherapy, radiation therapy or targeted therapy in the metastatic setting are being explored. Furthermore, PD-1 monoclonal antibodies are being tested in the adjuvant and neoadjuvant setting.

Gastric Cancer and Gastroesophageal Junction Cancer

Initial results of PD-1 pathway blockade in patients with gastric cancer were disappointing. Of seven patients with metastatic gastric cancer enrolled in the multicohort phase I trial of the anti-PD-L1 antibody BMS-936559, no objective responses were observed [12]. However, other PD-L1 inhibitors have shown promising activity in early phase trials, despite the small number of patients enrolled in those trials. In a multi-arm phase I trial of durvalumab or MEDI4736, 1 of 16 patients with advanced gastroesophageal cancer achieved a complete response, while 3 patients had partial responses (ORR 25%). Eight additional patients had stable disease, resulting in a disease-control rate (DCR) of 75% [94]. In addition, a phase I trial of atezolizumab (MPDL3280A) included one patient with metastatic gastric adenocarcinoma, who had a durable partial response lasting more than 48 weeks [95]. Based on these encouraging results, larger trials evaluating the efficacy of these inhibitors in advanced gastric and GEJ cancer are currently underway.

Monoclonal antibodies against PD-1 have been more rigorously evaluated in metastatic gastric adenocarcinoma. In KEYNOTE-012, a large multi-arm phase Ib trial investigating the activity of pembrolizumab in advanced solid tumours, 162 patients with previously treated metastatic gastric cancer were screened for PD-L1 positivity (defined as PD-L1 immunohistochemical staining in the stroma or in $\geq$1% of tumour cells). Of these patients, 40% (65) were found to be PD-L1-positive and 39 were enrolled in the trial. At a median follow up of 8.8 months, 13 (33%) patients remained on therapy and the ORR was 22% by central review. The median time to response was 8 weeks with a median duration of response of 40 weeks. Median PFS was 1.9 months; however, 26% of patients remained progression-free at 6 months. Median OS was 11.4 months, with a 6-month OS rate of 66%. In this preselected PD-L1-positive patient population, there was preliminary evidence that high PD-L1 expression was associated with ORR (1-sided $P=0.1$) [96]. Pembrolizumab is currently being evaluated in large phase III trials either in combination with cytotoxic chemotherapy in the first-line setting or as monotherapy in the second-line setting in patients with advanced or metastatic gastric or GEJ adenocarcinoma (NCT02494583, NCT02370498).

Table 6.2 Completed trials of PD-1/PD-L1 inhibitors in gastric and GEJ cancer

Phase	Regimen	Patient population	Results
I	Pembrolizumab (anti-PD-1 antibody)	Advanced solid tumours, including gastric or GEJ adenocarcinoma	ORR 22 %
I	Durvalumab (MEDI4736, anti-PD-L1 antibody)	Advanced solid tumours, including gastroesophageal adenocarcinoma	Of 16 patients, 4 had objective responses, including one complete response
I/II	Nivolumab (anti-PD-1 antibody)	Advanced solid tumours, including gastric or GEJ adenocarcinoma	ORR 14 % (ORR 27 % in PD-L1 positive patients vs. 12 % in PD-L1 negative patients)
I/II	Nivolumab 1 mg/kg + Ipilimumab 3 mg/kg (N1 + I3) vs. Nivolumab 3 mg/kg + Ipilimumab 1 mg/kg (N3 + I1) vs. Nivolumab monotherapy (N3)	Advanced solid tumours, including gastric or GEJ adenocarcinoma	ORR: N1 + I3 26 % vs. N3 + I1 10 % vs. N3 14 %; OS: N1 + I3 6.9 months vs. N3 + I1 4.8 months vs. N3 5.0 months

GEJ gastroesophageal junction, *ORR* objective response rate, *OS* overall survival

The efficacy of nivolumab has also been investigated in metastatic gastric and GEJ cancer. The phase I/II CheckMate-032 study included 59 patients with advanced gastric or GEJ cancer, regardless of PD-L1 status. In this heavily pretreated patient population, the confirmed ORR was 14 % and DCR was 32 %. Patients with PD-L1-positive tumours had an ORR of 27 %, while only 12 % of patients with PD-L1-negative tumours had objective responses. The median duration of response was 7.1 months and the 12-month OS rate for the entire cohort was 36 % [97]. A phase III trial of nivolumab monotherapy after failure of standard therapies is currently ongoing (NCT02267343).

The results of completed trials of PD-1 and PD-L1 inhibitors in gastric and GEJ cancer are summarised in Table 6.2.

Colorectal Cancer

Preclinical studies of PD-1 inhibition in colorectal cancer generated great enthusiasm for the use of these novel therapeutic approaches in this disease. Iwai and colleagues intravenously injected wildtype and PD-1 knockout mice (PD-$1^{-/-}$) with murine colon cancer cells, establishing a syngeneic mouse model of colon cancer. Dissemination of tumour cells to the lung was significantly decreased in the PD-$1^{-/-}$ mice. Similar results were observed when mice were treated with PD-1 monoclonal antibodies [98]. In a more recent study utilising the same syngeneic mouse models, response rates to PD-1, PD-L1 and CTLA-4 monotherapy were 25 %, 33 % and 50 % respectively. Combinations of PD-1 or PD-L1 inhibitors with CTLA-4

blockade had an additive effect with a response rate of 75 %. Further addition of the GVAX cancer vaccine to dual checkpoint inhibition led to complete eradication of the murine tumours [99].

Despite these promising preclinical data, the initial results of early phase trials of PD-1 and PD-L1 inhibition in colorectal cancer were disappointing. In the phase I trial of nivolumab in solid tumours, 19 patients with heavily pretreated metastatic colorectal cancer were included. However, no objective responses were observed in this cohort [11]. Additionally, no clinical activity was observed with the anti-PD-L1 monoclonal antibody BMS-936559 in a phase I trial, which included 18 patients with metastatic colorectal cancer [12]. Interestingly, in another phase I study of the PD-L1 antibody atezolizumab, one of four patients with metastatic colorectal cancer achieved a partial response [95]. Furthermore, the combination of atezolizumab with the anti-VEGF-A monoclonal antibody bevacizumab with or without cytotoxic chemotherapy was explored in a phase Ib study. Patients with refractory metastatic colorectal cancer were treated with atezolizumab and bevacizumab, while chemotherapy naïve patients were treated with atezolizumab, bevacizumab and cytotoxic chemotherapy (5-fluorouracil, leucovorin, oxaliplatin [FOLFOX]). Preliminary results from this trial showed an unconfirmed ORR of 7 % (1 of 14 patients) in the refractory cohort, and 48 % (11 of 23 patients) in the chemotherapy naïve cohort [100], which is similar to previously reported response rates with the combination of FOLFOX and bevacizumab in the first-line setting (ORR 45 %) [101].

Despite the disappointing results of PD-1 and PD-L1 blockade in unselected patients with colorectal cancer, a small subset seem to have better responses. Interestingly, among 33 patients with colorectal cancer that were enrolled in the early phase trials of PD-1 inhibitors, only one patient had an objective response. This patient, whose tumour was later found to be mismatch-repair deficient, achieved a complete response and remained disease-free for 3 years [102]. Based on this anecdotal case, a phase II clinical trial evaluating the effect of pembrolizumab in patients whose tumours had or did not have MMR-deficiency was initiated [103]. This study included 41 patients; 20 patients with dMMR colorectal and noncolorectal cancers and 21 patients with pMMR colorectal cancers. Among 10 patients with dMMR colorectal cancer, 4 (40 %) patients achieved objective responses and 5 (50 %) patients had stable disease. In contrast, no patient with pMMR colorectal cancer had an objective response and only 2 patients (11 %) experienced disease stabilisation. PFS at 20 weeks was 78 % in the dMMR colorectal cancer cohort and 11 % in the pMMR colorectal cancer group. Notably, patients with dMMR non-colorectal cancer had responses similar to those of patients with dMMR colorectal cancer (71 % [5 of 7 patients]). Based on these results, the clinical activity of pembrolizumab is currently being evaluated in a larger, single-arm phase II (NCT02460198) and a randomised phase III trial (NCT02563002) for patients with metastatic dMMR colorectal cancer. In addition, a phase II trial of durvalumab in dMMR colorectal cancer is currently enrolling patients (NCT02227667). Further research is needed to understand why immune checkpoint inhibition has largely failed in pMMR colorectal cancer and to develop strategies that will enhance the anti-tumour effect of immunotherapeutic approaches in this disease.

A summary of the completed trials of PD-1 and PD-L1 inhibitors is provided in Table 6.3.

Table 6.3 Completed trials of PD-1/PD-L1 inhibitors in colorectal cancer

Phase	Regimen	Patient population	Results
I	Nivolumab (anti-PD-1 antibody)	Advanced solid tumours, including CRC	Of 19 patients, no objective responses were observed
I	BMS-936559 (anti-PD-L1 antibody)	Advanced solid tumours, including CRC	Of 18 patients, no objective responses were observed
I	Atezolizumab (anti-PD-L1 antibody)	Advanced solid tumours, including CRC	Of 4 patients, one achieved a partial response
Ib	Atezolizumab (anti-PD-L1 antibody) + Bevacizumab +/– FOLFOX	Metastatic CRC (chemotherapy naïve and refractory)	ORR: 7 % in the refractory cohort (atezolizumab + bevacizumab), 48 % in the chemotherapy naïve cohort (atezolizumab + bevacizumab + FOLFOX)
II	Pembrolizumab (anti-PD-1 antibody)	Metastatic CRC, dMMR vs. pMMR	ORR: 40 % in the dMMR group vs. 0 in the pMMR group; PFS at 20 weeks: 78 % in the dMMR group vs. 11 % in the pMMR group

CRC colorectal cancer, *dMMR* mismatch repair deficient, *pMMR* mismatch repair proficient, *ORR* objective response rate

Pancreatic Cancer

Pancreatic cancer is considered a non-immunogenic tumour due to the paucity of T cell infiltrates in the tumour microenvironment. Therefore, immune checkpoint inhibitors that restore T cell function are unlikely to be effective in such an environment. The first trials of PD-1 and PD-L1 blockade were disappointing. In the multi-cohort phase I trial of the PD-L1 antibody BMS-936559, no objective responses were seen in patients with pancreatic cancer [12]. The only immune checkpoint inhibitor that has shown some activity in pancreatic cancer is the anti-PD-L1 monoclonal antibody durvalumab. Preliminary results of the multi-cohort phase Ib trial of durvalumab demonstrated an ORR of 7 % (2 of 29 patients) and a DCR of 21 % (6 of 29 patients) [104] (Table 6.4).

Hepatocellular Carcinoma

Following the encouraging results of CTLA-4 blockade in HCC [105], PD-1 pathway inhibition was tested in this disease. Clinical activity of PD-L1 blockade was initially observed in the multi-cohort phase I dose-expansion study of durvalumab. Of 21 HCC patients enrolled in the trial, 5 had HBV-associated disease and 4 had HCV-associated HCC. Of 20 evaluable patients, one patient achieved an objective response, while the DCR at 12 weeks was 25 % (5 of 20 patients) [104].

Table 6.4 Completed trials of PD-1/PD-L1 inhibitors in pancreatic ductal adenocarcinoma and hepatocellular carcinoma

Phase	Regimen	Patient population	Results
Ib	Durvalumab (anti-PD-L1 antibody)	Metastatic pancreatic cancer	ORR: 7%; DCR: 21%
I/II	Nivolumab (anti-PD-1 antibody)	Advanced HCC	ORR: 19%; ORR: 10% in HBV group vs. 36% in HCV group vs. 14% in the uninfected group; OS at 12 months: 62%

HCC hepatocellular carcinoma, *ORR* objective response rate

Further evidence of the efficacy of PD-1 blockade was seen in the phase I/II trial of nivolumab in patients with advanced HCC [106]. The study included 47 patients with advanced HCC with Child-Pugh score ≤B7 and progressive disease on, or intolerant of sorafenib. Patients were divided into three cohorts according to their hepatitis viral status. Of all patients, 11 (23%) were HBV-positive, 12 (26%) were HCV-positive and 24 (51%) were not infected by either virus. The majority of patients had extrahepatic metastases (33 of 47, 70%) and had received prior sorafenib (32 of 47, 68%). Of 42 evaluable patients, 8 achieved an objective response (2 CR and 6 PR), with responses extending beyond 12 months in 4 out of 8 responders. In addition, 20 patients (48%) had disease stabilisation. Notably, the OS rate at 9 and 12 months were 70% and 62%, respectively. Objective responses were seen in all three cohorts with 1 responder (10%) in the HBV group, 4 responders (36%) in the HCV group and 3 responders (14%) in the uninfected group (Table 6.4). The study has been expanded to include two additional cohorts, one comparing the efficacy of nivolumab with sorafenib, and the other one exploring the combination of nivolumab and ipilimumab for the treatment of patients with advanced HCC (NCT01658878). An additional phase III trial comparing the efficacy of nivolumab with sorafenib as first-line therapy for advanced HCC is currently recruiting patients (NCT02576509).

Biomarkers of Response to PD-1 Pathway Inhibition

It has become clear that only a subset of patients with gastrointestinal malignancies respond to PD-1/PD-L1 inhibitors. Therefore, to improve patient outcomes we need to optimise patient selection. Development of biomarkers that can predict which patients will derive benefit from these agents is an area of active investigation.

PD-L1 expression as a potential biomarker has been studied extensively in several malignancies. In the phase I study of nivolumab, PD-L1 expression on tumour cells was assessed by immunohistochemistry in 42 tumours. There was a significant correlation between PD-L1 expression and response to PD-1 therapy across multiple tumour types, including colorectal cancer. Of 25 patients with PD-L1–positive

tumours (defined as ≥5 % of tumour cells expressing PD-L1), 9 (36 %) responded to nivolumab, whereas no patients with PD-L1–negative tumours responded to therapy [11]. Herbst and colleagues reported a significant correlation between PD-L1 expression in the tumour-infiltrating immune cells and response to anti-PD-L1 therapy in several tumour types, including gastric cancer [95]. In this study, patients whose tumours expressed PD-L1 in ≥5 % of infiltrating immune cells had an ORR of 34 %, whereas 16 % of patients whose tumours expressed PD-L1 in ≤5 % of immune cells responded to anti-PD-L1 therapy. Similar findings were reported in melanoma with PD-L1 expression in the tumour cells being predictive of response to pembrolizumab, as well as prolonged PFS. However, 9–20 % of patients with PD-L1–negative tumours had a durable response to pembrolizumab, and median OS did not differ significantly between patients with PD-L1-positive and negative tumours [107]. These results demonstrate that, although PD-L1 expression correlates with higher response rates to PD-1 and PD-L1 inhibitors, patients without PD-L1 expression may also benefit from these therapies.

Few biomarkers studies in gastrointestinal malignancies have been reported. In the KEYNOTE-012 trial of pembrolizumab in metastatic gastric cancer, there was a trend towards an association between high PD-L1 expression and ORR (1-sided $P=0.10$) [96]. However, all patients in this study had PD-L1-positive tumours (immunohistochemical staining in the stroma or in ≥1 % of tumour cells). In contrast, in the CheckMate-032 study, which included patients with gastric tumours regardless of PD-L1 status, PD-L1 expression was associated with higher ORR to nivolumab (27 % vs. 12 %) [97].

Several technical and biological issues currently preclude the use of PD-L1 expression as a robust predictive biomarker. These include: (1) differing definitions of PD-L1 positivity threshold by immunohistochemistry, (2) variable detection antibodies, (3) use of archival versus fresh biopsies, (4) heterogeneous expression of PD-L1 in the primary tumour and metastatic sites, (5) evaluation of staining in the tumour cells or the infiltrating immune cells [108]. Further standardisation of PD-L1 immunohistochemical assays will be essential for the development of useful predictive models.

In addition to PD-L1, efforts to identify other biomarkers are currently ongoing. Herbst and colleagues utilised gene expression profiling to predict responses to anti-PD-L1 therapy. Tumours in responders to PD-L1 blockade demonstrated elevated expression of IFN-γ and IFN-γ-inducible genes (e.g. *IDO1* and *CXCL9*) [95]. Similar analysis was performed in gastric tumours from patients enrolled in KEYNOTE-012. Patients with low expression of IFN-γ and TCR-signalling signatures did not respond to pembrolizumab [109]. Furthermore, as noted above, high mutational burden is associated with better responses to immune checkpoint blockade [32, 33, 103]. However, further work is needed to determine which mutations are more likely to generate neoantigens that can induce a robust anti-tumour response. Lastly, establishing predictive biomarkers of resistance to PD-1 pathway blockade is also essential. Recent studies have explored the role of the WNT/β-catenin pathway in resistance to immune checkpoint inhibitors. Gene expression profiling across tumour types included in TCGA was used to classify tumours as

T-cell inflamed or non-inflamed. Genomic sequencing revealed that tumours lacking a T cell infiltrate had activation of the WNT/β-catenin pathway, by activating mutations in *CTNNB1*, inactivating mutations in *APC* or overexpression of other components of the pathway [110]. As the WNT/β-catenin pathway is commonly activated in colon cancer, these data may explain the lack of response of these tumours to immune checkpoint inhibition. These and other assays will be critical in our efforts to identify patients who will benefit most from immune checkpoint blockade.

Combination Approaches Involving PD-1/PD-L1 Blockade

Inhibitors of PD-1 or PD-L1 have achieved durable responses in some patients with gastrointestinal cancers. In order to enhance the anti-tumour benefit of these immune checkpoint inhibitors, we need to understand the mechanisms of immune escape and identify other therapies that may synergise with these agents. Rational combinations of PD-1 and PD-L1 inhibitors with other immunotherapies or conventional therapies are currently being explored in clinical trials (Fig. 6.1).

Combinations of PD-1/PD-L1 Blockade and Other Immune Checkpoint Inhibitors

Combinations of anti-PD-1/PD-L1 monoclonal antibodies and other immune checkpoint inhibitors may lead to more robust antitumour effects in gastrointestinal cancers. Indeed, in advanced melanoma, the combination of PD-1 and CTLA-4 blockade demonstrated a synergistic effect compared to either inhibitor alone [111]. The combination of PD-1 and CTLA-4 inhibition is currently being explored in gastric, colorectal, hepatocellular and pancreatic cancer. Preliminary analyses of the combination of nivolumab and ipilimumab in gastric, GEJ and dMMR colorectal cancer have been reported. In CheckMate-032, 160 patients with metastatic gastric or GEJ cancer were treated with nivolumab alone (3 mg/kg) or in combination with ipilimumab (two cohorts; nivolumab 1 mg/kg + ipilimumab 3 mg/kg or nivolumab 3 mg/kg + ipilimumab 1 mg/kg). The ORR was 14 % for the nivolumab alone group (N3), 26 % for the nivolumab 1 mg/kg + ipilimumab 3 mg/kg group (N1 + I3) and 10 % for the nivolumab 3 mg/kg + ipilimumab 1 mg/kg group (N3 + I1). Median OS was 5.0 months (95 % CI, 3.4–12.4 months) for the N3 group, 6.9 months (95 % CI, 3.6 months to NR) for the N1 + I3 group and 4.8 months (95 % CI, 3.0–9.1 months) for the N3 + I1 group [112]. The interim analysis of CheckMate-132 included 56 patients with dMMR metastatic colorectal cancer, treated with nivolumab 3 mg/kg (N3) or nivolumab 3 mg/kg and ipilimumab 1 mg/kg (N3 + I1). The ORR was 27 % for the N3 group and 15 % for the N3 + I1. Despite the higher response rate in the monotherapy group, the combination was superior with regard to PFS and OS. The 4-month PFS was 55 % for the N3 group and 80 % for the N3 + I1 group, while the

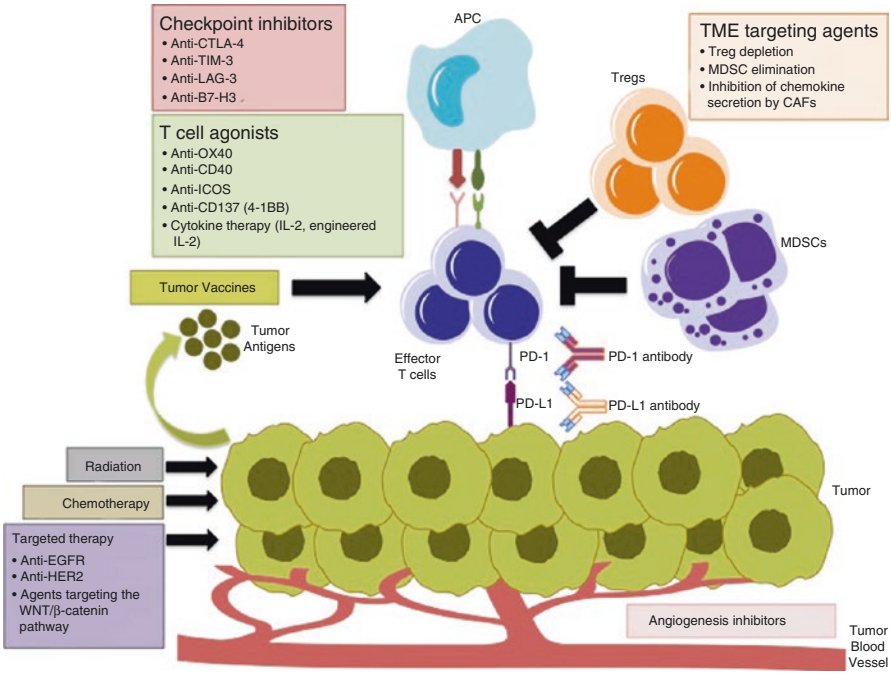

Fig. 6.1 Combination strategies with PD-1/PD-L1 inhibitors. Inhibitors of other immune checkpoints and T cell agonists can further increase T cell activity when combined with PD-1/PD-L1 inhibitors. Elimination of the immunosuppressive components of the tumour microenvironment (Tregs, MDSCs, VEGF) can enhance the anti-tumour effects of PD-1 blockade. Direct targeting of the tumour with radiation, cytotoxic chemotherapy or targeted therapy induces cancer cell death, increases tumour antigen presentation and promotes activation of effector T cells. Similarly, vaccines against tumour antigens can initiate an anti-tumour immune response by enhancing the influx of T cells into the tumour, and synergise with PD-1 pathway blockade. *APC* antigen-presenting cell, *Tregs* regulatory T cells, *MDSCs* myeloid derived suppressor cells, *TME* tumour microenvironment

5-month OS was 75% for the N3 group and 100% for the N3+I1 group [113]. These preliminary results demonstrate a promising clinical activity of immune checkpoint inhibitor combinations in gastrointestinal malignancies. Further research is needed to identify patients who are more likely to respond to these combinations compared to PD-1 monotherapy.

As noted above, several other co-inhibitory receptors, such as TIM-3, LAG-3, B7-H3, are expressed in gastrointestinal tumours and are associated with poor prognosis [54, 66, 68, 89, 90]. Preclinical studies in murine models of colon cancer have demonstrated synergy between PD-1 and LAG-3 blockade, leading to complete regression of the tumours in the majority of mice treated with this combination [114]. A large number of early phase trials are currently investigating the safety and efficacy of inhibitors targeting LAG-3 and other immune checkpoints in combination with PD-1 blockade in advanced solid tumours, including gastrointestinal malignancies (Table 6.5).

Table 6.5 Selected ongoing trials of therapeutic combinations with PD-1/PD-L1 blockade

Phase	Agents	Patient population	NCT identifier
A. Other immune check point inhibitors			
Ib/II	Durvalumab + Tremelimumab (anti-CTLA-4)	Recurrent or metastatic gastric and GEJ cancer	NCT02340975
II	Durvalumab + Tremelimumab (anti-CTLA-4)	Unresectable HCC	NCT02519348
I-Ib/II	PDR001 (PD-1 antibody) + MBG453 (anti-TIM-3 antibody)	Advanced or metastatic solid tumours	NCT02608268
I/IIa	Nivolumab + BMS-986016 (anti-LAG-3 antibody)	Advanced or metastatic gastric cancer, HCC, CRC	NCT01968109
I	Pembrolizumab + MGA271 (anti-B7-H3 antibody)	Advanced pancreatic cancer and B7-H3 expressing CRC	NCT02475213
B. T cell agonists			
I	Durvalumab + MEDI6383 (OX40 agonist)	Advanced solid tumours	NCT02221960
I	Durvalumab + Tremelimumab + MEDI0562 (OX40 agonist)	Advanced solid tumours	NCT02705482
I	Pembrolizumab + GSK3359609 (ICOS agonist)	Advanced solid tumours, including oesophageal cancer and CRC	NCT02723955
I	Nivolumab + Urelumab (CD137 agonist)	Advanced solid tumours	NCT02534506
I	Nivolumab + Varilumab (CD27 agonist)	Advanced solid tumours, including CRC	NCT02335918
I	Atezolizumab + RO7009789 (CD40 agonist)	Advanced solid tumours	NCT02304393
C. Targeted therapy			
Ib/II	Pembrolizumab + Trastuzumab (anti-HER2 antibody) or Cetuximab (anti-EGFR antibody)	HER2 overexpressing unresectable gastric and GEJ cancer, unresectable KRAS, NRAS and BRAF wildtype CRC	NCT02318901
Ib/II	Pembrolizumab + Margetuximab (anti-HER2 antibody)	Advanced or metastatic HER2+ gastric and GEJ cancer	NCT02689284
Ib/II	Pembrolizumab + Cetuximab (anti-EGFR antibody)	Metastatic KRAS and NRAS wildtype CRC	NCT02713373
D. Agents targeting the tumour microenvironment			
I	Pembrolizumab + Ramucirumab (anti-VEGFR2 antibody)	Advanced or metastatic gastric and GEJ cancer	NCT02443324
I	Durvalumab + Ramucirumab (anti-VEGFR2 antibody)	Advanced gastric and GEJ cancer, HCC	NCT02572687
I	Atezolizumab + Bevacizumab (anti-VEGF antibody) + chemotherapy	Advanced or metastatic gastric and GEJ cancer, metastatic pancreatic cancer, HCC	NCT02715531

Table 6.5 (continued)

I	Durvalumab + Mogamulizumab (anti-CCR4 antibody)	Advanced solid tumours, including pancreatic cancer	NCT02301130
I/IIa	Pembrolizumab + PLX3397 (CSF-1R inhibitor)	Advanced solid tumours, including pancreatic cancer	NCT02452424
I/II	Durvalumab + Epacadostat (IDO inhibitor)	Advanced solid tumours, including gastric and GEJ cancer	NCT02318277
I/II	Nivolumab + Epacadostat (IDO inhibitor)	Advanced solid tumours, including CRC	NCT02327078
I/II	Nivolumab + Ulocuplumab (anti-CXCR4 antibody)	Advanced pancreatic cancer	NCT02472977
E. Cytotoxic chemotherapy			
II	Pembrolizumab + cisplatin/5-FU	Recurrent or metastatic gastric and GEJ cancer	NCT02335411
I/IIA	Pembrolizumab + mFOLFOX6	Advanced or metastatic gastric and GEJ, CRC, biliary tract and pancreatic cancer	NCT02268825
Ib/II	Nivolumab + irinotecan/capecitabine	Advanced solid tumours, including CRC and pancreatic cancer	NCT02423954
Ib/II	Pembrolizumab + gemcitabine/ nab-paclitaxel	Metastatic pancreatic cancer	NCT02331251
F. Radiation therapy			
Ib/II	Neoadjuvant Pembrolizumab + chemoradiotherapy	Locally advanced GEJ or gastric cardia cancer that can be surgically removed	NCT02730546
Ib/II	Neoadjuvant Pembrolizumab + chemoradiotherapy	Resectable or borderline resectable pancreatic cancer	NCT02305186
II	Pembrolizumab + radiotherapy or ablation	Metastatic CRC	NCT02437071
II	Neoadjuvant Pembrolizumab + chemoradiotherapy	Stage II-III rectal cancer	NCT02586610
G. Vaccines			
II	Nivolumab + GVAX + CRS-207	Metastatic pancreatic cancer	NCT02243371
II	Adjuvant or Neoadjuvant Nivolumab + GVAX	Resectable pancreatic cancer	NCT02451982
II	Pembrolizumab + GVAX + stereotactic radiotherapy	Locally advanced pancreatic cancer	NCT02648282

GEJ gastroesophageal junction, *CRC* colorectal cancer, *HCC* hepatocellular carcinoma

Combinations of PD-1/PD-L1 Blockade and T-Cell Agonists

An alternative strategy to augment the efficacy of PD-1 inhibition is to increase T cell activity. Several co-stimulatory molecules, including members of the TNF receptor family (OX40, CD137 [4-1BB] CD40 and CD27) and members of the B7-CD28 family members (ICOS and B7-H3), lead to enhanced memory and effector T cell responses, increased T cell survival and regression of tumours in murine models of solid tumours [115–119].

Specifically, in preclinical models of colorectal cancer, activation of CD137 with an agonistic monoclonal antibody enhanced NK cell degranulation and cytotoxicity. Combined anti-EGFR and anti-CD137 therapy was synergistic and resulted in complete tumour resolution and prolonged survival [120]. In addition, the clinical impact of a CD40 agonist monoclonal antibody in combination with gemcitabine chemotherapy was evaluated in a phase I clinical trial for patients with chemotherapy-naïve advanced or metastatic pancreatic cancer. Of 21 patients enrolled in the trial, 4 patients (19%) achieved PR and 11 patients had stable disease. Median PFS was 5.6 months and median OS was 7.4 months. Gemcitabine monotherapy historically achieves an ORR of 5.4% with median PFS of 2.3 months and median OS of 5.7 months. Therefore, the combination of anti-CD40 and gemcitabine showed promising efficacy in patients with metastatic pancreatic cancer [121].

Based on these encouraging results, combinations of PD-1/PD-L1 inhibitors and T-cell agonists are currently being explored in early phase trials (Table 6.5).

Combinations of PD-1/PD-L1 Blockade and Targeted Therapies

Monoclonal antibodies targeting the human epidermal growth factor (HER) family receptors have been in clinical use in gastrointestinal malignancies for many years. Specifically, the anti-EGFR monoclonal antibodies, cetuximab and panitumumab, are approved for the treatment of *KRAS* wild-type advanced or metastatic colorectal cancer, while the anti-HER2 monoclonal antibody, trastuzumab, is approved as first-line therapy of metastatic, HER2-positive gastroesophageal cancer. In addition to blocking the EGFR/HER2 signalling pathway, these therapies have a significant impact on the tumour immune microenvironment by promoting antibody-dependent cellular cytotoxicity. Preclinical studies have demonstrated that the therapeutic effect of anti-EGFR and anti-HER2 monoclonal antibodies is dependent on CD8+ and NK cells [122, 123]. In addition, studies in immunocompetent mouse models of HER2-positive breast cancer suggest that PD-1 blockade can significantly improve the therapeutic efficacy of anti-HER2 monoclonal antibody therapy [123]. This potential synergistic effect of PD-1 inhibition and anti-EGFR or anti-HER2 therapy is currently being investigated in *KRAS* wild-type colorectal cancer and HER2-positive gastric and oesophageal cancer, respectively (Table 6.5).

Combinations of PD-1/PD-L1 Blockade and Agents Targeting the Tumour Microenvironment

Another approach to enhance the efficacy of PD-1/PD-L1 inhibitors is to combine them with agents targeting other immunosuppressive components of the tumour microenvironment.

Emerging data show that VEGF plays an important role in tumour immune evasion. In addition to promoting tumour angiogenesis, VEGF blocks the maturation of dendritic cells and therefore impairs antigen presentation, inhibits lymphocyte trafficking through the vasculature into tumours and enhances T cell exhaustion by upregulating immune checkpoint expression [124–128]. Angiogenensis inhibitors, particularly anti-VEGFA monoclonal antibodies, have been shown to augment the anti-tumour immune response and synergise with PD-1 blockade in both preclinical and early clinical studies [128, 129]. In a mouse model of colorectal cancer expressing high levels of VEGF-A, combined therapy with anti-PD-1 and anti-VEGF-A monoclonal antibodies led to a significant decrease in tumour volume, compared to either agent alone. Of note, anti-VEGF-A therapy decreased the proportion of tumour-infiltrating CD8+ T cells expressing immune checkpoints [128]. In patients with metastatic melanoma, combination therapy with bevacizumab and ipilimumab enhanced lymphocyte and macrophage trafficking leading to a more robust immune cell infiltration into the tumour, compared to ipilimumab monotherapy [129]. These studies provide the mechanistic foundation for combining antiangiogenic therapy with immune checkpoint blockade. Clinical trials evaluating the efficacy of combinatorial treatment with PD-1 blockade and monoclonal antibodies targeting VEGF-A or its receptor are currently ongoing in a variety of gastrointestinal malignancies (Table 6.5).

An additional approach to augment anti-PD-1 therapy is the depletion of Tregs. Chemokine receptor 4 (CCR4) is expressed selectively in effector Tregs, which promote immunosuppression. Preclinical in vitro studies demonstrated that CCR4 blockade led to the elimination of Tregs and the induction of T-cell responses specific to human tumour antigens. In addition, administration of the anti-CCR4 monoclonal antibody mogamulizumab to patients with adult T-cell leukaemia-lymphoma selectively depleted effector Tregs, enhanced immune responses and significantly reduced tumour cells [130]. Combination of mogamulizumab with either PD-L1 or CTLA-4 inhibitors is currently being tested in patients with metastatic pancreatic cancer (Table 6.5).

Critical drivers of the immune escape in gastrointestinal malignancies include tumour-associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs). These cells not only contribute to immune suppression, but also promote cancer cell proliferation, mediate resistance to cytotoxic chemotherapy and augment the metastatic potential of solid tumours [131, 132]. Colony stimulating factor 1 receptor (CSF1R) is expressed on TAMs and MDSCs and can be targeted with monoclonal antibodies. In preclinical models of pancreatic cancer, CSF1/CSF1R blockade was shown to decrease the number of TAMs and reprogram remaining

TAMs to support antigen presentation and bolster T cell activation within the tumour microenvironment. This in-turn led to reduced immune suppression and elevated interferon responses, which restrained tumour progression. However, inhibition of CSF1R also increased the levels of PD-L1 and CTLA-4 in these tumours, potentially limiting its long-term efficacy [133]. One rational strategy to overcome immune evasion is to combine PD-1/PD-L1 or CTLA-4 inhibitors with CSF1R blockade. This approach is under investigation in several solid tumours, including pancreatic and gastric cancer (Table 6.5).

Preclinical studies in melanoma have revealed that upregulated expression of indoleamine 2,3-dioxygenase (IDO) is a potential mechanism of resistance to immune checkpoint blockade. IDO is the enzyme that catalyses tryptophan degradation. As cytotoxic T-lymphocytes are exquisitely sensitive to tryptophan depletion, increased IDO expression leads to T-cell anergy and immune evasion [134]. The combination of IDO inhibitors with CTLA-4 or PD-1 blockade markedly improved tumour control in murine melanoma models [135, 136]. Promising results were reported in patients with advanced cancers treated with dual blockade. In a phase I trial of the IDO inhibitor epadacostat and PD-1 that included 19 patients with a variety of solid tumours, the ORR was 53% and the DCR was 79% [137]. These preclinical and encouraging early clinical data provided the rationale for the initiation of combination trials of IDO and PD-1/PD-L1 inhibitors in GEJ, gastric and colorectal cancer (Table 6.5).

An additional mechanism of immune evasion has been discovered in preclinical models of pancreatic cancer. Cancer-associated fibroblasts (CAFs) expressing fibroblast activation protein (FAP) produce chemokine (C-X-C motif) ligand 12 (CXCL12), which results in T cell exclusion from the tumour microenvironment. Inhibiting chemokine (C-X-C motif) receptor 4 (CXCR4), a CXCL12 receptor, induced rapid T-cell accumulation and acted synergistically with the anti-PD-L1 inhibitor to cause cancer regression [138]. A phase I/II trial of the CXCR4 monoclonal antibody ulocuplumab combined with nivolumab in patients with advanced pancreatic adenocarcinoma is currently ongoing (Table 6.5).

Combination of PD-1/PD-L1 Blockade and Cytotoxic Chemotherapy

Cytotoxic chemotherapy has been the mainstay of treatment in gastrointestinal malignancies. In addition to inducing DNA damage and cancer cell death, chemotherapeutic agents also directly affect the immune cells in the tumour microenvironment. Specifically, gemcitabine, an agent commonly used in pancreatic and biliary cancer, has been shown to increase cross-presentation of tumour antigens to CD8 cells leading to increased proliferation and cytotoxicity [139]. Furthermore, gemcitabine depletes MDSCs and restores IFN responsiveness in murine models of colon cancer [140]. Oxaliplatin augments dendritic cell maturation and function, but also downregulates PD-L1 and PD-L2 expression resulting in enhanced tumour

antigen recognition by antigen-specific T cells [141]. In contrast, the immune effects of 5-fluorouracil (5-FU) are primarily driven by depletion of MDSCs. Interestingly, 5-FU was shown to have a greater efficacy over gemcitabine in eliminating MDSCs, which in turn led to increased IFN-γ production by infiltrating CD8 T cells and enhanced anti-tumour responses in immunocompetent mouse models [142]. Based on these preclinical data, a large number of clinical trials are exploring combinations of chemotherapeutic agents and PD-1 inhibitors in gastric, pancreatic and colorectal cancers (Table 6.5).

Combinations of PD-1/PD-L1 Blockade and Radiation Therapy

Radiation therapy not only has a profound effect on the tumour immune microenvironment of the irradiated field, but can also induce a systemic anti-tumour immune response. There are multiple case reports of the abscopal effects of radiation therapy, where tumour shrinkage is observed outside the treatment field. Although this effect was attributed to a systemic inflammatory response provoked by radiation therapy, these events were rare and the exact mechanism of the tumour regression was difficult to elucidate. Two recent case reports highlighting the abscopal effects of radiation therapy in patients with melanoma provided some insight into the immunologic mechanism of tumour regression. In one report, the patient had abscopal regression of cutaneous metastases after radiation to the primary tumour and a complete response of all metastatic sites after combination of stereotactic brain radiation and ipilimumab [143]. In the second report, the patient had slow disease progression despite ipilimumab therapy, but then experienced a robust and durable response after combined palliative radiotherapy and ipilimumab [144]. Following radiotherapy, antibody levels against tumour-specific antigens rose, circulating activated T cells increased and MDSCs decreased. These anecdotal cases highlight the synergistic potential of combined radiation therapy and immune checkpoint inhibition. This combinatorial approach is currently being investigated in various gastrointestinal malignancies, including gastric, GEJ, pancreatic and colorectal carcinomas (Table 6.5).

Combinations of PD-1/PD-L1 Blockade and Vaccines

Immune checkpoint inhibition is unlikely to be efficacious in tumours that lack immune cell infiltrates. In these tumours, the administration of an antigen-specific vaccine can initiate an anti-tumour immune response by enhancing the influx of T cells into the tumour. The addition of immune checkpoint inhibitors may further augment this T cell response. A clinical study investigating the effect of neoadjuvant administration of the granulocyte-macrophage colony-stimulating factor (GMCSF) – secreting, allogeneic pancreatic cancer vaccine (GVAX) in patients

with resectable pancreatic cancer showed that vaccination increased T cell trafficking into the tumour microenvironment to form lymphoid aggregates. In addition, treatment with GVAX vaccine led to upregulation of PD-L1 expression, suggesting that patients with vaccine-primed pancreatic cancer may be better candidates than vaccine-naïve patients for immune checkpoint inhibition [145, 146]. Furthermore, in a syngeneic mouse model of pancreatic ductal adenocarcinoma, combination therapy with GVAX vaccine and PD-1 antibody blockade improved survival compared to PD-1 antibody monotherapy or GVAX therapy alone [146]. This approach is currently being investigated in ongoing trials, both in the neoadjuvant/adjuvant and the metastatic setting (Table 6.5). As additional vaccines are being developed in pancreatic cancer and other gastrointestinal malignancies [147], combinations with immune checkpoint inhibitors warrant evaluation in clinical trials.

Conclusions

Immune checkpoint inhibition represents a new avenue for the treatment of solid tumours. Monoclonal antibodies that inhibit the PD-1 pathway have led to marked responses in several malignancies. These agents are under investigation in gastrointestinal malignancies, with early phase trials showing promising results. Specifically, patients with gastric cancer, dMMR colorectal cancer and hepatocellular carcinoma have demonstrated robust and durable responses to PD-1 and PD-L1 inhibitors, while efficacy of these agents as monotherapy is more limited in pancreatic adenocarcinoma and pMMR colorectal cancer. Based on the encouraging data from the early phase trials, phase III trials evaluating the clinical activity of PD-1 blockade in select GI malignancies are currently ongoing.

One of the critical steps to improve anti-tumour responses to immune checkpoint inhibitors is to determine which patients will benefit most from these therapies. As several trials of PD-1/PD-L1 inhibitors are underway, correlative studies will be essential to guide the development of predictive biomarkers. To further enhance the efficacy of PD-1/PD-L1 inhibitors and overcome resistance to immune checkpoint blockade, development of rational therapeutic combinations is critical. These combinatorial approaches include combinations of PD-1/PD-L1 inhibitors with other immune checkpoint inhibitors, T cell agonists, agents targeting the tumour microenvironment, cytotoxic chemotherapy, radiation therapy, targeted therapy and tumour vaccines. These strategies are currently being tested in clinical trials.

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Chapter 7
The Role of the JAK/STAT Signalling Pathway in Immunoregulation of Gastrointestinal Cancers

Kris Vaddi

Introduction

Harnessing the immune system is a powerful approach for developing treatments with curative potential for a number of diseases, including cancer. Until recently, however, translating the success of targeted immunological treatments that has been observed in autoimmune diseases into treatments for cancer has eluded the oncology community. The failure of traditional vaccination approaches against tumour antigens to produce reliable, reproducible, and broadly active treatments in cancer settings has frustrated researchers for decades. The recent unprecedented success of immune checkpoint inhibitors in unleashing the power of the immune system in several cancers, including melanoma and non-small cell lung cancer, has brought the focus of cutting edge cancer therapy back to immune-based treatments. It also is becoming increasingly evident that further scrutiny of additional mechanisms that could be used in combination with a checkpoint blockade to optimise the efficacy of immune-targeted agents in immunogenic cancers and to develop effective immune-based therapies in nonimmunogenic cancers is warranted.

Tumour-associated inflammation is now recognised as a critical enabling factor in oncogenesis [24, 62]. A variety of host and environmental factors, including necrotic cell death, a predominant feature of many tumours, send proinflammatory signals into the surrounding tumour microenvironment, resulting in the recruitment of inflammatory cells of the immune system. Tumour-associated inflammation can aid tumour growth via multiple mechanisms, including (1) actively promoting tumour development by fostering angiogenesis, cancer cell proliferation, and tumour invasiveness and (2) eliciting a physiological immunosuppressive response to limit further inflammation with an unintended consequence of suppressing antitumour immunity. A variety of evidence suggests that a chronically inflamed

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state is associated with an immunosuppressive environment that limits the effectiveness of immune-based and other therapies in some disease settings [85]. Thus, new immune interventions that target tumour-promoting inflammation present an opportunity for combination therapies with checkpoint inhibitors and other vaccine-based treatments.

Cytokines and growth factors produced by various cell types within the tumour microenvironment, including infiltrating immune cells, tumour cells, and resident cells, mediate tumour-host cell communication. Precisely controlled communication is critical to the adequate generation of antitumour immunity. However, the same communication mechanisms can be subverted by cancer cells to evade detection by the host's immune system. Given the paramount importance of these mediators in controlling normal immune function, defence against infections, and autoimmune conditions, cytokines and growth factors have been extensively studied over the past several decades to better understand how they can be used for treating disease. We are just beginning to understand the role of cytokine networks in the context of cancer. Successfully leveraging cytokine biology to design new treatments that intercept or redirect tumour-immune cell communication to favour the host is imperative for bolstering novel immune-targeted treatment modalities.

The communication between cytokines/growth factors and responding cells starts with the binding of these mediators to their corresponding cell receptors. Such interaction triggers a cascade of intracellular events that involve various enzymatic and biochemical reactions, which ultimately result in major cellular responses that include phenotypic cell changes and alterations in the expression of a variety of intracellular and extracellular proteins. As such, understanding how to control the intracellular signalling pathways utilised by cytokines and growth factors has been a subject of extensive scrutiny in recent years. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is the signalling mechanism for many cytokines that play a role in the pathogenesis of gastrointestinal (GI) cancers. Therefore, leveraging the JAK-STAT pathway could aid in designing new treatments, improving the efficacy and safety of current treatments, and identifying the limitations of immune-based treatment approaches for patients with GI cancers. This section describes the components of the JAK-STAT pathway, the key cytokines/growth factors that signal through the pathway, the current understanding of how this pathway may influence the aetiology of GI cancers, and the current and potential future treatment approaches designed to modulate this pathway for treating patients with GI cancers.

JAK-STAT Signalling Pathway

The JAK-STAT pathway is an intricate signalling system that plays an essential role in a variety of processes that are critical for regulating cell interaction and gene expression and influences the transcription of tens of thousands of genes that are important for cell differentiation, proliferation, apoptosis, chromatin structure, and

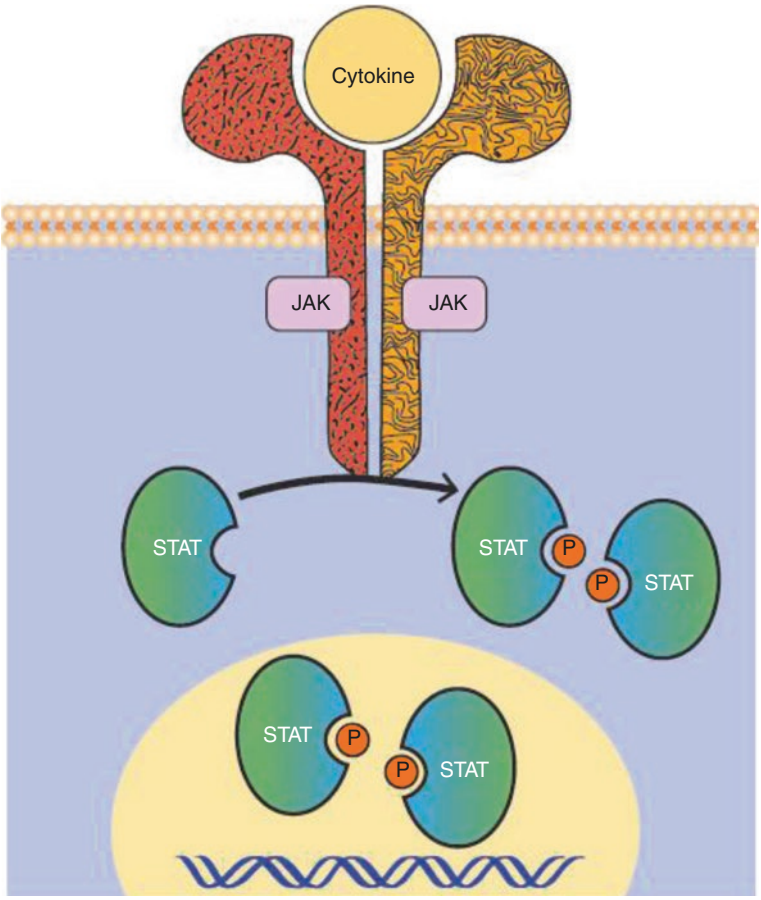

Fig. 7.1 The JAK-STAT signalling pathway. Upon binding to the membrane receptors, cytokines initiate downstream signalling cascades through the JAK-STAT pathway. Cytokine receptor homodimers or heterodimers phosphorylate and activate JAKs, which phosphorylate cytoplasmic STAT monomers. Upon phosphorylation, activated STAT dimers translocate to the nucleus and regulate the transcription of target genes

inflammation [132]. Signals from the cell membrane are transduced via the JAK-STAT pathway to the cell nucleus and regulate the transcription of target genes (Fig. 7.1). Prior to activation, unphosphorylated JAKs associate with the intracellular domain of cytokine and growth factor receptor subunits [132, 174]. Ligand binding and the subsequent formation of receptor subunit homodimers or heteromultimers brings the associated JAKs in close enough proximity for transphosphorylation and conformational changes that permit STAT binding [1, 174]. Activated JAKs phosphorylate cytoplasmic STAT monomers, which leads to nuclear translocation of STAT dimers. These dimerised STATs bind to DNA at specific regulatory sequences and thereby activate or repress the transcription of target genes.

The JAK-STAT pathway is a key intracellular mediator of cytokines that regulate the immune landscape [154]. Dysregulated JAK-STAT signalling, resulting in aberrant expression of STATs and genes regulated by STATs, is a hallmark of many GI cancers. JAK-STAT pathway activity in GI cancers is associated with oncogenesis, proliferation, survival, metastasis, angiogenesis, immune evasion [25, 33, 127], micro-RNA expression [76], and inflammation, which collectively help cultivate an immunosuppressive and tumour-enhancing environment [25]. Thanks to a well-defined ATP-binding pocket that is distinct from other kinases, JAK enzymes are attractive targets for designing highly selective small-molecule inhibitors to modulate STAT-driven molecular effects. A better understanding of JAK-STAT signalling in GI cancers will permit the development of pharmacologic agents with selective profiles that reduce tumour-related inflammation and improve antitumour immunity, as well as inhibit STAT-driven direct oncogenic effects. The objective of this section is to provide an overview of the JAK-STAT components with a focus on evidence concerning the potential role of this pathway in the pathogenesis of GI cancers.

JAK and STAT Family Members

JAKs were named after the Roman god Janus because they are "2-faced," having a kinase at the carboxyl terminus (at the JH1 domain) and a pseudokinase immediately adjacent at the JH2 domain; a total of 7 homology domains have been identified (JH1–JH7) [1]. The JAK family comprises the following four nonreceptor tyrosine kinases [132]: JAK1 [186], JAK2 [63, 186], JAK3 [82, 87], and tyrosine kinase 2 (TYK2) [42]. JAK1, JAK2, and TYK2 are ubiquitously expressed, whereas JAK3 is restricted primarily to hematopoietic cells [63, 192].

The STAT family comprises the following 7 transcription factors: STAT1–4, STAT5a, STAT5b, and STAT6 [132]. STAT proteins have a conserved tyrosine residue in the carboxy-terminus transactivation domain that is phosphorylated by JAKs and forms STAT dimers by interacting with the SH2 domain; STAT proteins also have coiled coil and DNA binding domains [1]. STATs are ubiquitously expressed, with the exception of STAT4, which is expressed primarily in the thymus and testes [66].

More than 50 cytokines and growth factors signal through various permutations of the 4 JAKs and 7 STATs, leading to a large number of potential signalling outcomes [174]. The downstream signalling effects of key cytokines and growth factors of interest in GI malignancies (Table 7.1; Fig. 7.2) and their potential influence on the tumour microenvironment are explored in a later section of the chapter.

Regulation of the JAK-STAT Pathway

Several intracellular components can regulate the activation of specific JAKs and STATs to modulate the central JAK-STAT signalling pathway. Certain signal-transducing adapter molecules (STAMs), including STAM1 and STAM2A, contain

an inducible tyrosine-based activation motif that allows phosphorylation by JAK1 and JAK3 [147]. The STAMs facilitate transcriptional activation of specific target genes, although the mechanism by which this occurs is unclear.

Signalling adapter proteins, including LNK, SH2B, and APS, possess SH2 and Pleckstrin homology domains that modulate JAK2 activation [13]. LNK binds

Table 7.1 JAK-STAT–dependent cytokine receptor systems implicated in solid malignancies

Cytokine	Cytokine receptors	JAK kinase	STAT
IL-6	IL-6R/sIL-6R/gp130	JAK1, JAK2, TYK2	STAT1, STAT2, STAT3, STAT4, STAT5
IL-4	IL-4/13R	JAK1, JAK2, JAK3, TYK2	STAT6
IL-10	IL-10/20R	JAK1, JAK2, TYK2	STAT1, STAT2, STAT3, STAT4, STAT5
IL-12	IL-12Rβ1/β2	JAK2, TYK2	STAT3, STAT4
IL-23	IL-23R/12Rβ1	JAK2, TYK2	STAT3, STAT4
GM-CSF	CD131 (βCR)	JAK2	STAT3, STAT5, STAT6

GM-CSF granulocyte/macrophage colony-stimulating factor, *IL* interleukin, *JAK* Janus kinase, *STAT* signal transducer and activator of transcription

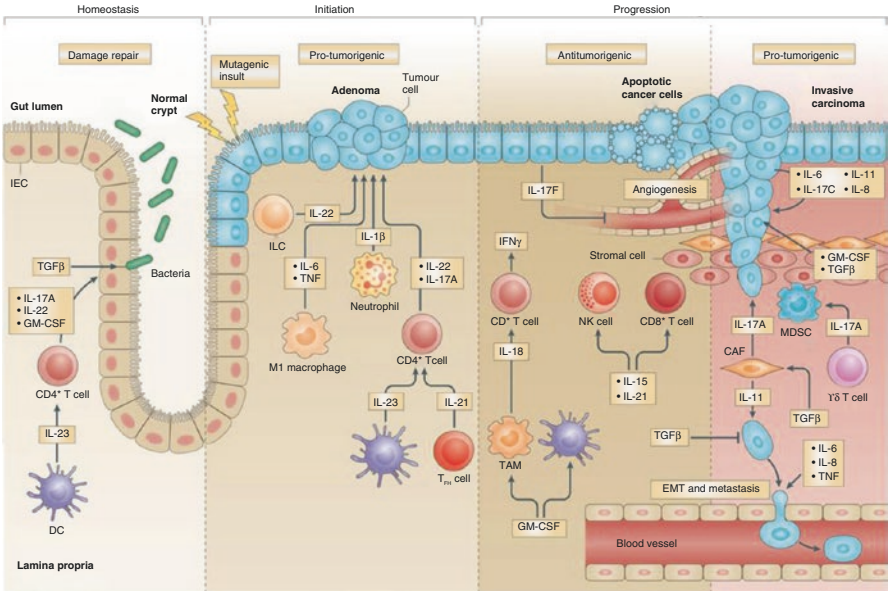

Fig. 7.2 The role of cytokines in the development of colorectal cancer [184]. Cytokines produced by a variety of cell types, including innate and adaptive immune cells and tumour cells, play an important role in the development of colorectal cancer. Some cytokines can impair tumour growth via inhibition of angiogenesis and cancer dissemination. However, in well-established tumours, many cytokines can promote tumour proliferation, invasion, and metastasis. This schematic depicts where and how cytokines may influence the pathogenesis of colorectal cancer

Cytokine	Cellular source(s)	Cellular responder(s)
TNF	Haematopoietic cells[27,28]	IECs[29] and cancer cells[31]
TGFβ	CAFs[43]	Cancer cells[43] and CAFs[41,42]
IL-1β	TAMs[46] and neutrophils[48]	IECs[48] and cancer cells[46,47]
IL-6	TAMs[54], CAF[55] and mesenchymal stem cells[56]	IECs[57] and cancer cells[55,57]
IL-11	CAFs[41]	Cancer calls[41,58]
IL-23	DCs[69] and TAMs (CD11b*GR1*)[61]	Haematopoietic cells[61]
IL-17A	CD4* αβT cells[59,67] CD8* Tc17 cells, ϒδ Tcells67, ILCs[66] and CAF[70]	Cancer cells[63], CICs[70] and MDSCs[67,69]
IL-17C	IECs[72]	IECs[72] and cancer cells[72]
IL-17F	IECs[74]	Endothelial cells[74]
IL-22	ILC3s[66] and CD4* T cells[78,81]	Cancer cells[66,78,81]
IL-18	TAMs and IECs[86]	IECs[84] and T cells[85,86]
IL-8	CD44* cancer stem-like cells[93]	CD11b* GR1* myeloid cells[94]
GM-CSF	Cancer cells[96-98]	Cancer cells[96], antigen-presenting cells[96] and monocytes[97]
IL-15	Cancer cells[102]	NK cells[99,100], CD8* T cells[99], B cells and T cells[102]
IL-21	CD4* T cells[105] and activated NK cells[105]	NK cells[104,105] and CD8* T cells[105]

Fig. 7.2 (continued)

phosphorylated JAK2 and the product of a somatic mutation, *JAK2*V617F, to inhibit downstream signalling [22, 54], whereas SH2B and APS have been associated with promotion of JAK2 signalling.

The suppressor of cytokine signalling (SOCS) family functions in a negative feedback loop in which SOCS proteins are expressed downstream of JAK/STAT signalling and act as negative regulators of cytokine signalling [13]. Most SOCS members function as E3 ubiquitin ligases, promoting ubiquitination and degradation of JAK-associated cytokine receptors and possibly JAKs as well. SOCS-1 and SOCS-3 prevent downstream signalling via direct binding to JAK1, JAK2, and TYK2.

Protein tyrosine phosphatases (PTPs), such as shatterproof-1 (SHP-1), SHP-2, PTP1B, T-cell PTP, and CD45, bind to and dephosphorylate JAKs or cytokine receptors, thereby reversing JAK activity [13]. Protein inhibitors of activated STATs (PIAS) inhibit STAT signalling by blocking STAT DNA binding or by recruiting histone deacetylases or other corepressors [48]. Conversely, the methyltransferase enhancer of zeste homolog 2 has been reported to methylate STAT3, which confers enhanced STAT3 activity [90].

Lessons from JAK Knockout Mice: Role of JAK-STAT in Immune and Bone Marrow Function

JAK-STAT signalling is a key regulator of immune cell growth, survival, and differentiation in innate and adaptive immunity, as evidenced by data from murine models and clinical observations [55]. *JAK1* and *JAK2* deletions are lethal in mice as a result of nursing-related complications [126, 136, 149]. *JAK3* and *TYK2* deletions are not lethal, but significantly influence lymphocyte biology and cytokine signalling that is essential to the differentiation and function of T cells and other immune cells. In *JAK1*$^{-/-}$ mice, a deficit in thymocyte production and maturation of B cells was observed, along with a lack of response of cells to interferon (IFN)-α and IFN-γ induction [149]. *JAK3* knockout mice also have thymocyte deficiencies and profound reductions in mature B and T cells [129, 138, 169], although no apparent nonimmunologic deficits have been observed [169].

Interestingly, the immunodeficient phenotype of *JAK3* knockout mice is similar to that of severe combined immunodeficiency syndrome in humans [129, 138, 169]. The effects of *JAK3* deficiency on T-cell subsets in mice include an increase in the susceptibility of nature killer (NK) cells and intestinal γδ T cells to apoptosis, induction of peripheral CD4^{+} T-helper (Th) cell anergy, and inhibition of Th1/Th2 cell differentiation [55]. In addition, *JAK3* knockout mice develop myeloid cell expansion and enhanced dendritic cell (DC) differentiation and cytokine production (e.g., interleukin [IL]-10, IL-12). *TYK2*$^{-/-}$ mice differ from *JAK3*$^{-/-}$ mice in that they show no obvious deficits in the numbers of lymphoid, myeloid, or monocyte cells [86, 157]. However, similar to *JAK3*$^{-/-}$ mice, TYK2$^{-/-}$ mice also have impaired Th1 differentiation [133], which can be attributable to its essential role in IL-12 signalling [86, 157]. In addition, these mice are particularly susceptible to infection, suggesting that TYK2 plays an essential role in host defence [86]. For example, IL-23–induced IL-17 production by peritoneal γδ T cells was reduced in *TYK2*$^{-/-}$ mice compared with wild-type mice, leading to impaired neutrophil infiltration and immune response against *Escherichia coli* infection [123]. Collectively, the data from JAK-STAT knockout studies confirm the importance of cytokines in regulating how the JAK-STAT pathway affects the immune system.

JAK-STAT Dysregulation in GI Cancers

JAK-STAT pathway dysregulation is an important mechanistic underpinning to the pathophysiology of many disorders (Fig. 7.3). Dysregulation of the JAK-STAT pathway is widely documented in GI cancers and may arise from cytokine-dependent or -independent mechanisms. Overexpression of cytokines resulting from

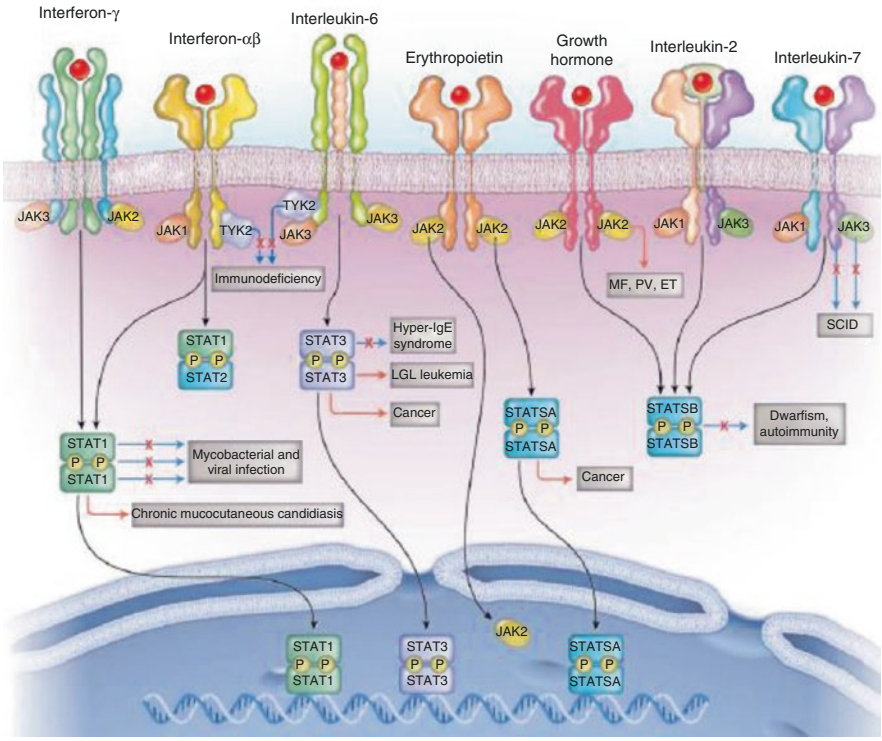

Fig. 7.3 Mutations of JAKs and STATs are associated with a variety of disorders [131]. Several *JAK* and *STAT* mutations result in dysregulation of the JAK-STAT signalling pathway and have been implicated in the pathogenesis of a variety of disorders. This schematic depicts where mutations in the JAK/STAT pathway can result in corresponding disorders

uncontrolled immune and inflammatory stimuli in the tumour microenvironment can lead to overactivation of receptor-mediated JAK family members. Cytokine-independent mechanisms could include inactivation of negative regulators (e.g., SHPs, PIAS) or activating mutations in *JAK* family members. In particular, persistent STAT3 activation promotes multiple aspects of GI tumour development [25, 33, 127]. A recent meta-analysis of 22 studies (N = 3585 total patients) that evaluated the prognostic significance of phosphorylated STAT3 (pSTAT3) in GI cancers reported that pSTAT3 expression was significantly associated with shorter overall survival (OS; hazard ratio [HR], 1.809; $P < 0.001$), disease-free survival (DFS; HR, 1.481; $P = 0.035$), and other clinicopathological features, including tumour cell differentiation (odds ratio [OR], 1.895; $P < 0.001$) and lymph node metastases (OR, 2.108; $P = 0.024$) [109]. The precise aetiology and role of abnormal JAK-STAT signalling in individual cancers remains unclear [168]. However, a variety of data are available that implicate key JAK-STAT pathway components in multiple tumour-specific settings.

Colorectal Cancer

In colitis-associated cancer (CAC), various members of the JAK-STAT pathway have been associated with tumour promotion, progression, and metastasis, as well as antitumour activity [205]. In addition, overexpression of PIAS3 was observed in colorectal cancer (CRC) tissue compared with normal tissue [108]. An analysis of population-based case–control studies (N = 5224) showed that mutations in various *JAK*s and *STAT*s were associated with increased mortality risks in patients with colon and rectal cancers [158]. However, other studies have reported contradicting prognostic evidence concerning pSTAT3 in CRC. In 104 French patients with advanced rectal cancer, pSTAT3 was an independent prognostic factor for improved OS (HR, 0.3; 95 % CI, 0.1–0.8; $P = 0.01$) [121]. In contrast, a study in 108 patients with colorectal adenocarcinoma reported that pSTAT3 expression was significantly correlated with shortened OS ($P < 0.001$) as well as with tumour invasion characteristics, including the depth grading of invasion, lymphatic invasion, and stages of Dukes' classification [104]. These contrasting results may have arisen from differential STAT3 expression in specific cell types. For example, an immunogenic tumour containing STAT3-positive lymphocytes may be reflective of an appropriate antitumour immune response, resulting in improved patient survival. However, a tumour containing STAT3-positive nonimmune cells originating from either host or cancer cells may reflect a highly metastatic and invasive stage of tumour development. Given the broad tumour heterogeneity among patients with CRC, further studies are warranted to better characterise the cellular origin of JAK-STAT activation to understand of the role and clinical significance of JAKs and STATs in CRC.

Pancreatic Cancer

A variety of data suggest that STAT3 and IL-6 have important roles in the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) [64]. Whereas inactive STAT3 is typical of nonmalignant pancreas tissue samples, activation of STAT3 via phosphorylation of tyrosine residue 705 has been observed in murine and human models of PDAC [64, 107]. IL-6, produced primarily by tumour-infiltrating macrophages, was demonstrated as the major driver of STAT3, the activity of which is mainly regulated by SOCS-3 in PDAC. In a study of transgenic mice, STAT3 was shown to induce the development of the acinar-to-ductal metaplasia via persistent expression of the transcription factor pancreatic and duodenal homeobox factor 1 (PDX-1) [120]. STAT3 is shown to be activated and overexpressed in ductal carcinoma cells compared with the ducts from chronic pancreatitis [153]. Functional inactivation of STAT3 in a subset of pancreatic cancer cell lines significantly inhibited cell proliferation in vitro and reduced tumour growth in vivo. In human pancreatic cancer, STAT3 has been shown to regulate vascular endothelial growth factor (VEGF) expression, angiogenesis, and metastasis [183], supporting a malignant phenotype of human pancreatic cancer.

Hepatocellular Carcinoma

Several *JAK1* gene mutations, including S729C, N451S, E483D, and S703I, have been identified in tissues from patients with hepatocellular carcinoma (HCC) [84, 194]. However, only S729C and S703I are believed to confer a gain-of-function role in the JAK-STAT pathway. Cells with the S703I mutation were capable of continual proliferation and were sensitive to the JAK1/JAK2 inhibitor ruxolitinib [194]. In a study that examined the molecular pathogenesis of HCC, enhanced activation of JAK-STAT components, including JAK1, JAK2, TYK2, STAT1, STAT3, and STAT5, were observed in HCC tumour tissues (n = 80) compared with normal liver tissue (n = 10) [27, 156, 188]. Furthermore, hypermethylation of SOCS-3 promoters (i.e., SOCS-3 inactivation) was observed in a subgroup of patients with survival of <3 years. This is consistent with an analysis that found significant correlations between abnormal pSTAT and SOCS-3 expression patterns and survival [188]. Expression of the JAK-STAT negative modulator SOCS-1 was also found to be substantially reduced in samples from 4 of 8 HCC tumours tested in one analysis (Nagai et al. 2001), providing an additional mechanism for the overactivation of JAK-STAT signalling. As in pancreatic cancer settings, HCC tumourigenesis and proliferation has been shown to be closely associated with IL-6-STAT3 signalling [127].

Gastric Cancer

Activated STAT3 has been observed in GI stromal tumours with activating mutations in *c-kit*, along with reduced tumour cell growth with pharmacologic blockade of JAK2 [134]. Consistent with this finding, activated STAT3 was increased in excised gastric cancers compared with healthy adjacent tissue, and was associated with disease stage [52]. In a meta-analysis of studies that evaluated the prognostic significance of pSTAT3 in GI cancers, elevated pSTAT3 had the most notable association with worse OS among patients with gastric cancer (HR, 2.264; $P < 0.001$) compared with other malignancies of the digestive tracts, including oesophageal cancer (HR, 1.825; $P = 0.092$), pancreatic cancer (HR, 1.716; $P = 0.196$), HCC (HR, 1.654, $P = 0.041$), and CRC (HR, 1.149; $P = 0.729$) [109].

Involvement of Cytokines and Growth Factors That Signal Through the JAK-STAT Pathway in GI Cancers

The tumour microenvironment is regulated by cytokines, chemokines, growth factors, enzymes, and angiogenic mediators from myriad cell types, including immune cells, endothelial cells, mesenchymal cells, and tumour cells [144]. A variety of these mediators are involved in the complex tumour microenvironment. The serum and tumour expression patterns of key cytokines and granulocyte/macrophage colony-stimulating factor (GM-CSF) are summarised in Table 7.2. This section

Table 7.2 Expression and roles of major cytokines in gastrointestinal cancers

Cytokine	Immune function	Inflammation	GI cancer	Expression and role
IL-6	Immune suppressing	Proinflammatory	Hepatocellular	Elevated serum expression [111, 113, 180]
			Colorectal	Elevated serum expression [113], associated with tumour stage, NLR [93], and reductions in OS and DFS [190] Elevated expression in tumour tissue [34]
			Gastric	Elevated serum expression, associated with poor prognosis [113]
			Pancreatic	Elevated serum expression [70, 113], associated with poor prognosis [113] Promotes cancer development and progression [70]
IL-4	Immune suppressing	Proinflammatory	Hepatocellular	Elevated serum expression [180]
			Colorectal	Elevated expression in polyp versus normal epithelial tissue [164] Patients with elevated serum levels of sIL-4R were less likely to have ≥3 polyps [31]
IL-10	Immune suppressing	Anti-inflammatory	Hepatocellular	Elevated serum expression [180], associated with poor prognosis [113]
			Colorectal	Elevated serum expression, associated with poor prognosis [113]
			Gastric	Elevated serum expression, associated with poor prognosis [113]
			Pancreatic	Elevated serum expression, associated with poor prognosis [113]
IL-12	Immune activating	Proinflammatory	Colorectal	Reduced serum expression [113]
			Gastric	Reduced serum expression [113]
			Hepatocellular	Reduced serum expression [113]

(continued)

Table 7.2 (continued)

Cytokine	Immune function	Inflammation	GI cancer	Expression and role
IL-23	Immune suppressing	Proinflammatory	Colorectal	Elevated serum [177] and tumour tissue expression [140] Serum levels higher in early versus advance disease [177]
			Gastric	Elevated serum expression [202], associated with poor prognosis [114] Elevated expression in tumour tissue [114]
			Pancreatic	Reduced serum expression [113, 179]
GM-CSF	Immune activating Immune suppressing	Proinflammatory	Colon	Elevated expression in tumour tissue [36], associated with good prognosis [124]
			Pancreatic	Elevated expression in tumour tissue [14]

CSS cancer-specific survival, *DFS* disease-free survival, *GI* gastrointestinal, *GM-CSF* granulocyte/macrophage colony-stimulating factor, *IL* interleukin, *NA* not available, *NLR* neutrophil-to-lymphocyte ratio, *OS* overall survival

discusses the key cytokines, including IL-6, IL-4, IL-10, IL-12, IL-23, and growth factor GM-CSF, that signal via the JAK-STAT pathway and are thought to play prominent roles in the pathogenesis in GI malignancies.

Interleukin-6

Interleukin-6 is a prototypic inflammatory cytokine that is produced by a variety of cell types, including hematopoietic cells, stromal cells, epithelial cells, and muscle cells. IL-6 can bind to corresponding membrane-bound receptors (IL-6R or CD126) or soluble receptors (sIL-6R). These complexes then bind to the gp130 IL-6 transducer (CD130), resulting in gp130 dimerisation, and phosphorylation and activation JAK1, JAK2, and Tyk2 [151]. Several studies have highlighted the effect of the IL-6/JAK/STAT signalling pathway on cancer initiation and progression, particularly in patients with CRC.

Increased expression of IL-6 in patients with CRC in serum and tumour tissue has been documented in a number of studies [51, 100]. In addition, IL-6 expression has been associated with increased tumour stage, size, and metastasis, and reduced survival in patients with CRC [96]. An association between serum levels of IL-6 and carcinoembryonic antigen has also been reported in patients with CRC [19]. Although data on IL-6 expression patterns in sporadic CRC are well established, the source of IL-6 expression in noninflammation–associated cancer is less clear. One explanation could be related to the infiltration of tumours with IL-6–secreting inflammatory cells as seen in patients with CAC.

Interleukin-6, secreted by lamina propria T cells and macrophages, was demonstrated to be important for the development in vivo in a murine model of CAC [16]. In the widely used mouse model of CAC using the mutagenic agents azoxymethane and dextran sulphate sodium, the authors observed intestinal tumour growth that was dependent on IL-6 trans-signalling in intestinal epithelial cells, possibly with downstream activation of STAT3. The tumour-promoting effect of IL-6 could be inhibited through treatment with anti–IL-6R antibodies or sgp130Fc, a designer variant of soluble gp130 that specifically blocks trans-signalling [16]. In addition, IL-6 was shown to mediate proliferative and antiapoptotic effects on malignant cells by activating STAT3 [58]. This is consistent with the finding that the majority of IL-6 target genes regulate cell cycle progression and the suppression of apoptosis [112]. During the acute inflammation phase, IL-6 was associated with immune response activation via recruitment of neutrophils and monocytes and subsequent transition to adaptive immunity via T-cell recruitment and differentiation towards Th2 and Th17 phenotypes [152].

Interleukin-6 has also been implicated in other inflammation-associated GI malignancies, including pancreatic cancer [201], HCC [81], and gastric cancer [181]. In patients with HCC, higher intratumoural Th17 cell density was associated with poor OS and DFS, suggesting that IL-6 may have immunosuppressive effects in GI malignancies [28, 198].

Interleukin-4

Interleukin-4 is a Th2 cell cytokine that regulates the immune response and microenvironment under normal physiological conditions and in cancer settings [162]. IL-4 binds to its high-affinity receptors on solid tumours, consisting of IL-4Rα and IL-13Rα1 chains, forming functional receptors in cancer cells that activate JAKs following ligand binding and heterodimerisation. Upon phosphorylation, 5 tyrosine residues (Y1–5) in the cytoplasmic tail of the IL-4Rα interact with signalling molecules to regulate the immune response through the JAK/STAT pathway [115]. Y1 is in the I4R sequence motif and interacts with the phosphotyrosine-binding domains of insulin receptor substrate 1 and 2. Y2–4 bind with the SH2 domain of STAT6, and Y5 is found in an ITIM consensus motif that brings SHP-1 to the activated receptor complex.

The biological effects of IL-4 in cancer cells include tumour proliferation, cell survival, cell adhesion, and metastasis. IL-4 is produced by several cell types, including mast cells, basophils, and activated T lymphocytes [110]. IL-4 is primarily known for regulating B and T lymphocytes [110]. Specifically, IL-4 can stimulate the proliferation and differentiation of these cell types and the expression of a variety of gene targets [110]. IL-4 has been shown to induce transient inflammatory responses in the colon tissue of mice via a STAT6-dependent mechanism [173]. Furthermore, IL-4 exerts carcinogenic effects that may be essential to the survival of colon cancer stem cells and mature cancer cells via STAT6-dependent regulation of antiapoptotic genes and related proteins (e.g., survivin) [37, 43]. This feature of IL-4 may also play a role in the chemoresistance mechanism observed in some patients with colon cancer via upregulation of proteins associated with multidrug resistance (e.g., P-glycoprotein) [43].

Endogenous IL-4 induces the immunosuppressive M2 phenotype of tumour-associated macrophages (TAMs) and Th2 differentiation [23, 203], which is often associated with poor prognosis in patients with GI malignancies [45]. Furthermore, IL-4 was shown to suppress the immune surveillance and support tumour growth and metastasis in patients with pancreatic cancer [142]. However, evidence suggests that exogenous IL-4 (i.e., not produced by immune cells) has an antitumour effect conferred in a biphasic fashion that involves innate immune cell accumulation and maturation, followed by $CD8^+$ T-cell activation [110].

Interleukin-10

Interleukin-10 is a potent anti-inflammatory cytokine that signals through a variety of JAK-STAT signalling components, including JAK1, TYK2, and STAT3 [105, 112]. In patients with CRC, IL-10 serum levels increased over time during tumour progression [130], and high preoperative serum levels of IL-10 have correlated with poor survival (HR, 2.561; 95 % CI, 1.11–5.91; $P = 0.023$) [119]. These data suggest

a tumour-promoting role for IL-10 in patients with CRC. However, IL-10 knockout mice were shown to be more susceptible to developing CAC than wild-type mice, whereas newborn IL-10 knockout mice treated with exogenous IL-10 showed no sign of intestinal inflammation or CAC [21]. Examples of the anti-inflammatory effects of IL-10 include downregulation of proinflammatory cytokine production by immune cells [11] and counteraction of IL-12–driven inflammation [69, 112]. In addition, IL-10 is an immunosuppressive cytokine that influences both the innate and adaptive immune responses [105]. In patients with HCC, IL-10 was shown to suppress antigen presentation, differentiation, and maturation of DCs, allowing tumour cells to evade immune surveillance mechanisms [15]. IL-10 has also been shown to help regulate the activities of regulatory T cells (Tregs) and the subsequent suppression of T-cell–mediated colitis and intestinal bowel disease, and prevent colon cancer [39, 116].

Interleukin-12 and Interleukin-23

Interleukin-12 and IL-23 are proinflammatory cytokines that have similar molecular characteristics, share a common subunit (IL-12p40), and signal via JAK2, TYK2, and a variety of STATs [112, 191]. IL-12 comprises the IL-12p40 subunit linked to the IL-12p35 subunit, which signals through the IL-12 receptor (IL-12R; includes β1 and β2 subunits) as a heterodimer. Upon binding to its receptor, IL-12 stimulates JAK2 and TYK2 activity, leading to phosphorylation of STAT1, STAT3, STAT5, and, in particular, STAT4 homodimers [182]. IL-23 comprises the IL-23p19 and the IL-12p40 (i.e., IL-12/23p40) subunits, which signal through IL-23R and IL-12Rβ1. Like IL-12, IL-23 activates JAK and STAT signalling molecules, predominantly STAT3. Despite their common features, IL-12 and IL-23 exhibit distinct effects on the immune response associated with cancer [191].

Endogenous IL-23 was suggested to support tumour growth, the expansion of Th17 cells, enhanced production of Th17 cell-related cytokines (e.g., IL-17), and reduced $CD8^+$ T-cell infiltration [78, 106]. IL-23–deficient mice have demonstrated enhanced cytotoxic T-cell activity, which reduced tumour incidence and growth [106]. In mouse models of colon cancer, STAT3 activation in Tregs was shown to upregulate IL-23 and downregulate IL-12 expression [101]. Evidence suggests that dendritic cells, macrophages, and neutrophils produce IL-23 during intestinal inflammation [10, 94]. A variety of hematopoietic cells in the intestine, including innate lymphoid, Treg, and Th17 cells, can react to IL-23 [3]. The net biological effect of IL-23 signalling may indirectly promote tumour cell survival. IL-23 has been reported to drive intestinal inflammation by inducing other proinflammatory cytokines, such as IL-6, IL-17, and IL-22. These cytokines may in turn activate tumour cell proliferation through STAT3 and nuclear factor kappa B (NF-kB). IL-23 has been associated with traditional hallmarks of chronic inflammation in GI cancers, including enhanced activities of MMP9, angiogenesis, and macrophage infiltration [106].

The effects of IL-12 in the context of cancer are thought to generally be protective. Some data suggest that IL-12 promotes antitumour immunity via activation and proliferation of NK cells, Th1 cells, and cytotoxic T cells [112, 170]. In mouse models of colon carcinoma, IL-12 induced tumour suppression via an IFN-γ–independent fashion [170]. Interestingly, unlike IL-23, IL-12 expression was not elevated in human colon adenocarcinoma [106]. Clinical studies have attempted to evaluate the effects of recombinant IL-12 in patients with different cancer settings, including colon cancer, but meaningful results have not been observed [170].

Granulocyte/Macrophage Colony-Stimulating Factor

Granulocyte/macrophage colony-stimulating factor belongs to the hematopoietic growth factor family of cytokines and has significant functional homology with IL-3 and IL-5 related to a common β-receptor subunit [185]. The ligand specificity of GM-CSF is preserved by distinct α-receptor subunits. The α-subunits are specific for each cytokine and bind their specific ligand with low affinity. The βc-subunit forms a high-affinity receptor with all 3 α-subunits, despite its lack of capacity to bind the cytokines by itself. The βc-subunit is not only required for the formation of the high-affinity receptor complex but also crucial for signal transduction.

GM-CSF is elevated in the majority of PDAC patient samples. In a study evaluating the tumour tissue expression, 14 out of 16 patient samples were positive for GM-CSF (i.e., ≥75% of pancreatic intraepithelial neoplasias within a section exhibited ≥50% stained cells per lesion) [143]. Upregulated GM-CSF expression was also observed in invasive PDAC lesions, suggesting that overexpression of GM-CSF persists throughout disease progression. In addition, GM-CSF expression was not detected in lesions from non-PDAC cases, including chronic pancreatitis, pancreatic dermoid cyst, pancreatic endocrine neoplasm, and serous cystadenoma.

The effects of GM-CSF on the JAK-STAT signalling pathway are central to macrophage differentiation, tumour transformation via epithelial to mesenchymal transition, and tumour angiogenesis [4]. GM-CSF–activated STAT3 is involved in epithelial-mesenchymal transition regulation, and JAK2 and STAT3 are believed to play a role in blood vessel development [4]. In an elegant series of experiments using a genetically engineered mouse model of PDAC, two independent groups led by Drs. Pylaveya Gupta and Bayne demonstrated that Kras-induced GM-CSF production promotes the development of pancreatic neoplasia, promotes suppressive myeloid cells, and negatively modulates T-cell tumour immunity [14, 143]. In pancreatic ductal cells harbouring oncogenic Kras, GM-CSF production is associated with the expansion of a heterogenous Gr1+CD11b+ myeloid cell population, including myeloid-derived suppressor cells, monocytes, and immature myeloid cells [143]. Several lines of evidence suggest that Gr1+CD11b+ cells are immunosuppressive. Of particular relevance is that tumour cell-derived GM-CSF can orchestrate an immunosuppressive crosstalk between Gr1+ CD11b+ immature myeloid cells and $CD8^+$ T cells [14]. Abrogation of tumour-derived GM-CSF led to reduced Gr-1+

CD11b+ cell infiltration and failure of implanted tumours to grow; however, $CD8^+$ T-cell depletion was able to restore tumour growth. The potential relevance of this immune modulatory mechanism to advanced stages of pancreatic cancer is further supported by the reciprocal relationship between $CD8^+$ T cells and Gr1+CD11b+ cell infiltrates observed in pancreatic tumours from the murine PDAC model.

The role of GM-CSF in other GI malignancies is not well characterised and may be multifactorial. In CRC, GM-CSF has been proposed to induce tumour suppression via separate immune-mediated and immune-independent pathways [172]; abolishing GM-CSF resulted in marked increases in tumour volume and weight in vivo. In addition, T-lymphocyte infiltration and T-cell–mediated cytolytic effects were higher in GM-CSF–expressing tumours compared with controls [172], suggesting that findings in PDAC may not be applicable to other GI cancer settings. Further studies are needed in carefully controlled experiments utilising patient-derived tissues to determine the clinical settings in which GM-CSF blockade or addition could be beneficial.

Regulation of the Tumour Microenvironment by JAK-Signalling Cytokines

Bone marrow-derived cells of the innate and adaptive immune systems are recruited to the tumour microenvironment during the inflammatory response and may play different roles in promoting or suppressing GI carcinogenesis [41, 60, 144]. JAK-signalling cytokines represent a key mechanism by which immune and inflammatory cells interact with the tumour and tumour stroma to influence tumour initiation, promotion, and metastasis (Fig. 7.4). Individual cellular components are explored in this section to further understand the potential impact of JAK-STAT modulation on the tumour microenvironment.

Inflammatory Component of GI Tumours

Tumour-associated inflammation is an important component of oncogenesis in a variety of cancers [62]. Several types of inflammatory cells infiltrate preceding or following tumour formation and may be induced by cancer treatments, including chemotherapy and radiation [60]. Many types of GI malignancies, including CRC, pancreatic cancer, HCC, and gastric cancer, may arise from chronic inflammatory disorders [145]. In CRC, inflammatory signals (e.g., via IL-6), are essential to the transformation of adenomas to carcinomas via activation of antiapoptotic and proliferative factors, including STAT3 [59]. The activation of several oncogenes, including KRAS, p53, APC, and β-catenin, were suggested to promote inflammation in GI malignancies, primarily through dysregulated NF-kB and STAT3 signalling [38, 40, 167].

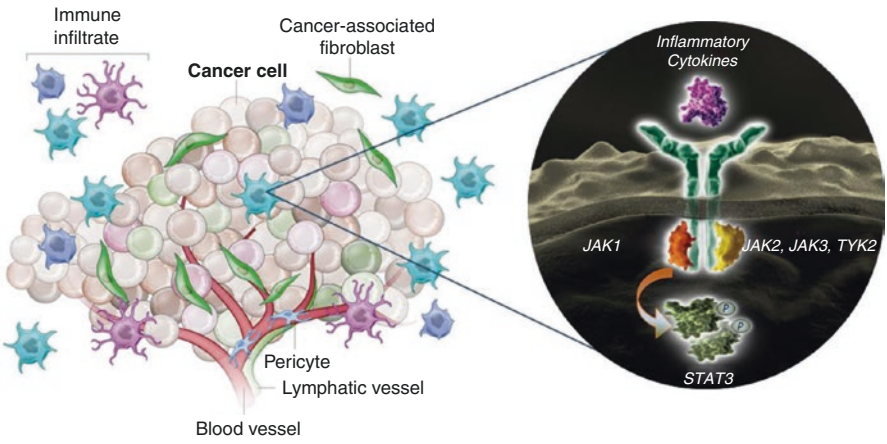

Fig. 7.4 Tumour-associated inflammation orchestrated by cytokines and JAK-STAT signalling (Juntila 2013). The JAK-STAT signalling pathway is central to signal transduction of inflammatory cytokines in the tumour microenvironment and the regulation of tumour-associated inflammation, including immune cell infiltration and new blood vessel development

In the microenvironment of solid tumours, including GI malignancies, inflammation is involved in complex mechanisms that directly and indirectly affect multiple aspects of tumour biology, including tumour initiation, tumour development, and angiogenesis [60]. A variety of evidence suggests that tumourigenesis and inflammation operate in tandem. Reactive nitrogen and oxygen species and tumour-associated cytokines produced from activated inflammatory cells may induce DNA damage and epigenetic changes, including stem cell-like phenotypes in tumour progenitor cells, which contribute to the genetic instability that promotes tumour initiation [60, 89, 146]. In addition, chronic inflammation can perpetuate this instability via diminished DNA mismatch repair mechanisms, DNA damage-induced apoptosis, and cell-cycle control [89, 146]. These changes can induce proinflammatory cytokine production and further inflammatory responses [60].

Cytokines and enzymes produced by inflammatory cells in the GI tumour microenvironment also support tumour survival, proliferation, invasion, and metastasis, which are primarily regulated via NF-kB and STAT3 signalling pathways [61]. For example, the proinflammatory cytokine IL-6 was demonstrated to regulate the survival of malignant pancreatic cells via upregulation of antiapoptotic genes, including *BcL-2* and *BcL-xL*, and the proliferation of pancreatic cancer cells was shown to be regulated via IL-4, IL-6, and IL-8. Studies have also demonstrated that other proinflammatory cytokines (e.g., IL-1α, IL-1β, transforming growth factor beta [TGF-β]) play important roles in the invasive and metastatic mechanisms of pancreatic cancer cells. In a model of colon cancer, matrix metalloproteinases 2 and 9 (MMP2, MMP9) produced by immature myeloid cells (iMCs) were also associated with tumour invasiveness [95].

The "angiogenic switch," or promotion of intratumoural blood supply in solid tumours, including GI malignancies, is essential for tumourigenesis and can be turned

on via tumour-associated inflammatory responses [102]. Triggered by a hypoxic tumour microenvironment, hypoxia-inducible factor-1α upregulates the expression of different proangiogenic factors, including VEGF, angiopoietin, and other proinflammatory cytokines/chemokines [102]. In pancreatic cancer, the angiogenic effect is primarily mediated via proinflammatory IL-6 and IL-8 signalling [64].

Triggers of inflammation in GI cancers can also be indirect, stemming from environmental or infectious origins. For example, associations between infection and risk of cancers have been observed between hepatitis B and C viruses and HCC and between Schistosoma or Bacteroides species and colon cancer [60]. In addition, obesity has been proposed to induce HCC and pancreatic cancer in some patients because of obesity-related chronic inflammation [88, 137]. Finally, inflammatory cytokines and growth factors, including cyclooxygenase-2 and VEGF, have been described as components of the mechanisms through which nicotine promotes GI carcinogenesis [80].

Innate Immune Components of GI Cancers: Myeloid Cells

Myeloid-derived suppressor cells (MDSCs), TAMs, and tumour-associated neutrophils (TANs) are among the most abundant cells of the innate immune system in the tumour microenvironment [144, 175]. MDSCs consist of a heterogenous population of activated iMCs that are produced excessively in tumour-bearing mice and human cancer settings [144]. Significant tumour infiltration of iMCs in mouse models of colon cancer and oesophageal squamous cancer suggest that MDSCs are implicated in the pathogenesis of GI malignancies [160, 165]. Under pathological conditions, components of the tumour microenvironment alter the normal differentiation process by expanding and activating iMCs to MDSCs [49]. A variety of cytokines and growth factors can utilise the JAK-STAT signalling pathway to induce MDSC expansion and activation in GI malignancies, including VEGF, GM-CSF, IFN-γ, IL-4, IL-10, IL-12, and IL-13. JAK2/TYK2-STAT3, JAK1/JAK3-STAT6, and JAK1-STAT1 are major components of signalling pathways involved in the expansion and activation of MDSC populations. Upon activation, MDSCs produce immunosuppressive factors, including TGF-β, IL-10, IL-12, arginase, nitric oxide synthase (iNOS or NOS2), nitric oxide, and reactive oxygen species that inhibit cytotoxic T cells, NK cells, and DCs while promoting Th2 and Treg cells [49, 68, 144, 195].

Under specific chemokine/cytokine and growth factor signalling, peripheral blood monocytes are recruited and differentiated into macrophages with two principal phenotypes, the classically activated M1 and alternatively activated M2 [23, 91]. IL4/IL-13 and IL-10 activate STAT6 and STAT3, respectively, and induce M2 and M2-like phenotype, whereas IFN-γ and lipopolysaccharide activate STAT1 and other transcription factors that lead to M1 phenotype polarisation [23]. The different roles of M1 and M2 in carcinogenesis and inflammation are also dependent on the types of cytokines and chemokines they express [50]. M1 macrophages are typi-

cally characterised by a low expression of immunosuppressive IL-10, a high expression of IL-12 and Th1-attractant chemokines (e.g., CXCL9, CXCL10), and the production of proinflammatory cytokines (e.g., IL-12, IL-23). Therefore, the M1 phenotype has been proposed to play a crucial role in enhancing Th1 responses and mediating cytotoxic activities against tumours [50]. In contrast, M2 macrophages are characterised by elevated IL-10 levels and other molecules involved in the recruitment of Th2, Treg, and other immune cells that promote tumour growth, invasion, and metastasis, as well as angiogenesis, while inhibiting T-cell–mediated antitumour activities [50]. In addition, the M2 phenotype has been associated with inflammation resolution [23].

Tumour-associated neutrophils are mature neutrophils in the tumour microenvironment that are different from their granulocyte precursors, as evidenced by findings from transcriptomic analyses [44, 91]. Emerging evidence has suggested that neutrophils participate in both procarcinogenic and anticarcinogenic processes via specific cytokines and other mediating molecules in different types of cancers, including GI malignancies [50]. The combination of IL-6 and G-CSF was shown to augment STAT3 signalling in neutrophils in vivo and may promote tumour growth and angiogenesis [193]. Similar to the phenotype polarisation observed with macrophages, TANs may adopt either an N1 or N2 phenotype, with a skewed differentiation towards the latter [44]. The proinflammatory N1 phenotype was proposed to amplify intratumoural $CD8^+$ T-cell activities via production of T-cell–attracting cytokines (CXCL9, CXCL10, CCL3) and proinflammatory cytokines (IL-12, TNF-α, GM-CSF, and VEGF) [44]. In contrast, N2 neutrophils suppress T-cell–effector activities via production of high arginase levels and do not promote inflammation [44]. Findings from a mouse model of colon carcinoma indicated that $CD8^+$ T-cell depletion led to decreased TANs in the tumour microenvironment, suggesting a possible mechanism by which T cells may attract and activate TANs [44].

Both the antitumour immunity and tumour-promoting effects of DCs have been implicated in the pathogenesis of solid tumours, including GI malignancies [30]. At the interface of innate and adaptive immune systems, increased tumour-infiltrating DCs was correlated with increased infiltration of $CD8^+$ and $CD4^+$ lymphocyte subsets, highlighting the essential role of DCs in antitumour activities via recruitment of cytotoxic T cells [35]. However, it was demonstrated that tumour-derived factors, including IL-4 and GM-CSF, activated the JAK2/STAT3 signalling pathway in iMCs and impaired the differentiation process of DCs, which could potentiate immune evasion [125]. In vitro and in vivo studies in human pancreatic carcinoma cells demonstrated that tumour-derived cytokines, including TGF-β, IL-10, and IL-6, reduced DC survival and proliferation and promoted differentiation toward the immunosuppressive plasmatoid DC phenotype versus the protective myeloid phenotype [17]. The immunosuppressive and procarcinogenic effects of tumour-associated DCs are primarily dependent on the production indoleamine 2,3-dioxygenase enzymes, which inhibit effector T cells; TGF-β, which expands Tregs; and angiogenic factors that promote new blood vessel development in the tumour microenvironment [20, 30].

Adaptive Immune Components of GI Cancers: Lymphoid Cells

Lymphoid-derived cells, including T-cell subsets, are members of the adaptive immune system and have essential roles in the tumour microenvironment [60]. Several T-cell subtypes are present in the tumour microenvironment and play an important role in carcinogenesis, including $CD8^+$ cytotoxic T cells, $CD4^+$ Th cells, Tregs, and NK cells [45, 60]. A T-cell–mediated adaptive immune response is a key driver of antitumour activities, primarily via cytotoxic and Th cells [5].

The differentiation of $CD4^+$-naive T cells into Th1, Th2, Th17, or Treg cells depends on the cytokine environment and the corresponding affect on STAT proteins [203]. IL-12 and IFN-γ induce Th1 differentiation via STAT4 and STAT1/2 heterodimers, whereas IL-4 and IL-2 induce the Th2 phenotype via STAT5 and STAT6 [203]. Th cells can mediate an antitumour response through multiple mechanisms, including the regulation of adaptive and innate immune cells [97]. They can directly or indirectly enhance cytotoxic T-cell growth through IL-2 signalling, activate APCs, and reactivate memory T cells. Cytokines produced by Th cells can also recruit macrophages and eosinophils to the tumour site, which produce cytotoxic factors, including superoxide and nitric oxide [73]. In GI malignancies, Th2 cells can induce inflammation and support tumour growth [144]. In murine models of colitis-associated colon cancer, IL-4 produced by Th2 was found to induce tumour proliferation [99]. The signature cytokines of Th2, including IL-4 and IL-13, can also contribute to tumour formation and progression via M2 phenotype induction of TAMs [23].

Major JAK signalling cytokines involved in T-cell differentiation include IL-6, IL-21, and IL-23. All 3 of these cytokines have been shown to activate STAT3 and induce Th17 development, whereas Treg development is dependent on IL-2–driven STAT5 activation [203]. In addition to being induced locally, Tregs can also be recruited from peripheral organs to the tumour microenvironment [45, 204]. Studies have demonstrated elevated Th17 cell levels in the peripheral blood of patients with gastric and pancreatic cancer, suggesting a role in carcinogenesis [103, 197]. Th17 cells also produce a wide array of other cytokines, including TNF-α, IL-21, IL-22, GM-CSF, and IFN-γ, that can regulate the tumour microenvironment [6]. In patients with early-stage GI cancers, Th17 and Treg-cell densities were elevated in the tumour microenvironment compared with intraepithelial lymphocytes from healthy gastric mucosa [118]. Interestingly, as the tumours progressed, a decrease in Th17 cells and an increase in Treg cells was observed [118]. This observation is consistent with the hypothesis that Tregs suppress adaptive and innate antitumour immunity to enable tumour evasion [5, 60, 116]. Multiple suppressive mechanisms of Tregs have been described, including inhibition of effector T-cell proliferation and APC function in a juxtacrine or paracrine fashion [5, 204] and suppression of antitumour activities via the production of immunosuppressive cytokines [176].

NK cells are a subset of T cells that are known for their direct and indirect antitumour effects via activation of tumour cell apoptosis or induction of Th cells [175]. Nearly all aspects of NK cell biology are dependent on a core set of cytokines and

associated receptors that signal via virtually all components of the JAK-STAT pathway, including IL-2, IL-12, IL-15, IL-18, and IL-21 [150]. Several clinical trials have evaluated these cytokines as therapeutic targets in the context of GI malignancies, including IL-12 in colon cancer and IL-21 in CRC. NK cells also produce a mixture of cytokines, including IFN-γ, that promote a Th1 response [57]. Other chemokines produced by NK cells can also promote the antigen-presenting function of other immune cells in the inflammatory tumour microenvironment [57]. However, these functions can be dysregulated by immunosuppressive cells, including MDSCs, TAMs, Tregs, and tumour-associated fibroblasts, and associated mediators that exist in the tumour microenvironment [175].

Therapeutic Approaches to Modulate JAK-STAT Pathway for the Treatment of GI Cancers

Overactive JAK-STAT signalling [156] resulting from tumour intrinsic or extrinsic mechanisms, gain of function mutations, or abnormal cytokine stimulation has important tumour-promoting effects, including tumour cell proliferation, invasiveness, metastatic potential, and antiapoptotic effects. Given the success of treatments targeting mutated kinases (e.g., Bcr-Abl, epidermal growth factor receptor [EGFR], B-raf, ALK, or those that are less critical for normal immune functions, such as VEGF receptor kinase, BTK, and PDGF), it is tempting to approach JAK inhibitors in a similar manner (i.e., maximal and around-the-clock inhibition). However, considering the critical roles that the JAK-STAT pathway plays in regulating normal physiological processes (e.g., immune and bone marrow functions), the goal of JAK-targeted therapies should be to achieve normalisation rather than complete inhibition. To achieve this goal, the use of highly selective inhibitors with well-defined dosing regimens is required to limit treatment-related side effects while maximising efficacy.

This section considers the published preclinical and clinical data regarding the activity of small molecule inhibitors of JAK-STAT signalling in GI cancers and also explores the potential to combine JAK-STAT–targeted therapies with immune checkpoint inhibitors for the development of optimised, comprehensive immunomodulatory treatment approaches for GI cancers.

Small-Molecule JAK or STAT Inhibitors

Several direct inhibitors of JAKs and STATs have been evaluated for a variety of disorders, but only a limited number of trials have included patients with GI cancers. However, existing preclinical evidence support antitumour activity for JAK inhibitors in models for CRC [8, 155, 178], pancreatic cancer [56, 72, 166], HCC [47, 187], and gastric cancer [83].

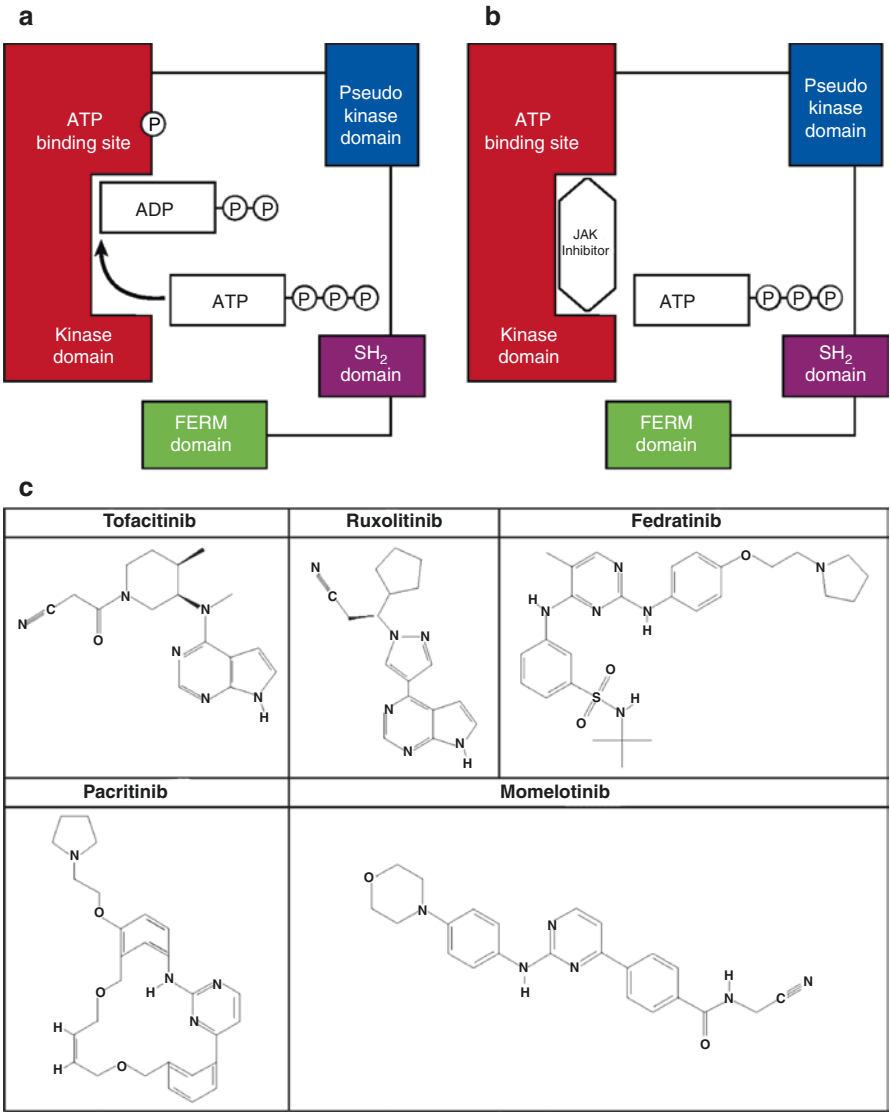

Fig. 7.5 Janus kinase structure and inhibitors. JAK includes an ATP-binding site where ATP must interact for signalling to progress through the JAK/STAT pathway (**a**). Blocking the ATP-binding site with a JAK inhibitor prevents JAK phosphorylation and activation (**b**). Several JAK inhibitors (**c**) are approved for use in certain disease settings (tofacitinib and ruxolitinib) or are in ongoing clinical development (fedratinib, pacritinib, and momelotinib)

Typical JAK inhibitors are ATP mimetics that bind to the catalytic domain of the enzyme and competitively inhibit ATP from binding, thereby inhibiting the phosphotransfer reaction (Fig. 7.5). Tofacitinib (Xeljanz®, Pfizer Inc, NY, NY) [189] and ruxolitinib (Jakafi®, Incyte Corporation, Wilmington, DE) [79] are 2 JAK inhibitors that have been approved by the US Food and Drug Administration (FDA) for use in

humans. In addition, several other JAK inhibitors are being evaluated in ongoing trials. The effectiveness of these inhibitors in patients with GI cancer remains unclear. Tofacitinib has inhibitory activity against JAK1, JAK2, and, JAK3 and is approved for patients with moderately to severely active rheumatoid arthritis who have had an inadequate response or intolerance to methotrexate Tofacitinib is also being tested for other autoimmune conditions, such as alopecia areata, atopic dermatitis, psoriasis and psoriatic arthritis, ankylosing spondylitis, systemic lupus erythematosus, ulcerative colitis, and keratoconjunctivitis sicca; there is no evidence, however, of ongoing trials in patients with cancer.

Ruxolitinib is a JAK1/JAK2 inhibitor that is approved for patients with intermediate or high-risk myelofibrosis (MF), including primary MF, post-polycythemia vera (PV) MF, and post-essential thrombocythemia MF, and patients with PV who have had an inadequate response to or are intolerant of hydroxyurea [79]. In addition, ruxolitinib is in clinical trials for patients with multiple other haematological and solid cancer indications, including leukaemia, non-small cell lung cancer, and breast cancer.

In vitro studies were performed to evaluate the role of JAKs and the potential utility of ruxolitinib in a number of CRC cell lines [7], including DLD-1 and RKO, which phosphorylate JAK1 and JAK2 at basal levels. Ruxolitinib inhibited JAK1 and JAK2 in DLD-1 cells but only JAK1 in RKO cells, and inhibition of JAK1 but not JAK2 was proportional to cell death. In addition, ruxolitinib conferred caspase-dependent cell death, which was abrogated by treatment with z-VAD. Considering that the inhibition of JAK1 phosphorylation seemed sufficient for ruxolitinib-mediated apoptosis in this analysis, further research is warranted to determine if a selective JAK1 inhibitor could be an effective therapy against CRC.

Ruxolitinib has also been evaluated in vitro using models of HCC tumourigenesis [187]. Ruxolitinib effectively inhibited the expression of pSTAT1 and pSTAT3, which are important downstream targets of the JAK/STAT signalling pathway, and was associated with a marked reduction in HCC cell proliferation and colony formation. Interestingly, ruxolitinib was not associated with notable increases in apoptosis, suggesting that ruxolitinib confers an anticancer effect mediated via inhibition of proliferation as opposed to induction of apoptosis. Given the presence of activating *JAK1* mutations in some patients with HCC [194], ruxolitinib was evaluated in *JAK1*-mutant models alongside a *JAK1*-WT PDX model as a control. In mice with the $JAK1^{S703I}$ mutation, treatment with ruxolitinib was associated with 48% tumour growth inhibition, suggesting that $JAK1^{S703I}$ may play a critical role in HCC tumourigenesis in this model. In addition, treatment with ruxolitinib significantly reduced (50%) STAT3 phosphorylation. Collectively, these results suggest that further research is warranted to determine if ruxolitinib could be an effective treatment option in patients with HCC, and in particular patients with specific *JAK1* mutations.

In a double-blind, randomised phase 2 study, ruxolitinib combined with capecitabine was compared with capecitabine alone in patients with metastatic pancreatic cancer who had experienced treatment failure with gemcitabine [74]. In the intent-to-treat population, the HR for OS and progression-free survival (PFS) were 0.79 (95% CI, 0.53–1.18; $P=0.25$) and 0.75 (95% CI, 0.52–1.10; $P=0.14$),

respectively. In a prespecified subgroup analysis of patients with inflammation (serum C-reactive protein >13 mg/L), OS was significantly greater with ruxolitinib compared with placebo (HR, 0.47; 95% CI, 0.26–0.85; $P=0.011$). The frequency of grade ≥3 adverse events was similar between ruxolitinib and placebo arms (74.6% vs 81.7%, respectively). However, in a larger phase 3 study (JANUS 1) of ruxolitinib or placebo in combination with capecitabine in patients with advanced or metastatic pancreatic cancer who had failed or were intolerant of first-line chemotherapy and had evidence of systemic inflammation [75], preliminary results did not indicate that ruxolitinib was sufficiently efficacious; JANUS-1 and the similarly designed phase 3 JANUS 2 study were subsequently discontinued [77].

In a 2-part phase 3 study, momelotinib is being evaluated in combination with nab-paclitaxel and gemcitabine in patients with untreated metastatic PDAC. The lead-in phase aims to evaluate the safety and pharmacokinetics and define the maximum tolerated dose (MTD) of momelotinib, and the randomised treatment phase will evaluate the efficacy (objective response rate, OS, and PFS), safety, and tolerability of the combination of momelotinib administered at the MTD versus matched placebo combined with chemotherapy (Clinical trials.gov, NCT02101021).

Other JAK and STAT inhibitors are under clinical investigation for a wide range of cancers and other disorders. Lestaurtinib (Cephalon, Inc., Frazer, PA) inhibits the activity of JAK2, as well as the tyrosine kinases FLT3 and TrkA [53, 67, 159] and is in clinical trials for patients with myeloproliferative neoplasms, leukaemia, neuroblastoma, psoriasis, multiple myeloma, prostate cancer, and chronic beryllium disease. Momelotinib (Gilead Sciences, Foster City, CA) is a JAK1/JAK2 inhibitor [135] that is in clinical development for multiple indications, including myeloproliferative neoplasms, metastatic pancreatic ductal adenocarcinoma, and non-small cell lung cancer. Pacritinib (Cell Therapeutics, Inc., Seattle, WA) is a JAK2 inhibitor that was placed on clinical hold by the FDA in February 2016 for life-threatening side effects, including intracranial haemorrhage, cardiac failure, and cardiac arrest.

The potential for developing pharmaceutical agents targeting STAT family members is extensively discussed in the literature, but the discovery and development of selective small-molecule STAT inhibitors has been challenging. Unlike JAKs, STATs do not possess a well-defined binding pocket that allows structure-based drug design. OPB-31121 is a chemical that was shown to strongly induce growth inhibition of various tumour cell lines through STAT inhibition. Although the exact mechanism of action associated with OPB-31121 has not been fully elucidated, this chemical was shown to inhibit tumour growth in mice and to inhibit phosphorylation of STAT1, STAT3, and STAT5 in gastric cancer cell lines [92]. Further analyses in Hep G2 cells demonstrated that IL-6 stimulation induced JAK2 phosphorylation, which was not inhibited by OPB-31121, whereas STAT3 phosphorylation was strongly inhibited [65]. In HEL cells with activating *JAK2* mutations, STAT3 and STAT5 phosphorylation was inhibited at early time points after OPB-31121 administration. Inhibition of JAK2 phosphorylation was not observed, although phosphorylated JAK2 decreased 24 h after OPB-31121 administration, most likely due to cell death-related degradation of JAK2. In H1650 cells carrying constitu-

tively activated STAT3 induced by mutated EGFR, OPB-31121 reduce STAT3 phosphorylation, indicating that the STAT3 phosphorylation effect could occur independently of the type of upstream kinases. OPB-31121 is currently in clinical trials for patients with advanced solid and hematologic tumours.

Other approaches to target STAT inhibition, including STAT antisense oligonucleotides and dominant-negative STAT proteins, have also been investigated with limited success [26]. In a mouse model of melanoma using the dominant-negative STAT3 variant, STAT3β gene therapy inhibited tumour growth and regression [128]. Despite relatively low transfection efficiencies (approximately 10–15 %), STAT3β-induced antitumour effect was associated with melanoma cell apoptosis, suggesting an effect on "bystander" cells (i.e., genetically untransduced tumour cells). Additional strategies for achieving STAT inhibition have been proposed, including (1) antagonists of receptor-ligand interaction (e.g., cytokine antagonists, receptor-neutralising antibodies), (2) alteration of STAT-interacting proteins (e.g., PIAS, SOCS), (3) inhibition of STAT-activating serine kinases, (4) activation of STAT-specific phosphatases, (5) modification of STAT-regulated genes associated with tumour progression, and (6) small-molecule inhibitors that interfere with STAT dimerisation or DNA binding [26]. In addition, the peptides that block STAT3 dimerisation and DNA-binding activity both in vitro and in vivo can inhibit cell transformation mediated by activated STAT3, and provide rationale for exploration of peptidomimetics with pharmacologic properties [171].

Cytokine Targeted Therapies

Immunomodulatory cytokines such as IFN-α, IL-6 and IL-10 signal prominently through the JAK-STAT pathway and may be effective targets for GI cancer therapies. Pegylated (PEG)–IFN-α variants in combination with the antiviral medication ribavirin was shown to inhibit viral replication [46, 117], which may reduce the risk of hepatitis B or C infection and the associated development of HCC [139]. In a murine HCC model, PEG–IFN-α suppressed tumour growth in vitro and in vivo [9]. In addition, IFN-α–based therapies may improve survival in patients with HCC after curative therapy [71].

A number of neutralising antibodies that block the biological functions of cytokines implicated in GI malignancies have been approved for the treatment of other disease states (Fig. 7.6), some of which are currently in clinical trials for GI malignancies. Tocilizumab (Actemra®, Genentech, San Francisco, CA) [2] is an IL-6R antagonist indicated for the treatment of rheumatoid arthritis, polyarticular and systemic juvenile idiopathic arthritis. A phase 2 clinical trial is planned to evaluate tocilizumab in combination with chemotherapies in patients with advanced or metastatic pancreatic cancer (Clinicatrials.gov identifier: NCT02767557). Other IL-6–directed therapies approved for clinical use or in late stage clinical trials include siltuximab (Sylvant®, Janssen Biotech, Horsham, PA) [163] for multicentric Castleman's disease, clazakizumab in phase 2b trial for moderate to severe rheumatoid arthritis

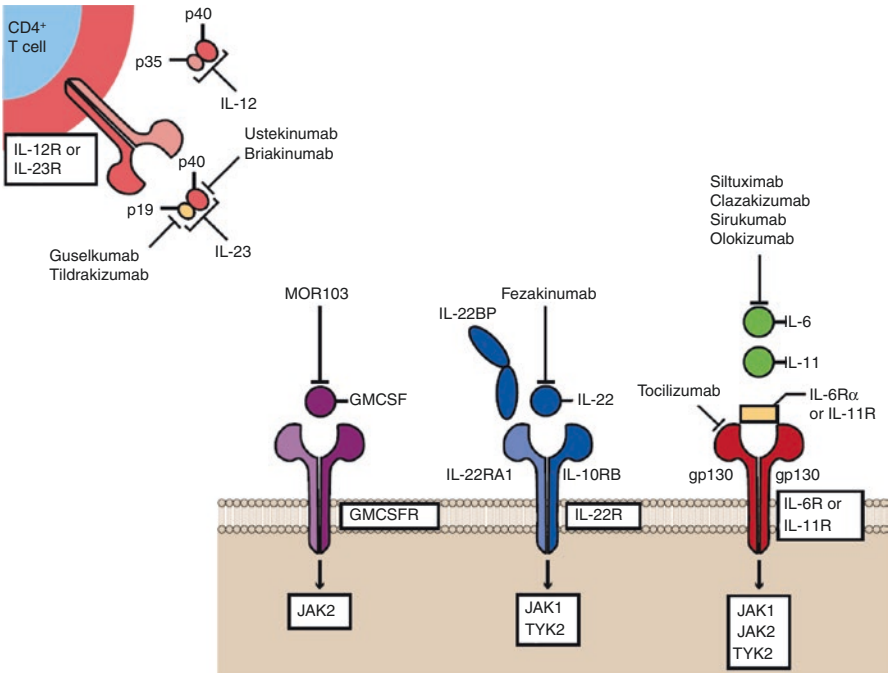

Fig. 7.6 Major cytokine signalling pathways in colorectal cancer and associated monoclonal antibodies in clinical development. This schematic depicts the four primary cytokine/receptor complexes that may play key roles in the pathogenesis of colorectal cancer (IL-12/23, GMCSF, IL-22, and IL-6/11) and corresponding monoclonal antibodies that have been developed against these complexes. Clinical investigations of these agents are necessary to evaluate their antitumour properties in the GI cancer setting

(NCT02015520), and olokizumab in phase 3 trials for rheumatoid arthritis (NCT02760368, NCT02760433, NCT02760407). In addition, sirukumab is in phase 3 trials for rheumatoid arthritis (NCT01606761, NCT01689532, NCT01604343, NCT02019472, NCT01856309) and giant cell arteritis (NCT02531633).

Four antibodies targeting various components of IL-12/23 system are currently approved or in clinical trials for various inflammatory indications. Ustekinumab (Stelara®, Janssen Biotech, Horsham, PA) [161] targeting the p40 subunit of IL-12/23 receptor, is currently approved for chronic plaque psoriasis, psoriatic arthritis and is in phase 3 clinical trials for ulcerative colitis (NCT02407236), Crohn's disease (NCT01369355, NCT01369342, NCT01369329), and axial spondyloarthritis (NCT02438787, NCT02407223). Briakinumab, another monoclonal antibody targeting the p40 subunit of IL-12/23, is in phase 2 and 3 clinical trials for multiple sclerosis (NCT00086671) and psoriasis (NCT00626002, NCT0691964). Antibodies targeting the p19 subunit of IL-23 that are currently in clinical trials for psoriasis include guselkumab (NCT02207244, NCT02207231, NCT02343744, NCT02203032, NCT02325219, NCT02319759) and tildrakizumab (NCT01729754, NCT01722331).

While there are no ongoing clinical trials for any of these agents in GI malignancies, emerging preclinical evidence on the role of these cytokines in cancer pathogenesis may prompt future clinical investigation. Similarly, other agents, including MOR103 (anti-GM-CSF) and fezakinumab (anti-IL-22), may also offer interesting novel approaches to evaluate the role of the cytokines in future clinical trials.

Potential Impact of JAK-STAT Pathway Modulation on Checkpoint Inhibitors

Immune checkpoint inhibitors target negative regulators of the immune response to promote antitumour immunity. The key immune checkpoint targets currently in clinical development act against PD-1/PD-L1, CTLA-4, and indoleamine 2,3-dioxygenase (IDO) [122, 141]. PD-1 and CTLA-4 are membrane-bound receptors expressed by T cells that downregulate T-cell activity after interacting with their membrane-bound ligands, PD-L1 and B7/CD80, respectively [141]. IDO is characterised as a tryptophan-catabolising enzyme. IDO-expressing antigen-presenting cells promote T-cell anergy and activation of regulatory T cells as a result of local consumption of tryptophan and production of kynurenine [122].

Several preclinical studies have suggested that the JAK-STAT pathway plays a role in regulating the activity of immune checkpoint inhibitors. Initial observations that $CD8^+$ T-cell–derived IFN-γ is a major driver of IDO1 and PD-L1 expression in tumour cells led to the current paradigm in tumour immunology that tumour cells may escape from immune control via an "adaptive resistance" mechanism mediated by T-cell–secreted IFN-γ [148]. These data support the idea that blocking IFN-γ signalling by JAK inhibition may reverse the local immunosuppressive effects of IDO and PD-L1. In vitro analyses also demonstrated that IL-6/IL-12 upregulated PD-1 expression in a STAT-dependent pathway [12], whereas JAK inhibition inhibited IFN-γ–induced upregulation of PD-L1 expression in tumour cell lines [18]. In addition, JAK2 was identified as a common signalling node to relay tumour cell-mediated signals induced by IFN-γ and EGFR. In a recently published study, overexpressed wild-type EGFR was significantly associated with PD-L1 and JAK2 expression in a large cohort of head and neck cancer specimens [32]. Specifically, PD-L1 expression was dependent on both EGFR and JAK2/STAT1. In addition, inhibition of JAK2 blocked PD-L1 upregulation in tumour cells, leading to enhanced tumour immunogenicity. Collectively, these data suggest a novel role for JAK2/STAT1 in tumour immune evasion, suggesting that therapies targeting this signalling node may be useful for limiting PD-L1 expression in a variety of cancers, including GI cancers.

Another IL-12 family cytokine, IL-27, was associated with upregulation of IDO and PD-L1 expression via STAT1 and STAT3, respectively, in cell culture assays [29, 199]. Furthermore, STAT5 signalling was required for expression of the CTLA-4 membrane-bound ligand B7/CD80 in cutaneous T-cell lymphoma cells [200], and mice lacking SOCS-3 were associated with upregulation of CTLA-4 [196].

Given the potential for the JAK-STAT pathway to regulate PD-1/PD-L1, CTLA-4, and IDO1 immunomodulatory pathways, preclinical studies were conducted to evaluate combinations of JAK inhibitors with IDO1 and PD-1 blockers [98]. Treatment with single JAK inhibitor agents, including ruxolitinib and INCB039110 (JAK1 selective), was shown to inhibit tumour growth in the immunocompetent syngeneic PAN02 pancreatic cancer model, which is driven by oncogenic JAK signalling, but not in immunocompromised mice. These results indicate that the antitumour effects of these agents require an intact immune system. JAK inhibition was also observed to synergise with IDO1 inhibition or anti–PD-L1 antibodies to block tumour growth. In addition, both JAK inhibitors modulated the levels and activity of tumour-infiltrating cells. The dose-dependent decreases in pSTAT that occurred with JAK inhibition coincided with observed changes in both inflammatory cytokines and IFN-γ–related genes. Based on these encouraging preclinical results, phase 1 clinical trials are currently under way to evaluate the JAK1 inhibitor INCB039110 plus the IDO inhibitor epacadostat (ClinicalTrials.gov identifier, NCT02559492) and INCB039110 plus the PD-1 inhibitor pembrolizumab in patients with advanced solid tumours (ClinicalTrials.gov identifier, NCT02646748).

Conclusions

In the 20 years since the discovery of JAK-STAT pathway as a key signal transduction mechanism for a wide range of cytokines and growth factors, clinicians and researchers have made exciting progress in exploiting this pathway for the treatment of a number of disease states. The pleiotropic nature of this mechanism and its overactivation in many disease states, including GI cancers, suggest that the promise of modulating this pathway has yet to be fully realised. Its importance in tumour intrinsic and extrinsic aspects and immune microenvironment also demands that appropriate combination therapies be developed to optimise the effectiveness of JAK-STAT modulators. In recent years, there has been an increasing appreciation of the role of key intervention points in immune networks to harness the immune system for improving cancer treatment options. Checkpoint blockers have validated the hypothesis that reversing tumour-driven subversion of immune control can be effective in restoring antitumour immunity. In addition, tumour-associated inflammation is now believed to be a key driver of tumour growth. Anti-inflammatory mechanisms are evoked following localised inflammation in tumours, which may have unintended consequences of suppressing antitumour immunity. Cytokines are critical regulators of inflammatory, immune, and anti-immune responses associated with tumours, and the JAK-STAT pathway is an established cornerstone of how cytokines mediate myriad immunologic responses. As our understanding of the specifics concerning immunomodulation and carcinogenesis expands and evolves, it is plausible to hypothesise that pharmacologic treatments targeting the JAK-STAT pathway may provide important breakthroughs in optimising treatment for some patients with GI cancer.

Conflict of Interest Statement Kris Vaddi is a consultant to Incyte Corporation.

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Chapter 8
Hypermutated Colorectal Cancer and Neoantigen Load

Mark A. Glaire and David N. Church

Introduction

While the importance of the immune system in modulating the growth and dissemination of human malignancies has been recognised for more than half a century [12], the recent clinical application of immune checkpoint inhibitors has helped substantially advance our understanding of the dynamic interplay between the tumour and the host immune response. Early studies of drugs targeting the immunosuppressive molecules CTLA-4, PD-1 and PD-L1 revealed that these agents were able to induce remarkable, and prolonged responses in a subset of melanomas [41, 115], non small-cell lung cancers (NSCLCs) [9, 31, 105] and bladder cancers [9, 105]. However, these results were not generalisable to many other tumour types, including unselected colorectal cancers (colorectal cancer, CRC) in early trials [9, 105]. Immune checkpoint inhibition enables T cells to respond to mutated peptides – or neoepitopes – presented by tumour cells [87]. The observation that melanomas, NSCLCs and bladder cancers are highly mutated suggested that the preferential benefit of these drugs in these cancers might be a consequence of an enrichment of antigenic, mutated neoepitopes in these tumour types. This postulate has been supported by translational studies, which have demonstrated that the number of antigenic mutations in tumours – that is, those mutations that generate peptides predicted to be efficiently presented by the patients' HLA class I molecules – correlates with clinical benefit from immune checkpoint inhibition in both melanoma and NSCLC [83, 98]. More recently, the correlation between mutation burden and response to immune checkpoint inhibitors has been extended to

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colorectal cancers, with the demonstration that hypermutated, mismatch repair-deficient (MMR-D) tumours show remarkable responses to these agents [55].

In this chapter, we review both the causes of hypermutation in colorectal cancer, and the current evidence regarding the mechanistic basis by which this elicits an antitumour immune response. While our focus is predominantly on mismatch repair deficiency, we also discuss other causes of hypermutation, such as mutation of the proofreading exonuclease domain of the DNA polymerase *POLE*, and highlight exciting data that suggest that hypermutation per se may not be required for an antitumour immune response.

The Biology of Colorectal Cancer

Historical Perspective

Most colorectal cancers are sporadic, though a small proportion (3–4 %) are caused by Mendelian high-penetrance conditions such as Lynch syndrome – a condition in which carriers carry a deleterious germline mutation in one of the mismatch repair (MMR) genes (discussed further below). The relative ease with which both adenomatous polyps (the precursor lesions to invasive colorectal malignancy), and colorectal cancers can be resected has meant that the biology of colorectal preneoplasia and neoplasia has been one of the best described among all cancers [29]. Until recently, colorectal cancers have typically been divided into two types according to their molecular phenotype. Most are characterised by aneuploidy and chromosomal instability (CIN), while tumours from patients with Lynch syndrome, and approximately 15 % of sporadic cancers are defined by an alternative mechanism of genomic instability, as a consequence of mismatch repair deficiency [62].

In a landmark paper published in 1988, Vogelstein and colleagues proposed a model for the biology of the adenoma-carcinoma sequence in CIN tumours [112]. In this schematic, sequential driver mutations in tumour suppressor genes and oncogenes mediate progression from pre-cancer to non-invasive, invasive and ultimately metastatic malignancy. This model – often colloquially referred to as the "Vogelgram" – involves early inactivating mutation of *APC*, followed by activating mutations in the small GTPase *KRAS*, loss of chromosome 18q and loss of *TP53* [112]. The corresponding model for mismatch repair deficient cancers involves early inactivation of mismatch repair components, either by loss of the wild-type gene copy as a result of loss of heterozygosity in patients with Lynch syndrome, or by methylation of the *MLH1* promoter, with consequent gene silencing in sporadic cases [44, 47]. Following this, tumours acquire subsequent mutations in tumours suppressors such as *BAX*, *IGF2R*, *TGFBR2* and *PTEN*, often due to mutation of repetitive microsatellites within these genes that are at substantially increased risk of alteration in the context of defective MMR. The biology and consequences of defective mismatch repair are discussed in greater detail in Sect. 4.1. While these models represent a simplification of what is a far more complex underlying biology,

they have framed many if not most of the subsequent studies that have advanced our understanding of colorectal cancer pathophysiology. Indeed, in the pivotal recent transcriptomic-based Consensus Molecular Classification of colorectal cancer [36], MMR-D tumours mainly cluster in CMS1, while CIN tumours are found in subtypes CMS3, CMS4, and particularly CMS2 subtypes.

Recent Advances

Compared with the Sanger sequencing that informed the Vogelstein model discussed above, next generation sequencing (NGS) technologies provide an immeasurably more powerful platform with which to interrogate tumour biology. Therefore it is unsurprising that, as has been the case with other malignancies, their application to colorectal cancer has substantially advanced our understanding of the molecular pathogenesis of this disease.

Perhaps the most comprehensive effort to date is that from The Cancer Genome Atlas group of investigators in the US. This collaborative effort has performed whole exome sequencing, transcriptomics, methylomic and copy number analysis on 33 cancer types, using paired tumour and normal tissue from 11,000 individuals [68].

The TCGA colorectal cancer study, reported in 2012, performed exome sequencing of 224 colorectal tumours [102]. This pivotal analysis confirmed that colorectal cancers could broadly be classified into on the basis of their mutational load into non-hypermutated and hypermutated cancers. Non-hypermutated colorectal cancers, defined as exhibiting a mutational rate of <8.24 per 10^6 bases and with a median of 58 non-silent mutations per tumour exome, represented the majority of tumours (84 %). The remaining, hypermutated cancers – which mostly displayed mismatch repair deficiency – had a substantially greater mutation load of >12 non-synonymous per 10^6 bases The rate of somatic copy number alterations was far greater in the non-hypermutated group, than the hypermutated group [102].

Analysis of the frequency of specific mutations shows that while hypermutated and non-hypermutated share genetic traits in their development, there are important differences. Consistent with the Vogelstein model of colorectal carcinogenesis, the most frequently mutated genes in the non-hypermutated group were *APC* (81 %), *TP53* (60 %) and *KRAS* (43 %). In hypermutated CRCs, the three commonest were: *ACVR2A* (63 %), *APC* (51 %) and *TGFBR2* (51 %). *TP53* was mutated in approximately 20 % of hypermutated cancers, and *TGFBR2* aberrations a very infrequent event in non-hypermutated [102]. In keeping with its recognised importance in colorectal cancer biology, deregulation of the WNT signalling pathway was common in both non-hypermutated and hypermutated tumours evident in 92 and 97 % of tumours respectively. However, deregulation in other signalling pathways highlights the different pathogenesis of these two groups. Hypermutated cancers were characterised by a down regulation of TGF-β signalling, present in 87 % of hypermutated cancers compared with 27 % of non-hypermutated tumours, and an up

regulation of RTK-RAS signalling (80 % vs. 59 %), and less frequent loss of function within the p53 pathway (47 % vs. 64 %) [102]. The distinct biology of mismatch repair deficient colorectal cancers is discussed further below.

Mutational Landscape of Colorectal Cancer

The preceding discussion regarding somatic mutations in colorectal cancers has focused on pathogenic variants that confer a selection advantage to cells during cancer initiation and progression – generally referred to as "driver" mutations. Unsurprisingly, given their importance, driver mutations have been the primary focus of oncology research. However, they typically account for only a minority of somatic variants in most colorectal (and other) cancers. The majority of mutations occur in genes that are unimportant for neoplastic processes, and have been widely regarded as being evolutionarily neutral – a class of variants commonly referred to as "passenger" mutations. Collectively the combination of driver and passenger mutations in a tumour constitute a record of the mutational processes that have operated during the life of that cancer and are referred to as the tumour "mutanome". It is now clear that this genomic mutational landscape, including both the number and spectrum of mutations, provides a wealth of information regarding not only the underlying tumour biology, but also the likely prognosis of that cancer and its response to therapy.

Although they vary in importance between, and within tumour types, the predominant mechanisms that contribute to somatic mutations in cancers broadly include: (i) inaccuracy of DNA replication; (ii) mutagen exposure; (iii) enzymatic DNA modification and; (iv) defects in DNA repair mechanisms [3, 40]. Considering only single nucleotide variants (SNVs), and the flanking bases immediately 5' and 3' to the substitution there are 96 possible triplet mutational combinations. Collating both the SNV frequency and type as documented by this 96-channel readout generates a mutational signature for a tumour, which in effect provides a record of the mutagenic forces that have operated during the carcinogenic process. In a landmark study, Alexandrov and colleagues used the 96-channel signature to analyse the mutational spectrum of 30 cancer types [3]. In addition to demonstrating that there is considerable variation in the total number of mutations between cancers, they demonstrated 21 distinct mutational signatures, several of which can be directly correlated with specific aetiological factors. For example, melanomas have the highest absolute mutational burden, and in particular a prevalence of mutational signature 7 – characterised by a high prevalence of C>T mutations at T<u>C</u>C trinucleotides caused by UV light induced DNA damage. In sporadic colorectal cancers, mutagen exposure has not been demonstrated to play a major role and accordingly, with the exception of mismatch repair deficient tumours, colorectal cancers have a lower mutational burden than melanoma and lung cancers. Furthermore, the commonest mutational signature in CRCs, detected in 77 % of cases was Signature 1B (C>T mutations predominantly occurring at N<u>C</u>G trinucleotides), which correlated

strongly with age at diagnosis and is likely the result of spontaneous deamination of 5-methyl-cytosine. In contrast, the two other notable signatures in CRCs, numbers six and ten, defined smaller subsets of tumours with a substantially higher mutational burden, collectively accounting for almost 70 % of mutations across all cases. Signature 6 is characterised by a predominance of N<u>C</u>G > N<u>T</u>G mutations and is associated with mismatch repair deficiency. Signature 10 is characterised by an exceptional number of SNVs (>100/Mb), and predominance of T<u>C</u>T > T<u>A</u>T and to a lesser extent N<u>C</u>G > N<u>T</u>G mutations. This signature is associated with a high total number of mutations, and results from mutations within the proofreading exonuclease domain of the DNA polymerase *POLE* [3].

Hypermutated Colorectal Cancers

Colorectal cancers are a heterogeneous group of tumours and whilst it has long been known that there exists a subset with substantially more mutations, TCGA analysis provided a comprehensive evaluation of the degree of hypermutation and the frequency at which it occurs. As mentioned above 16 % of CRC are hypermutated, pertaining to a mutation rate $>12 \times 10^6$ bases and a median of 728 non-silent mutations. Of these hypermutated tumours, 77 % exhibited microsatellite instability, which is a hallmark of mismatch repair deficiency. This was most commonly associated with hypermethylation of *MLH1*. The remaining hypermutated tumours constituted a group that did not show MSI, had the greatest mutational load and possessed somatic mutations in *POLE* [102]. The biology of the hypermutated mismatch repair deficient, and ultramutated *POLE* proofreading domain mutant colorectal cancer subgroups are discussed further in the following sections.

Mismatch Repair Deficiency

Accurate DNA replication is essential for maintaining genomic integrity [52, 53]. While DNA replication in itself is subject to errors, under normal circumstances several surveillance and repair mechanisms serve to limit the mutation rate to roughly one mutation for every $10^{7–8}$ bases replicated, or one error per genome duplication [52, 66]. Probably the most relevant of these mechanisms to cancer is the DNA mismatch repair system, which is responsible for the correction of base-base mismatches and insertion-deletion loops (IDLs) created during DNA replication [53].

Successful repair of base mispairs and IDLs by the mismatch repair system depends upon recognition of erroneous DNA replication, excision of the mispaired base or loop, and replacement and realignment of the DNA strands. In humans, the repair process begins with the binding of the MutS complex to the aberrant DNA. MutS has two principle forms: MutSα, a heterodimer of MSH2

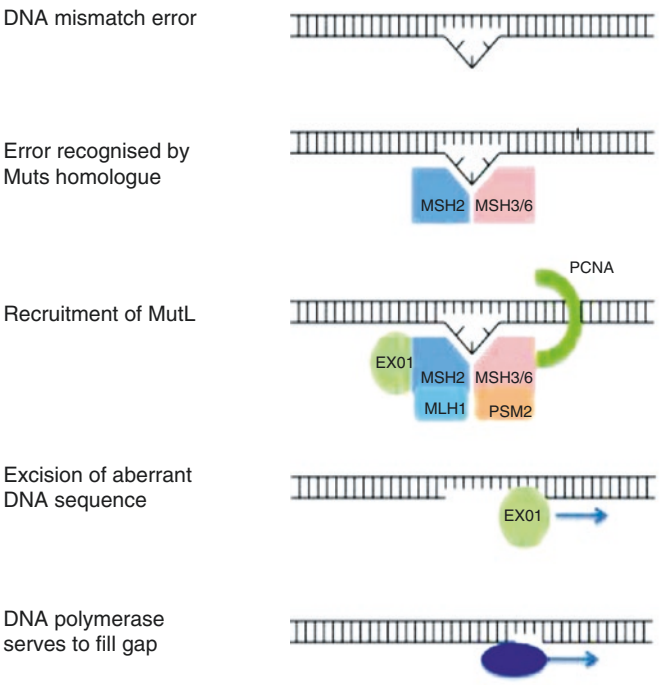

Fig. 8.1 DNA mismatch errors commonly occur at unstable genomic regions with multisatellite repeats. The repair of such DNA mismatch errors is the function of the MMR system. This process begins with recognition of DNA sequence errors by MutS homologues, which then recruits the MutL and associated peptides. The aberrant DNA bases are then excised and replaced by the replicative DNA polymerase. Abbreviations: *Exo1* exonuclease, *PCNA* proliferation cell nuclear antigen

and MSH6 proteins, is responsible for the repair of base-base mismatches and small IDLS [27, 74]. MutSβ, a heterodimer of MSH2 and MSH3, predominantly recognises larger IDLs [1]. Binding of MutS to the erroneous DNA sequence initiates recruitment of MutL complexes, of which the most biologically active is MutLα – a heterodimer of MLH1 and PMS2. MutL forms a ternary complex with MutS and the aberrant DNA strand to facilitate the recruitment of further proteins, including Exo1 and PCNA that participate in DNA repair. The offending DNA sequence is excised and replaced by the actions of DNA polymerase (Fig. 8.1) [46, 53].

Much of our understanding of MMR function, and the pathology resulting from loss of function has been collated from preclinical models, which have indicated a degree of functional overlap between the mismatch repair components. MSH2 appears to be essential for the initial recognition of DNA errors: *Msh2*$^{-/-}$ mice have a strong mutator phenotype at a cellular level, and develop lymphoma at an early age [23]. Furthermore, mice with conditional *Msh2* loss in the intestine develop multiple intestinal tumours [50]. *Msh6* null mice, similar to *Msh2*$^{-/-}$, are

prone to lymphoma development, but do not exhibit the same predisposition to intestinal tumourigenesis; whilst *Msh3*$^{-/-}$ mice are far less prone to cancer. However, combining *Msh6* and *Msh3* ablation resulted in intestinal tumour formation and a phenotype similar to Msh2 deficiency [24, 28]. These findings broadly correlate with observations in human cancers. In HNPCC families, *Msh6* germline mutations are uncommon, and *Msh3*$^{-/-}$ mutations are not seen. This suggests a degree of redundancy between MutSα and Mutβ. Simultaneous loss of both is required for a MMR-D state and cancer development [24]. Similar murine knockouts have also delineated the role of MutL proteins. Mice with *Mlh1* deficiency develop multiple intestinal adenomas and adenocarcinomas, in addition to other epithelial cancers and lymphoma. *Psm2*$^{-/-}$ mice, however, only showed predisposition to lymphoma and sarcoma development [79]. It is suggested that PSM2 loss can be compensated by the function of MutSβ, a heterodimer of *Psm1* and *Mlh1* [81].

While loss of MMR function may be evolutionarily beneficial for microbes by increasing genetic variation with subsequent selective advantage, as evidenced above, its consequences in mammals tend to be highly detrimental. Inherited mutations in mismatch repair genes results in Lynch syndrome, an autosomal dominant condition that confers a predilection to malignancies of the colon and rectum, endometrium, stomach, ovary, prostate and several other sites [60]. The lifetime risk of colorectal cancer for patients with Lynch syndrome is between 20 and 50 % depending on the inherited variant and sex of patient [26, 35, 80]. Several large studies have shown that it accounts for approximately 3 % of all CRCs in unselected series [37, 73]. Patients with Lynch syndrome inherit a mutation in single copy of one of the mismatch repair genes, most frequently *MSH2* though any of the genes (*MLH1*, *PMS1* or *PMS2*) may be mutated. During adult life a somatic cell within the colon loses the wild-type copy as a result of a "second hit" – comprising either loss of heterozygosity, somatic mutation or hypermethylation of the gene promoter region [44, 104]. In sporadic CRCs, MMR deficiency is almost always caused by hypermethylation of the MLH1 promoter with consequent silencing of gene expression [113].

Consistent with its essential role in the maintenance of genomic stability, loss of MMR function leads to an increased frequency of mutations across the genome (typically 10–100 mutations per Mb), referred to as "hypermutation", and increased risk of carcinogenesis [7, 75]. Genomic regions at particular risk of mutation as a result of defects in mismatch repair include long repetitive mononucleotide or dinucleotide sequences, known as microsatellites [51]. These microsatellites are particularly susceptible to small insertion and deletion (indel) mutations as a result of polymerase slippage during DNA replication, and the MMR system is the predominant mechanism by which these errors are recognised and repaired. Failure to do this causes phenotype referred to as microsatellite instability (MSI) [58, 76]. Clinically, mismatch repair deficiency has typically been diagnosed by immunohistochemistry for mismatch repair protein expression, or by PCR of microsatellite markers to confirm instability [54, 73, 92, 109]. It has recently also been shown that MMR deficiency can be identified by quantification of mutational load using NGS

cancer panels. In a study that used either 341 or 410 gene panel assay, a cut off values >20 mutations but <150 proved a 100 % sensitive and 100 % specific method for the detection of MMR-D [99].

Clinical Features of MMR-D Colorectal Cancers

In addition to their distinct biology and mutational profile compared to other tumours, MMR deficient CRCs also display distinctive clinical and pathological characteristics. They typically occur in the proximal colon [103], and are poorly differentiated [2]. Perhaps most relevant to this Chapter, MMR-D cancers are also associated with a substantially increased number of tumour infiltrating lymphocytes (TILs), and particularly $CD8^+$ cytotoxic lymphocytes when compared to other CRCs [2, 25, 97, 117].

The association of mismatch repair deficiency with colorectal cancer prognosis has been the subject of much research. Though most early studies were underpowered to detect a difference in outcome, a landmark meta analysis published in 2004 by Popat and co-workers combined data from 32 studies to confirm that MMR-D was associated with a significantly reduced risk of cancer recurrence in stage II/III colorectal cancer, with a hazard ratio of 0.67 for disease-free survival [77]. Subsequent studies have both confirmed this result, and revealed that the impact of MMR-D on prognosis appears to vary according to the stage of disease. In stage II disease, MMR-D tumours have roughly half the risk of recurrence of other cancers, while in stage III disease the reduction is more modest (HR of ~0.8) [6, 43, 85, 96]. Interestingly, the 4 % of stage IV tumours with MMR-D have a dismal prognosis, with an outcome that is worse than that of any other molecular subtype in several studies [32, 36, 106].

It has been generally believed that the favourable prognosis of early stage MMR-D colorectal cancers is a direct consequence of the enhanced immune response they appear to elicit – a conclusion supported by recent studies which have demonstrated that the strength of the adaptive immune response correlates with decreased metastases and increased survival time [30, 70]. However, this begs the question as to how these tumours are able to grow into clinically detectable cancers when they appear to induce such a potent immunological reaction? A highly plausible answer to this apparent paradox was provided by an elegant recent study, which demonstrated that in addition in increased cytotoxic markers, MMR-D tumours also display substantial upregulation of immune checkpoints, such as PD-1, PD-L1 and CTLA-4, which counteracts the host cytotoxic immune response [56]. The possibility suggested by these data – that these cancers would be particularly good candidates for treatment with immune checkpoint inhibitors was explored in a prospective clinical trial conducted by the same group [55], the exciting results of which are discussed further subsequently.

POLE Proofreading Domain Mutation

In addition to the mismatch repair system, eukaryotes have evolved several other mechanisms that function to suppress potentially deleterious mutagenesis. One of these that has recently emerged as important in human cancers is the proofreading exonuclease activity intrinsic to the replicative DNA polymerases Pol δ and Pol ε, encoded by *POLD1* and *POLE* in humans [82]. During DNA replication, proofreading of the newly synthesised strand serves to recognise and excise mispaired bases incorrectly incorporated by the polymerase catalytic domain, following which the correct base can be inserted, and replication continued [82]. While studies in yeast and in mice have confirmed that loss of either Pol δ and Pol ε proofreading activity results in a mutator phenotype and causes cancer [65, 95], evidence that such defects are relevant in human tumours has been limited. However, in 2012 both TCGA and another study demonstrated that a subset of highly mutated, but mismatch repair proficient colorectal cancers harboured recurrent mutations within the *POLE* exonuclease domain [89, 102]. In parallel with this report, a study of Individuals with intestinal polyposis and family history of colorectal cancer but without Lynch or other predisposition syndromes, demonstrated that these patients harboured germline mutations in the exonuclease domain of *POLD1* or *POLE* [71].

Both the somatic and germline *POLE* exonuclease domain mutations affect highly conserved residues close to the DNA binding interface, and causality of several of these variants has been further supported by the demonstration that they confer a mutator phenotype in yeast (see Rayner et al. for a recent review). In addition to colorectal cancer, somatic *POLE* exonuclease domain mutations also occur in 6–15 % of endometrial cancers [14, 19, 20, 100] and rarely in tumours of the stomach, pancreas and brain where they are also associated with ultramutation, microsatellite stability and characteristic mutation spectrum [82, 93].

Recent studies have shown that endometrial cancers with ultramutation caused by *POLE* proofreading domain mutations have an excellent prognosis, despite a strong association with high-grade histology – a recognised poor prognostic factor. Interestingly, like MMR-D colorectal cancers, *POLE*-mutant endometrial tumours show evidence of a striking cytotoxic T cell response, and strong upregulation of immune checkpoints [110]. Given these data, the results of similar study of the consequences of *POLE* proofreading domain mutation in colorectal cancer are eagerly awaited.

Determinants of the Anti-tumour Immune Response

To date, most mechanistic data pertaining to the interaction between tumours and the host immune response, and the mechanism of action of immune checkpoint inhibition relates to the ability of host T cell compartment to recognise and target malignant cells [86, 87]. The postulate that such discrimination could occur as a consequence of

recognition of tumour antigens as non-self was first suggested by seminal studies performed more than half a century ago. Sarcomas were induced in a mouse using methylcholanthrene, then subsequently excised and used to produce a tumour cell suspension. Inoculating syngeneic mice with this suspension resulted in rapidly growing tumours, which were then also removed. When these same "exposed" mice were re-inoculated with the same tumour mixture, there was evidence of resistance and in some cases complete tumour regression [34]. Subsequent work confirmed these original findings: showing that it is possible to induce anti-tumour immunity [78]. Importantly, it was shown that the acquired immunity was specific to the tumour cells, and not a consequence of genetic heterozygosity between the donor and recipient mice [21].

The T-cell is central to the adaptive immune response, and has numerous vital functions in both tumour recognition and elimination. T-cell maturation and development occurs in the thymus, where they begin life as double negative ($CD4^-$ $CD8^-$) precursor thymocytes. Somatic rearrangement of the T-cell receptor (TCR) loci, first TCRβ and then TCRα, leads to the generation of T-cells with unique TCRs. Following TCRαβ heterodimer expression, thymocytes then undergo positive and negative selection. Those cells expressing Major Histocompatibility Complex (MHC) class II restricted receptors generally become $CD4^+$ cells, while those expressing class I restricted are skewed towards $CD8^+$ characterisation. Thymocytes that exhibit strong reactivity to self-antigens are clonally deleted, a process referred to as negative selection or central tolerance. The activation of T-cells occurs through interaction of the TCR with peptides presented by Major Histocompatibility Complexes (Major Histocompatability Complexes, MHC) of either class I or II that are present on cell surfaces. The specific part of a peptide that acts as an antigen and is capable of eliciting an immune response is referred to as an epitope. This process is aided by the co-receptor CD4 in T-helper cells, and CD8 in cytotoxic T-cells [33].

MHC class I molecules are expressed on the surface of most cells and are capable of being directly "read" by $CD8^+$ cytotoxic T cell, a process which can result in direct killing of the offending cell. In contrast, MHC II is found on professional Antigen Presenting Cells (APCs) and serve to stimulate $CD4^+$ T helper cells (Th). Under normal conditions, T cells do not react with self-peptides presented by HLA class I. However, foreign antigens presented by class I molecules may be recognised as non-self and induce a T cell response.

Current evidence suggests two principal mechanisms by which tumour peptides can act as antigens. The first category includes peptides that arise from aberrant expression of non-mutated self-proteins in cancer cells. The best described of these are referred to as cancer testis (CT) antigens, because their expression is restricted to the testis under normal conditions. The testis is an immunologically privileged site, and presentation of antigens at this site is associated with T cell tolerance due to expression of co-inhibitory molecules. Outside the testis, cancer testis antigens are usually not expressed, but aberrant expression occurs in several human cancers. Because these sites lack immune privilege T-cell tolerance is incomplete, and consequently expression of CT antigens by tumour cells represents a potential immune

target. Much research has focused on vaccination against CT antigens commonly expressed in certain tumours, including MAGE1 in melanoma and New York oesophageal squamous cell carcinoma (NY-ESO-1) antigen in various tumours such as melanoma, bladder, prostate and hepatocellular [18, 45]. While the results of early studies were mixed, recently this approach has been associated with some success [17, 22, 45, 61]. However, there is little evidence that CT antigens play a substantial role in colorectal cancer, and they will not be further discussed in this Chapter. In contrast, most current and emerging evidence indicates a far more important contribution for the second class of tumour antigens, which include peptides encoded by DNA sequences that are normally absent from the human genome, a group collectively referred to as neoantigens.

Neoantigens in Cancer

There are two principal mechanisms by which novel antigens are generated in cancers. The first occurs in the substantial fraction of human cancers that are a consequence of viral infections. While the hijacking of normal cellular processes by cancer-causing viruses enables the cellular proliferation and survival that leads to malignancy, the resultant tumours often display epitopes derived from viral open reading frames integrated in the host DNA [48, 67, 72, 114]. Because these viral sequences are not normally found in the human genome they can be recognised as non-self, and targeted by T cells. Many chronic viruses have evolved mechanisms to suppress this immune response, including downregulation of MHC class I molecules on infected cells [4, 101], and immunosuppression through upregulation of PD-1 [5] and IL-10 [10]. However, recent data strongly suggest that an immune response against viral epitopes may account for the favourable prognosis of several viral-associated malignancies, including those in the upper GI tract such as Epstein Barr Virus (EBV)–associated gastric cancer [13]. However, like CT antigens, there is little evidence that viruses contribute to the pathogenesis of colorectal cancer, or that viral antigens contribute to its prognosis. The second mechanism relates purely to the novel peptides that arise from tumour-specific somatic DNA mutations, often referred to as tumour neoantigens. Because such neoantigens are completely absent from noncancerous tissues, T-cells reacting to them are not subject to the rigours of central and peripheral tolerance, and consequently are capable of eliciting a more vigorous immune response. Furthermore, their tumour-restricted expression means that therapies targeting these variants should be highly tumour-specific with lower rates of off target deleterious side effects. The last few years have witnessed substantial advances in the understanding of the determinants of immunogenicity of neo-antigens, and rapid progress in the ability to identify and predict these variants in cancers, largely as a consequence of improvements in sequencing technology and bioinformatics. These advances are reviewed in the subsequent sections (Fig. 8.2).

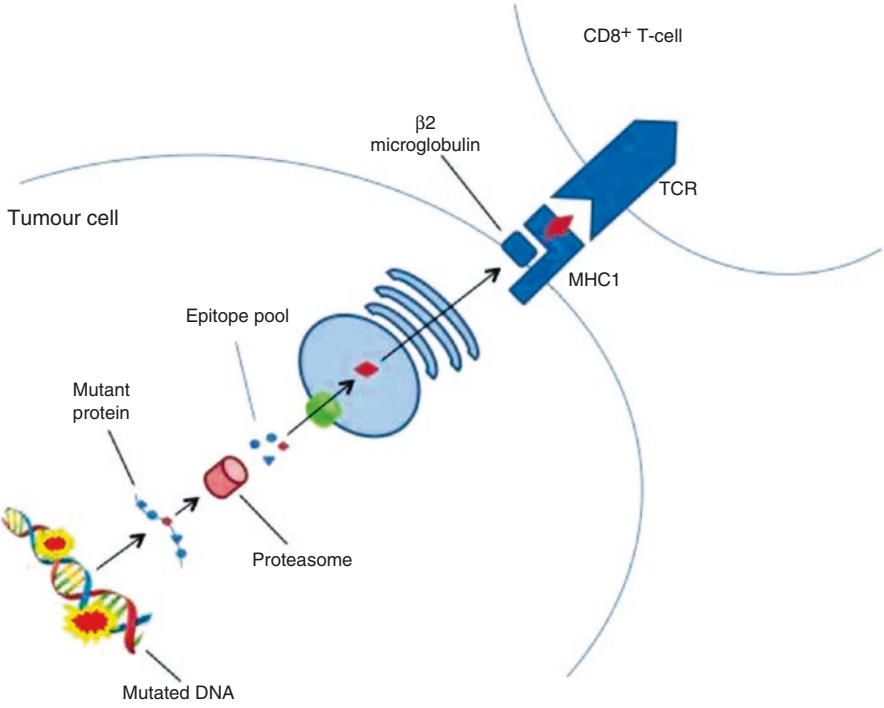

Fig. 8.2 Simplification of neoantigen presentation to CD8+ cells. Tumours harbour numerous non-synonymous, protein altering, DNA mutations that provide a portfolio of possible novel epitopes. These mutated peptides undergo intracellular processing, first being catabolised by proteasomes in the cytoplasm. In the Endoplasmic reticulum, these putative epitopes are bound to MHC class I molecules: the most selective step in determining the antigenicity of a mutant peptide. This protein complex is then translocated to the cell surface where it is presented to the T-cell receptor of a CD8+ cytotoxic T-cell

Identification of Tumour Neoantigens

The ability to predict which tumour mutations are likely to generate antigenic epitopes results from a combination of advances in understanding the mechanisms of peptide presentation by MHC molecules, the advent of next generation sequencing, and bioinformatic tools and pipelines to combine both for individual tumours.

Peptide binding with HLA-1 appears to be the most discerning step in the antigen presentation process. Each individual MHC molecule presents a unique binding specificity to a given peptide. The genomic region encoding for MHC is highly polymorphic, and to date more than 3000 allelic variants have been discovered. Of the 1500 known HLA class 1 molecules, less than 5 % have been examined in relation to their binding with more than 50 peptides [59, 90]. Because experimentally characterisation of the binding specificities of peptides for HLA molecules is laborious, the availability of a data driven bioinformatic approach would be of great

benefit. NetMHCPan, developed by Nielsen and colleagues, is one such tool [69]. Using peptide and primary HLA sequencing as inputs, this artificial neural network (ANN) is able to predict the affinity of any peptide for HLA-A or HLA-B molecules. The authors validated this *in silico* approach by predicting the affinity of 500,000 random peptides for both HLA molecules. Of these, they selected 10–15 with high affinity (>50nM) and confirmed the interaction using an *in vitro* assay. NetMHCPan was able to predict binding with 86 % accuracy. Further validation was carried out on peptide binding with previously unstudied HLA molecules, and with known HIV immune epitopes. This approach has also been validated for use in non-human primates [42].

As discussed previously, next generation sequencing technology provides unprecedented ability to examine the mutational landscape of cancers. In particular, study of tumour exomes, that is the 1–2 % of the genome that codes for protein, is now both relatively inexpensive and scalable, albeit in specialist centres. With this wealth of available data, the next step has been to understand how the tumour mutational landscape defines the neoantigen repertoire. Although a typical tumour genome may contain 10–100 s of SNV or indel mutations that result in amino acid substitutions, only a small percentage of these result in the expression of neoantigens [39]. To identify these, most investigators have used an *in silico* approach informed by the understanding of MHC I binding preferences as described.

In an early study that predated the development of NGS approaches, Segal and colleagues sought to predict the neoantigen repertoire of 11 CRC and 11 breast cancer samples, from somatic mutations in roughly 13,000 cancer genes. Focusing solely on theoretical binding of tumour-derived 9mer peptides with HLA-A*0201 (the most common class I allele in Caucasian populations), they found that CRC and breast cancer possessed an average of seven and ten predicted antigenic novel epitopes respectively; corresponding to approximately 1 antigenic epitope for every ten nonsynonymous mutations [88]. As humans have up to six different MHC I alleles (2 loci for each of HLA-A, B and C) the authors suggested that their conservative estimate could be multiplied sixfold, resulting in a possible average of 40–60 novel epitopes in typical colorectal cancers. While this study provided an intriguing insight into the possible antigenicity of mutations in cancer genes in these common tumour types, it was necessarily limited by the technologies of the time. For example, the absence of gene expression data made it uncertain as to whether all these mutant peptides were in fact expressed, and the absence of individual HLA typing meant that the binding affinities could only be calculated against a single representative class I molecule.

Building on these results, Brown and colleagues developed a bioinformatic tool, HLAminer to extract permit four digit HLA typing from TCGA RNAseq data. They combined these data with exome sequencing from patients with six common tumour types (lung, ovary, breast, brain, colorectal and kidney) analysed by TCGA. For each missense mutation, they calculated the binding affinity for the patients HLA class I molecules using NetMHCPan. Informed by the results of the preclinical studies discussed earlier, they defined antigenic mutations as those that gave rise to peptides that bound with high affinity (<500nM). This approach sug-

gested that an appreciable fraction of missense mutations were antigenic, and revealed substantial variation in the number of antigenic variants both between and within different tumour types. Importantly, the number of immunogenic mutations correlated significantly with $CD8^+$ cytotoxic response and survival rates, and interestingly, the strongest association was observed among the 170 cancers included in this study [11]. An extremely comprehensive subsequent study by Rooney and colleagues also used TCGA data to interrogate the burden and correlates of antigenic mutations in multiple cancer types. To perform their analysis, the authors developed a novel bioinformatic tool, Polysolver, which permits HLA typing with four digit accuracy from exome sequencing [84]. In a separate publication [94], the group demonstrated that Polysolver was able to call HLA type with substantially greater accuracy than HLAminer and comparable to conventional serological and PCR typing strategies [94]. The group subsequently employed Polysolver to type the class I HLA alleles in over 7000 cases comprising 18 different types of tumour from TCGA, and in parallel used exome sequencing data to calculate all possible peptides predicted to be generated by all missense and indel mutations in these patients' tumours. Restricting their analysis to variants in expressed genes they demonstrated that on average 50 % of non-silent mutations were predicted to generate an predicted neoepitope, defined as a peptide predicted to bind the patient's class I alleles with affinity of <500nM. The number of antigenic mutations displayed a strong positive correlation with the level of tumour immune cytolytic activity, as quantified by the geometric mean of the cytotoxic effectors granzyme A (GZMA) and perforin (PRF). After noting that the number of antigenic mutations in tumours correlated very strongly with their total mutation burden (Spearman rho = 0.91), the authors were able to demonstrate that in both renal and colorectal cancers, the observed number of predicted neoepitopes was significantly lower than predicted based on the frequency of silent mutations in these cancers, suggesting immune selection against antigenic variants. Importantly, this depletion was lost when neoepitopes were estimated based on randomly shuffled, rather than individual HLA genotypes [84].

Functional Validation of Predicted Neoepitopes

While the power of in *silico* approaches to predict antigenicity has been demonstrated by the studies discussed above, it is important to note that only a small fraction of mutations predicted to be antigenic by bioinformatic pipelines are confirmed to be so when analysed in functional studies [87]. Castle and colleagues performed next generation sequencing of B16F10 cells, a mouse melanoma cell line that is able to grow when inoculated into immunocompetent syngeneic hosts, and identified 962 mutations in 563 expressed genes. 50 of these variants were selected for functional study, based on expression and predicted antigenicity, although this approach did

not include the predicted binding of the peptides for MHC class I molecules. Using a long peptide vaccination strategy in mice, 16 of these variants proved to induce an adaptive immune response when inoculated *in vivo*. Importantly, vaccination with several of these peptides protected against tumour growth following subsequent inoculation of B16F10 cells [16].

One such strategy to improve the accuracy of neoantigen prediction is to combine the sequencing and bioinformatic predictions with the use of mass spectrometry analysis. Examining the mouse MC38 colorectal cancer cell line, Yadav and colleagues used whole exome sequencing to identify 4285 non-synonymous mutations, 1290 of which were in expressed genes. 170 were considered candidate neo-epitopes in that they were predicted to bind MHC class I molecules using NET MHCPan [116]. In parallel with the informatics approach, the authors employed an additional strategy to confirm the immunogenicity of candidate immunogenic peptides. Using mass spectrometry, they discovered that only of the seven mutated peptides were found to be presented on MHC I. These seven epitopes were then subjected to further assessment using computational structural modelling to assess MHC binding affinity, and interaction between TCR and the mutated peptides. These experiments suggested that of the seven peptides, only three would meet the criteria to stimulate a T-cell response. Indeed, inoculation of mice with these three selected peptides induced a $CD8^+$ T-cell response, conferring immunity to tumour grafts and an immune mediated growth control of already established tumours [116].

While most studies have focused on the role of cytotoxic T cells in recognition of neoantigens, emerging evidence suggests that T helper cells also serve an important role in both this and the antitumour immune response more broadly. Activated via MHC II, $CD4^+$ Th cells participate in augmenting $CD8^+$ response and recruiting natural killer (NK) cells. Using a similar approach to selecting MHC I class presented mutations, melanoma intra-tumour $CD4^+$ cells could be strongly induced in response to autologous neo-epitopes [57]. Notably, a recent report suggests that MHC II class restricted epitopes may be far more abundant in tumours than MHC I class I restricted peptides. In analysis of melanoma, mammary and colorectal murine cancer models Kreiter and colleagues demonstrated that more than 80 % of nonsynonymous mutations were MHC II class restricted. These conclusions were corroborated by analysis of tumours from TCGA samples. The relative abundance of $CD4^+$ responsive mutations may be due to less stringent length and sequence criteria for MHC II binding when compared to MHC class I restricted peptides. Additionally, incorporating these MHC II mutant epitopes into a poly epitope RNA based vaccine generated efficient tumour control in mice, inducing a combined $CD4^+$ and $CD8^+$ immune response [49]. It would appear that there exists a more extensive range of MHC II epitopes; specific $CD4^+$ cell targeting of these mutations is central to an effective anti-tumour immune response [108]. Figure 8.3 summaries the experimental process to identifying and validating potential tumour associated neoantigens.

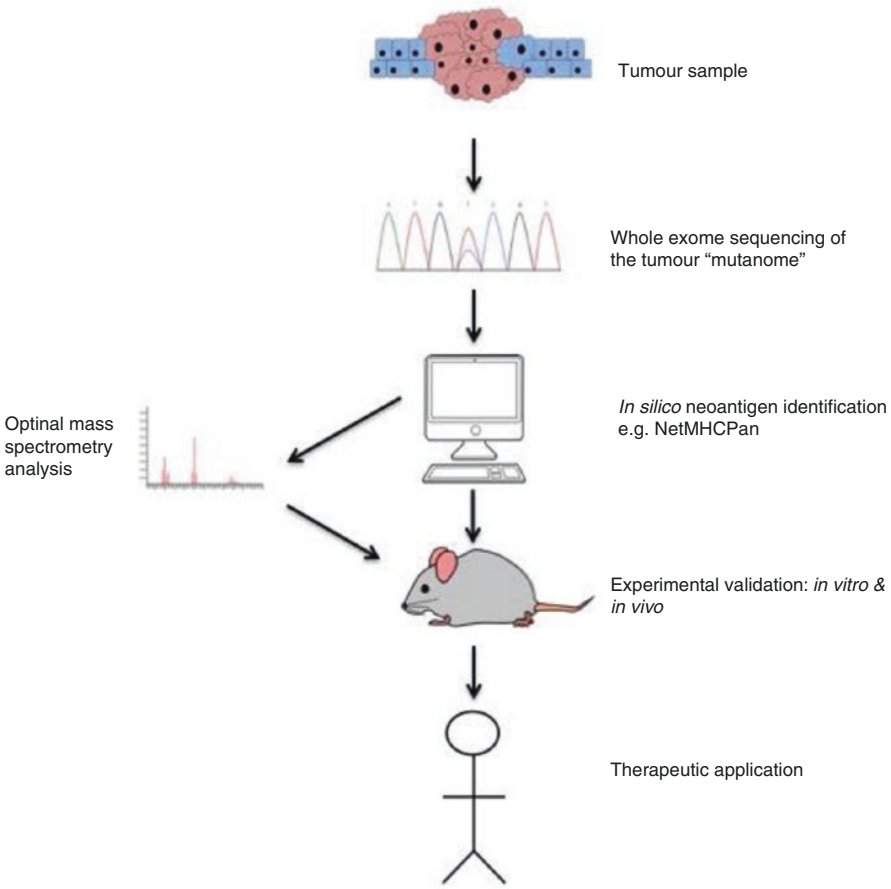

Fig. 8.3 Tumour associated neoantigen identification pipeline. Representative tumour samples are subjected to whole exome sequencing to identify possible neoantigen forming DNA mutations. In silico analysis selects candidate neoepitopes based on the predicted binding with HLA molecules. The use of additional algorithms such as HLAminer or Polysolver to define the patient specific HLA allele further refines this process of selection. Additionally, mass spectrometry adds another possible filtering step. The candidate neoantigens are then experimentally validated before possible therapeutic application, which includes vaccination or autologous T-cell transplantation

Neo-Epitopes in Colorectal Cancers

Given the correlation between the total mutation burden of cancers and the number of predicted neoepitopes they are predicted to generate, it is unsurprising that most attention has focused on the subset of immunogenic, hypermutated, mismatch repair deficient tumours.

In a recent study, Mlecnik and colleagues demonstrated that increased intratumoural immune gene expression in mismatch repair deficient colorectal cancers

was associated with a significantly greater number of predicted neo-epitopes in this molecular subset. Interestingly, similar to the previous study by Rooney and co-workers, they also noted that the number of neo-epitopes in mismatch repair deficient tumours was less than predicted by the frequency of silent mutations, suggesting that antigenic variants were subject to negative selection in vivo [64]. This analysis also suggested that the enhanced immunogenicity of mismatch repair deficient tumours may also result at least partly from their enrichment of frameshift mutations, as these variants were predicted to generate more neoepitopes per mutation than missense variants.

In general, the prediction that mismatch repair deficient cancers are to likely generate substantially more neo-epitopes than mismatch repair proficient tumours is mirrored by their apparently enhanced immunogenicity. However, notable exceptions exist – that is some mismatch repair deficient tumours are predicted to generate many neo-epitopes but fail to demonstrate significant immune response, while a subset of mismatch repair proficient cancers appear strongly immunogenic despite seeming to harbour a paucity of neoepitopes. Indeed, in an elegant recent study from the Rosenberg group, exome sequencing was performed on metastases from patients with several different tumour types including colorectal cancer. They proceeded to examine whether somatic mutations from tumours were recognised by tumour infiltrating lymphocytes using a tandem minigene approach, and confirmed reactivity of several mutations, including a *KRAS*^{G12D} mutation in a patient with colorectal cancer. Importantly, none of the colorectal cancers examined in this study were hypermutated (the number of mutations per colorectal cancer sample ranged from 58 to 134) [107].

While this study clearly demonstrates that non-hypermutated colorectal cancers may harbour reactive neoepitopes, the paucity of responses to anti-PD1 therapy in unselected colorectal cancers (one of 33 patients in trials conducted by Topalian and colleagues [8, 105]) contrasts with the dramatic responses among mismatch repair deficient tumours [55]. Whether this discordance is a reflection of the lower probability that a non-hypermutated tumour harbours a highly antigenic variant, or the cumulative effect of multiple modestly antigenic neoepitopes in hypermutated cancers awaits definition.

Immunoediting of Neoepitopes

Until recently, the interaction between the host immune system and malignancy was often referred to as immunosurveillance – a term that implied that the immune response had a purely protective role in preventing cancer development. However, during the last few years it has been broadly accepted his term fails to capture what is clearly a complex, dynamic interaction between the two [86]. The seminal studies of MCA-induced murine sarcomas discussed earlier demonstrated that the immune response influences not only the kinetics of tumour growth, but also the neoantigen landscape of the developing malignancy. In an elegant series of experiments, the

investigators demonstrated that sarcomas grown in *Rag2*$^{-/-}$ immunodeficient mice were frequently rejected when transplanted into immunocompetent wild-type mice, while those transplanted from either wild-type or *Rag2*$^{-/-}$ into *Rag2*$^{-/-}$ recipients grew progressively. Furthermore, in this study the authors were also able to demonstrate that tumour rejection was a T-lymphocyte dependent process, and to identify the specific rejection antigens by exome sequencing. Taken together, these results provide convincing evidence of the protective role of lymphocytes against tumour development, and the ability of the host immune response to sculpt the mutational landscape of the developing tumour – a process they referred to as "immunoediting" [91].

The process of immunoediting has been hypothesised to involve three key phases: elimination, equilibrium and escape. Elimination is the process by which the immune system is able to detect and remove nascent cancer cells. While direct *in vivo* evidence to support this stage is lacking, it has been inferred from numerous murine models in which facets of the adaptive or innate immune system – such as lymphocytes, or immune effector molecules – have been selectively ablated (Reviewed in: [111]). Tumour cells that escape initial elimination may then enter a steady state of equilibrium, during which the immune system prevents tumour progression, resulting in dynamic modelling of the tumour immune landscape. It has been speculated that tumours may exist in this state for some time before becoming clinically evident. Overt disease eventually results when tumour cells are able to circumvent the controlling effects of the immune system: escape. The final product, the cancer and its neoantigen profile, is the result of the accumulation of mutations and continuous immune pressures: this Darwinian selection produces clones that are "invisible" to the immune system. It has in fact been proposed that this final stage – the evasion of immunosurveillance – is the seventh hallmark of cancer [38, 120]. Current evidence suggests two principal methods by which this is achieved: by reducing the capacity for immune cell recognition, or alternatively through the creation of an immunosuppressive tumour microenvironment (TME). The former is often achieved by mutation or decreased expression of components in the antigen presentation machinery, including β2 microglobulin or the HLA genes, while the latter appears most commonly due to upregulation of immune checkpoints or other immunosuppressive molecules such as PTGS2 [63, 118, 119]. The process of immunoediting is shown in Fig. 8.4.

As discussed before, the depletion of neoepitopes in hypermutated mismatch repair deficient colorectal cancers provides convincing, if correlative, evidence of immunoediting of these tumours [64, 84]. Furthermore, both mutation of B2M and downregulation of HLA expression appear to be common events in mismatch repair deficient cancers, and a recent study has shown that these tumours are also characterised by robust upregulation of immune checkpoints including PD1 and PDL1 [56].

Targeting Neoantigens for Therapy

The enrichment of neoepitopes among hypermutated colorectal cancers is of substantial clinical relevance and is currently the subject of considerable investigation. As noted previously, because these variants are tumour-specific and absent from

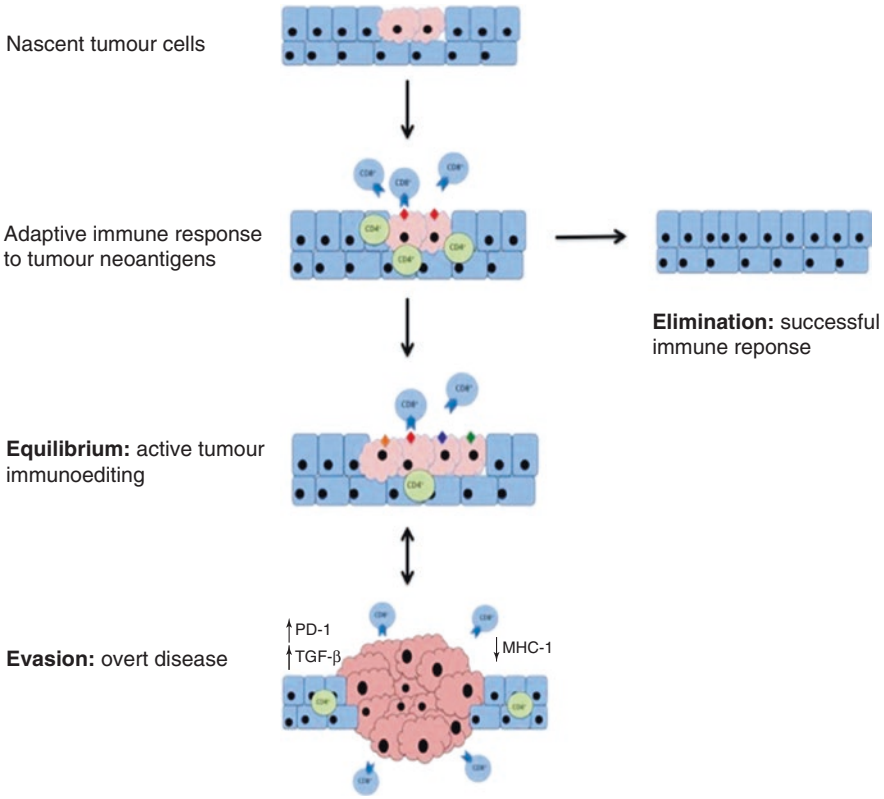

Fig. 8.4 The process of immunoediting. Nascent tumour cells display neoantigens that are recognised by the adaptive immune response, and results in CD4+ and CD8+ T-cells response (other facets of the immune system also participate but are not shown here). This immune response results in two possible outcomes: elimination, in which the tumour cells are completely eradicated; or alternatively a state of equilibrium develops, during which constant immune pressure serves to sculpt the neoantigen landscape by selecting against immunogenic clones and promoting the development of tumour cells invisible to the immune system. This process may continue for an extended time, and the tumour becomes only becomes clinically relevant when immune evasion occurs. In this third, and final, stage the highly edited non-immunogenic tumour is able to escape its immunological restraints: leading to invasion and dissemination

healthy tissues targeting them may provide a highly favourable therapeutic index and avoid the deleterious side effects of unselective traditional cytotoxic chemotherapeutics. Although most attention has focused on the use of immune checkpoint inhibitors, other therapeutic opportunities include the development of a tumour specific vaccines, and Adoptive Cell Therapy (ACT).

The striking benefit of PD1 blockade in patients with mismatch repair deficient colorectal cancers has been mentioned previously. In this landmark study, profound and seemingly durable responses were observed in seven of nine (78 %) patients with mismatch repair deficient tumours, compared with two of 18 (11 %) mismatch repair proficient cancers [55]. As expected, exome sequencing demonstrated that the number of somatic mutations in mismatch repair deficient cancers substantially

exceeded that in mismatch repair proficient tumours (mean of 1782 mutations vs. 73 mutations; $P < 0.001$), as did the number or likely neoantigens, predicted using the patients' individual HLA haplotype (mean 578 vs. 21 neoepitopes). Importantly, despite the small size of this study, high numbers of somatic mutations and tumour neoepitopes were significantly associated with longer progression-free survival following immune checkpoint blockade, and also demonstrated a trend towards improved response rate [55].

Study of vaccination strategies are less clinically advanced, although this strategy has been demonstrated to be feasible in several mice models, and limited reports suggest that this approach may also have therapeutic potential in humans. In one study, patients with metastatic melanoma were inoculated with a tailored vaccine consisting of tumour mutant epitopes predicted in silico to bind MHC I molecules. Vaccination produced a strong neoantigen T-cell response [15]. Similarly, adoptive cell therapy is another strategy that holds promise, to target tumour neoantigens. In a seminal study involving one patient with metastatic cholangiocarcinoma, an in silico and functional studies were used to identify and expand autologous tumour-reactive CD4+ T-cells. Re-infusion of these resulted in tumour regression and disease stabilisation [108]. This study proves the feasibility of a pipeline in identifying neoantigens that may be clinically relevant. While this approach remains highly labour intensive, it arguably represents the ultimate in personalised therapy, and further research in the field is eagerly awaited.

Conclusions

The combination of advances in sequencing technology, bioinformatics and the advent of immune checkpoint inhibitors has provided an unprecedented opportunity to investigate the immunogenic consequences of somatic mutations in cancers. It is clear that hypermutated mismatch repair deficient tumours constitute a highly immunogenic tumour subset with an excellent prognosis in early stage disease. The apparent paradox that these tumours have a particularly poor prognosis when metastatic may be explained in part by the immunoediting they are likely to have undergone, and partly by the substantial upregulation of immune checkpoints they display – though the remarkable efficacy of immune checkpoint inhibition against these cancers raises the possibility that the latter is the dominant mechanism of immune escape. While mechanistic confirmation that the distinct clinical behaviour of these tumours is a consequence of the enrichment of antigenic neoepitopes they are predicted to display is currently lacking, this is the subject of current research, and the study of non-hypermutated colorectal cancers strongly suggests that at least a proportion of the mutations they harbour are likely to generate a T cell response.

However, several questions remain unanswered. For example, is the behaviour of hypermutated colorectal cancers a consequence of a limited number of highly reactive neoepitopes, or a larger number of modestly antigenic mutations? What are the mechanistic determinants of antigenicity in tumour-derived peptides? And can

immunotherapeutic approaches be used to benefit the majority of patients, whose colorectal cancers that are not hypermutated, for whom current checkpoint blockade appears only modestly efficacious at best? Determining the answers to these questions will be a priority for future studies, along with translating their results into the clinic, for the benefit of colorectal cancer patients.

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Chapter 9
Antibodies for Treatment of Metastatic Colorectal Cancer

Volker Heinemann and Sebastian Stintzing

Introduction

Substantial progress has been achieved in the treatment of metastatic colorectal cancer (mCRC) during the last two decades and overall survival (OS) has been prolonged from 12 months to more than 30 months in recent studies (Table 9.1) [1]. This major step towards increased treatment efficacy was made possible not only by the introduction of new chemotherapeutic agents such as irinotecan and oxaliplatin, but also by the availability of targeted agents directed against the epidermal growth factor receptor (EGFR) as well as against the vascular endothelial growth factor (VEGF).

The EGFR Pathway

The EGFR constitutes the link between the extracellular space and the intracellular signal transduction, which regulates nuclear processes involved in cell growth, differentiation, survival, cell cycle progression, angiogenesis, and drug sensitivity. It is a member of the erbB family of receptor tyrosine kinases, which also include erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). The EGFR- transmembrane protein is composed of three components: an extracellular ligand-binding domain, a lipophilic transmembrane domain, and an intracellular tyrosine kinase domain. Apart from erbB2, specific ligands have been identified for each of the erbB receptors. Among these, the epidermal growth factor (EGF) and the transforming growth factor-α (TGF-α) selectively bind to the EGFR. Extracellular ligand binding induces activation of the transmembrane receptors, subsequent homo- or heterodimerisation between the

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Table 9.1 Randomised first-line studies investigating the effect of anti-EGFR agents in RAS wt patients

Study	Refs.	Regimen	No of patients	ORR (%)	OR (P-value)	PFS (mo)	HR PFS (P-value)	OS (mo)	HR OS (P)
CRYSTAL	[6]	FOLFIRI + Cet FOLFIRI	178 189	66.3 38.6	3.11 (<0.001)	11.4 8.4	0.56 (<0.001)	28.4 20.2	0.69 (0.0024)
OPUS	[7]	FOLFOX + Cet FOLFOX	38 49	58 29	3.33 (0.0084)	12 5.8	0.53 (0.0615)	198 17.8	0.94 (0.80)
PRIME	[8]	FOLFOX + Pani FOLFOX	259 253	60 47	NR (0.003)	10.1 7.9	0.72 (0.004)	26.0 20.2	0.78 (0.04)
COIN[a]	[20]	FU/LV or Cape + Ox FU/LV or Cape[b] + Ox + Cet	367 362	57 64	(0.049)	8.6 8.6	0.96 (0.60)	17.9 17.0	1.04 (0.67)
NORDIC[a]	[21]	FLOX FLOX + Cet	97 97	47 46	0.96 (0.89)	8.7 7.9	1.07 (0.66)	22.0 20.1	1.14 (0.48)
FIRE-3	[1]	FOLFIRI + Cet FOLFIRI + Bev	199 201	65.3 58.7	1.33 (0.18)	10.3 10.2	0.97 (0.77)	33.1 25.0	0.697 (0.0059)
CALGB 80405	[10]	FOLFOX/FOLFIRI + Cet FOLFOX/FOLFIRI + Bev	270 256	68.6 53.8	1.75 (<0.01)	11.4 11.3	1.1 (0.31)	32.0 31.2	0.9 (0.40)
PEAK	[77]	FOLFOX + Pani FOLFOX + Bev	88 82	63.6 60.5	NR	13.0 9.5	0.65 (0.029)	41.3 28.9	0.63 0.058

Legend: [a]Patients with KRAS wild-type tumours

[b]67 % Cape-based therapy

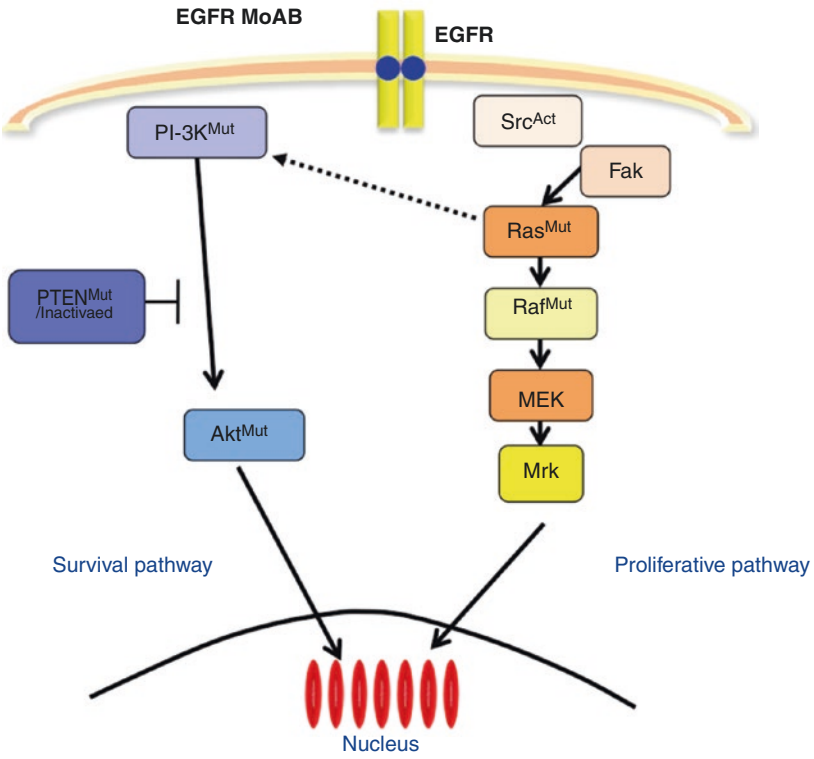

Fig. 9.1 Pathways of EGFR-mediated signal transduction

different receptors and intracellular autophosphorylation at the tyrosine kinase domain, which, in turn, activates downstream signalling pathways [2, 3] (Fig. 9.1).

Upon activation of the EGFR, several pathways of signal transduction have been identified. Activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway induces phosphoprotein expression of p-MEK and p-ERK1/2. In fact, preclinical and clinical data suggest that MAPK plays a key role in the regulation of cell growth, differentiation, proliferation, apoptosis, angiogenesis and cellular invasiveness [3, 4]. EGFR-mediated signalling via PI3 kinase (PI3K) causes activation of AKT, which results in the expression of phosphoproteins such as p-AKT, p-GSK3, and p-P70S6K. The PI3K/AKT pathway is regulated by phosphatase protein homologue to tensin (PTEN) the deficiency of which has been associated with an unsuppressed activation of signal transduction.

Anti-EGFR Directed Agents: Cetuximab and Panitumumab

Presently, two anti-EGFR antibodies, cetuximab and panitumumab, are used in the treatment of mCRC. Cetuximab is a monoclonal chimeric (mouse/human) IgG1 antibody targeting the domain III of the extracellular part of the EGFR [5]. By

contrast, panitumumab is a fully human IgG2 antibody binding to slightly different epitopes of the extracellular domain of the EGFR [5]. Both antibodies are approved in first- and further-line treatment of colorectal cancer in combination with FOLFOX or FOLFIRI and as monotherapy [6–8].

In the year 2006 it became clear that only patients without KRAS exon 2 mutations had a benefit from anti-EGFR treatment [9]. Further studies also revealed that mutations in KRAS exons 3 and 4 and NRAS exons 2–4 needed to be excluded prior to use of anti-EGFR agents. Thus, recent studies such as PRIME and FIRE-3 established extended RAS mutational analyses (including KRAS exons 2 and 3, as well as NRAS exons 2, 3, and 4) as a standard assessment prior to any anti-EGFR treatment in mCRC [6, 8].

Within the subgroup of RAS wild-type tumours, tumour response rates of 60–70 %, median PFS times of about 10 months and median survival times beyond 30 months have been reached in first-line treatment [1, 6, 8, 10]. In pretreated patients, significant benefits were reached by the combined use of FOLFIRI plus anti-EGFR agents with regard to objective response rate (ORR) and performance free survival (PFS) (Table 9.2).

Mechanism of Action

Binding of cetuximab to the receptor prevents ligand binding, induces receptor internalisation and causes direct inhibition of the receptor tyrosine kinase activity [11]. This in turn blocks downstream signal transduction via the PI3K/Akt and RASRAF/MAPK pathways inducing pro-apoptotic mechanisms and inhibiting cellular proliferation, angiogenesis and metastasis [12, 13]. As an IgG1 antibody cetuximab may also induce antibody-dependent cell-mediated cytotoxicity (ADCC). However, the clinical relevance of ADCC with regard to antitumour efficacy is likely to be rather low [11]. Cetuximab has single-agent activity in mCRC refractory to irinotecan, oxaliplatin and fluoropyrimidines [14] and was shown to restore chemosensitivity in irinotecan-refractory mCRC patients [15, 16]. Response and survival of cetuximab-treated patients strongly relates to the severity of an acneiform skin rash [14].

Effect of Anti-EGFR Agents in First-Line Therapy (Table 9.1)

The efficacy of anti-EGFR agents in first-line treatment was demonstrated by two randomised phase III studies. The CRYSTAL study compared FOLFIRI plus cetuximab to FOLFIRI alone, while the PRIME study evaluated the addition of panitumumab to FOLFOX vs FOLFOX alone [6, 8]. Both studies retrospectively selected for all RAS wild-type patients and showed a consistent improvement in ORR, PFS and OS achieved by the addition of anti-EGFR agents.

Table 9.2 Randomised second-line studies investigating the effect of anti-EGFR agents

Study	Refs.	Pretreatment	Regimen	No of patients	ORR	OR (P)	PFS	HR PFS (P)	OS	HR OS (P)
EPIC[a]	[22]	Fluoropyrimidine + oxaliplatin	FOLFIRI + Cet FOLFIRI	648 650	16.4 4.2	NR (<0.0001)	4.0 2.6	0.692 (<0.0001)	10.7 10.0	0.975 (0.71)
191[b]	[23]	Fluoropyrimidine-based therapy (66 % oxaliplatin 19 % Bev)	FOLFIRI + Pani FOLFIRI	303 294	35 10	(<0.001)	5.9 3.9	0.73 (0.004)	14.5 12.5	0.85 (0.12)

Cet cetuximab, *Bev* bevacizumab
Legend: [a]Unselected patients
[b]KRAS wild-type

The positive results observed in selected populations of the CRYSTAL- and the PRIME study are contrasted by two further studies with negative outcome. The COIN study also employed capecitabine plus oxaliplatin as a chemotherapy backbone [20], while the NORDIC study used a bolus 5-FU regimen plus oxaliplatin, the so-called FLOX regimen [21]. This observation may lead to the conclusion that the addition of cetuximab to regimens based on bolus or oral fluoropyrimidines did not improve treatment efficacy. While, a prospective verification of this statement has not been performed, the present recommendation is to use anti-EGFR agents only in combination with infusional regimens such as FOLFIRI or FOLFOX when a fluoropyrimidine needs to be part of the protocol.

Effect of Anti-EGFR Agents in Second-Line Therapy (Table 9.2)

The anti-EGFR agents cetuximab and panitumumab were also investigated in second-line treatment. The EPIC study was performed in fluoropyrimidine/oxaliplatin pretreated patients and compared FOLFIRI plus cetuximab to FOLFIRI alone [22]. The 191-patient study included fluoropyrimidine pretreated patients, 66 % of whom had also received prior oxaliplatin [23]. Also this randomised study chose FOLFIRI as a chemotherapy backbone and evaluated the benefit from the addition of panitumumab. Both studies demonstrate a significant improvement of ORR and PFS induced by the addition of anti-EGFR agents. A survival benefit was, however, not achieved.

For the EPIC study it needs to be mentioned that unselected patients were included and that the evaluation of antitumour activity was performed regardless of RAS mutational status. Also cross-over treatment may have affected the survival results. In the 191-study, subsequent use of EGFR mAbs was reported in 31 % of KRAS-wt patients in the FOLFIRI arm compared to 10 % in the panitumumab-FOLFIRI arm.

Activity of Anti-EGFR Agents in Chemorefractory Patients

In chemotherapy-refractory mCRC, best supportive care plus anti-EGFR agents was compared to best supportive care (BSC) alone in two large randomised studies [17, 18]. In patients not selected for RAS status, addition of panitumumab to BSC induced a significant benefit with regard to ORR (10 % vs 0 %) and PFS (HR 0.54, $p < 0.0001$). By contrast, no difference in OS was detected (HR 1.00) which was explained by similar activity of panitumumab after 76 % of BSC patients had entered the cross-over study [17].

A further study investigated the addition of cetuximab to BSC [18]. In a retrospective analysis of patients with KRAS wild-type tumours, addition of cetuximab was associated with a marked improvement of ORR (12.8 % vs 0 %), PFS (3.7 vs

1.9 months; HR 0.40, p < 0.001) and OS (95. vs 4.8 months; HR 0.55, p < 0.001). By contrast, no benefit was reported in the subgroup of patients with KRAS-mutated tumours [18].

Head-to-Head Comparison of Cetuximab and Panitumumab

The ASPECCT study was the first randomised trial performing a head-to-head comparison of single-agent panitumumab to cetuximab in patients with chemotherapy-refractory mCRC [19]. Using a non-inferiority design the study indicated that in patients with KRAS exon 2 wild-type disease, panitumumab was non-inferior to cetuximab providing a similar overall survival benefit (HR 0.97), comparable response rates (22 % vs 20 %) and a comparable spectrum of grade 3–4 toxicities. In contrast to cetuximab, which is a humanised monoclonal antibody, panitumumab is fully human, causing less allergic reactions. Therefore, expectedly, a smaller rate of all-grade infusion reactions was observed in the panitumumab- compared to the cetuximab arm (3 % vs 14 %). While panitumumab is registered for application at 2- or 3 week intervals, the label prescribes a weekly dosing for cetuximab.

Mechanisms of Resistance Against Anti-EGFR Agents

Numerous mechanisms of resistance against anti-EGFR agents are presently known (Fig. 9.2). In view of the multitude of possible alterations that may lead to relapse, Misale and coworkers stated that biochemically most of them appear to converge to activate the EGFR-RAS-MAPK pathway [24].

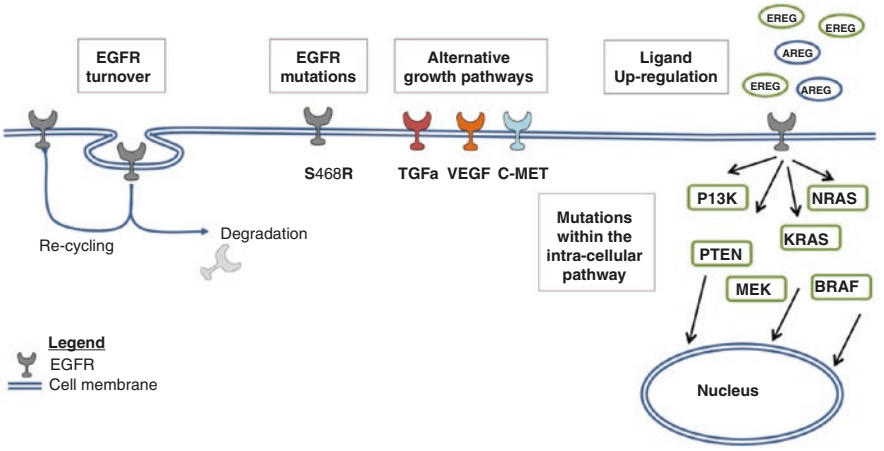

Fig. 9.2 Mechanisms of anti-EGFR resistance

Among the intrinsic mechanisms leading to primary resistance, specifically KRAS- and NRAS mutations have to be named. BRAF V600E mutation is clearly a negative prognostic factor. Its relevance as a predictive factor, however, is presently under discussion. Referring to the AKT/mTOR pathway by contrast, neither PIK3CA mutation nor PTEN deletion are likely to gain relevance as predictive factors.

Acquired resistance to anti-EGFR agents includes newly emerging RAS mutations, HER2 amplification, MET amplification, and EGFR gene mutations.

EGFR Gene Mutations

Activating mutations of the *EGFR* gene have been reported in NSCLC and were linked to the clinical efficacy of tyrosine kinase inhibitors such as gefitinib. It appears, however, that *EGFR* gene mutations are rare in colorectal cancer and have no clinical relevance with regard to the activity of anti-EGFR therapy [25, 26]. Barber et al., investigated a large cohort of 239 CRC patients and found only one mutation [25].

Mutation of the EGFR Ectodomain

Mutations of the EGFR ectodomain represent an important mechanism of acquired (secondary) resistance to anti EGFR antibodies [27, 28]. Initially, a mutation leading to the substitution of serine by arginine at position 492 of the EGFR gene had been described. The S492R mutation prevents binding of cetuximab to the EGFR and thus mediates resistance to cetuximab. Since the EGFR domain III epitopes only partially overlap, the S492R mutation does not induce resistance to panitumumab [27, 29]. The S492R mutation evolves during treatment with cetuximab and was not observed in 505 tumours previously not exposed to anti-EGFR agents [29]. Screening for this mutation should therefore only be performed during or after anti-EGFR therapy.

More recently, a novel EGFR exon 12 mutation was described in one out of three panitumumab-treated patients. This G465R mutation introduced a positive charge in both epitopes and therefore abrogated binding of cetuximab and panitumumab leading to cross-resistance to both agents [27].

RAS-Dependent Signal Transduction

Rat sarcoma oncogene (RAS) proteins play an important role in the intracellular signal transduction downstream of the EGFR. They constitute a family of proto-oncogenes encoding small G-proteins with a molecular weight of 21 kDa (p21). Three different members contribute to the RAS gene family known as Harvey-Ras (*HRAS*), Kirsten-ras (*KRAS*), and Neuroblastoma-RAS (*N-RAS*). These genes are located on different chromosomes. The expression levels of the respective genes vary in different tissues [30, 31].

The KRAS protein is located at the inner cell membrane and has GTPase activity [32]. Extracellular binding of ligands to the EGFR causes activation of the downstream signal transduction cascade to the nucleus. In a first step, the intracellular tyrosine kinase domain of the EGFR is phosphorylated which in turn induces a transient activation of the RAS protein. While in its inactive state, RAS is bound to guanosine diphosphate (GDP), activation occurs by conversion of GDP to guanosine triphosphate (GTP).

Clinical Relevance of RAS Mutations at Codons 12 and 13

Since anti-EGFR agents showed efficacy only in a subgroup of mCRC patients, intensive research was devoted to clarify mechanisms of intrinsic resistance. In fact, retrospective evidence from randomised studies soon provided compelling evidence that activating mutations downstream of EGFR were a primary cause of constitutive resistance. Initially, the KRAS mutation status, and later an extended RAS status also including NRAS, moved into the centre of clinical attention.

KRAS and NRAS are closely related members of the RAS oncogene family. Mutations occur in either gene at codons 12 and 13 in exon 2, at codons 59 and 61 in exon 3, and at codons 117 and 146 in exon 4 (Table 9.3) [8]. They result in increased levels of guanosine triphosphate-bound RAS proteins which contribute to an intrinsically activated signal transduction within the MAPK pathway.

In mCRC, approximately 50% of tumours show a KRAS- or NRAS mutation [8, 33]. These mutations typically tend to be mutually exclusive. Up to 90% of activating mutations of the K*RAS* gene are detected in codons 12 and 13. About 70% of KRAS exon 2 mutations occur in codon 12, and 30% in codon 13 [34]. The most frequent types of *KRAS* mutations in colorectal cancers are G-to-A transitions and G-to-T transversions. Codons 12 and 13 code for two adjacent glycine residues located in the proximity of the catalytic site of RAS [35–37].

Different RAS mutations result in an exchange of different amino acids at these catalytic sites and therefore may be responsible for different levels of intrinsic GTPase activity reduction. As a consequence, variable RAS mutations may imply variable effects on the biology of disease. For example, the presence of codon 12 glycine-to-valine mutations has been associated with a more aggressive tumour biology in advanced CRC. Compared KRAS and NRAS, mutations of HRAS are very infrequent in mCRC.

Table 9.3 Analysis of gene mutations in KRAS exon 2 wild-type tumours

Variable	PRIME Study[a] [8] Gene mutations (n = 641)	FIRE-3 [33] Gene mutations (n = 475)
KRAS exon 3, codon 59 and 61	4%	4.0%
KRAS exon 4, codon 117 or 146	6%	5.9%
NRAS exon 2, codon 12 or 13	3%	3.6%
NRAS exon 3, codon 59 and 61	4%	2.1%
NRAS exon 4, codon 117 or 146	0%	0.2%

[a]Douillard et al. [8], KRAS/NRAS exon 3, codon 59 not determined

Clinical Relevance of RAS Mutations Beyond KRAS Exon 2

A retrospective analysis of the PRIME study revealed that 17 % of patients who were originally categorised as KRAS exon 2 wild-type had other RAS mutations in KRAS codons 61, 117, and 146 or in NRAS codons 12, 13, and 61. Such other RAS mutations were associated with negative outcome in patients receiving FOLFOX4 plus panitumumab [8]. An exploratory analysis further suggested that also RAS codon 59 mutation might be a negative predictor of anti-EGFR treatment efficacy. The analysis of outcome data from the PRIME study indicated a disadvantage for patients with RAS-mutant tumours that were treated with FOLFOX plus panitumumab compared to FOLFOX alone [8].

Also a post hoc analysis of the OPUS phase II study suggested that patients with RAS mutant mCRC were potentially harmed by the addition of cetuximab to FOLFOX4 [7]. This data led to the conclusion that the addition of anti-EGFR mABs to FOLFOX not only failed to induce a benefit in patients with RAS mutant tumours, but also was associated with a negative effect on outcome.

Data from the CRYSTAL study helped to understand the effect of anti-EGFR therapy in RAS mutant patients treated with an irinotecan-based chemotherapy. A post hoc analysis of the CRYSTAL study using a 5 % mutant/wild-type cutoff identified 15 % of other RAS mutations in patients previously typed as KRAS exon 2 wild type. Also this evaluation indicated that cetuximab was not active in RAS mutant tumours. However, the addition of cetuximab to FOLFIRI did not induce a negative effect in this subgroup [6].

Conversely, the data from several studies clearly suggest that the exclusion of patients with other RAS mutations from a KRAS exon 2 wild-type treatment population defines a subgroup that is more likely to benefit from the addition of anti-EGFR mAbs to chemotherapy with FOLFOX or FOLFIRI in the first-line setting (Table 9.4) [6, 8].

Based on the retrospective analyses from the above referenced studies, it was concluded that molecular testing of tumours for all activating mutations in KRAS and NRAS is essential in selecting appropriate first-line treatment. This led to the

Table 9.4 RAS testing improves selection of tumours sensitive to anti EGFR mABs

Study	Refs.	Comparison	Biomarker	n	HR	Range
CRYSTAL	[6]	Cet + FOLFIRI vs FOLFIRI	KRAS RAS	666 367	0.80 0.69	0.67–0.95 0.54–0.88
PRIME	[8]	Pani + FOLFOX vs FOLFOX	KRAS RAS	656 512	0.83 0.78	0.70–0.98 0.62–0.99
FIRE-3	[1]	Cet + FOLFIRI vs Bev + FOLFIRI	KRAS RAS	592 400	0.77 0.70	0.62–0.96 0.54–0.90
CALGB/SWOG 80405	[10]	Cet + Chemo vs Bev + Chemo[a]	KRAS RAS	1137 526	0.92 0.90	0.78–1.09 0.70–1.10

Cet cetuximab, *Bev* bevacizumab
Legend: [a]Chemo, FOLFOX or FOLFIRI

restriction of anti-EGFR directed agents to patients with RAS wild-type tumours. As a consequence, an extended analysis of the RAS mutational status including KRAS and NRAS at exons 2–4 is strictly required in all patients prior to the clinical application of anti EGFR agents such as cetuximab.

Optimal Threshold for Detection of RAS Mutation

The significance of low prevalence RAS mutations in relation to the effectiveness of EGFR antibody therapy in mCRC is not entirely clear [38, 39]. Accordingly, the optimal threshold that differentiates RAS mutant from wild-type tumours still needs to be defined (Table 9.5).

To explore this question, different cutoffs ranging from 0.1 to 20 % were applied in the CRYSTAL study [6]. The data suggested that patients with low prevalence mutations (between 0.1 and <5 % mutant to wild-type) derived a treatment benefit from the addition of cetuximab to FOLFIRI. This conclusion was in line with another retrospective analysis of 95 patients with mCRC who had received EGFR antibody therapy, which indicated that the PFS of patients with tumours with low prevalence KRAS mutations (<5 %) was comparable to that of patients with KRAS wild-type tumours [40].

The data further suggested that higher fractions of mutated cells were associated with increasing resistance to EGFR antibody treatment. This would be in line with the hypothesis that acquired resistance to such agents may result, at least in part, from the outgrowth of small numbers of cells with pre-existing RAS mutations [6, 38].

The data available from the retrospective analysis of the CRYSTAL study were therefore consistent with <5 % mutant sequences being a clinically appropriate cut-off to define eligibility for FOLFIRI plus cetuximab first-line treatment.

Table 9.5 RAS mutation rates in first-line studies

Study	Refs.	Eval. Pts.	Method	Cut-off	Other RAS mutations, %
CALGB/SWOG 80405	[10]	670	BEAMing	1 %	15.3
OPUS	[7]	118	BEAMing	5 %	26.3
CRYSTAL	[6]	430	BEAMing	5 %	14.7
FIRE-3	[1]	475	Pyrosequencing	5 %	16.0
PRIME	[8]	620	Dideoxy sequencing/ WAVE	20 %/1 %	17.4
PEAK	[77]	221	Dideoxy sequencing/ WAVE	20 %/1 %	23.1

Legend: BEAMing, beads, emulsion, amplification, and magnetics; WAVE, WAVE-based Surveyor Scan Kits (Transgenomic)

Acquired Resistance to Anti-EGFR Therapy

Despite initial sensitivity, patients almost invariably develop resistance to anti-EGFR agents, mostly within less than 12 months of treatment. Several mechanisms of acquired resistance to anti-EGFR therapy are known. Misale and coworkers analysed metastases from patients who developed resistance to panitumumab or cetuximab [41]. They reported secondary KRAS mutations in 60 % (six out of ten) of cases. In none of these cases KRAS mutations had been detectable before initiation of therapy. In addition, no mutations were detected in patients undergoing chemotherapy alone. The authors concluded that treatment with anti-EGFR antibodies, but not with cytotoxic chemotherapy, was associated with acquisition of new KRAS mutations [41].

Subsequent investigations performed in patient-derived xenografts support the hypothesis that dual EGFR- and MEK inhibition may prevent the development of resistance if used a an upfront combination. This is explained by the finding that the combined EGFR-MEK blockade triggers Bcl-2 and Mcl-1 downregulation and initiates resistance [42].

Liquid biopsy is evolving as a technique most helpful in the early detection of RAS mutations in the tumour. The underlying concept is that circulating tumour DNA (ctDNA) can be found in the majority of mCRC patients. It is therefore expected that also mutant Ras genes are released into the circulation where they can then be detected by sensitive methods such as BEAMing (beads, emulsion, amplification, and magnetics) [43]. Based on BEAMing analyses from serial plasma samples, Misale and coworkers demonstrated that KRAS mutant alleles were detectable in the peripheral blood of cetuximab-treated patients as early as 10 months before disease progression was defined radiographically. Importantly, the same KRAS variants were observed in the liquid biopsies as in the post treatment tumour biopsies.

These analyses were nicely complemented by a report from Diaz and coworkers who observed the occurrence of KRAS mutations in the plasma of 38 % of patients (with originally KRAS wild-type tumours) exposed to panitumumab alone [44]. The mutations typically occurred in the plasma 5–6 months after start of treatment. Mathematical modelling suggested that the mutations were already present in expanded subclones before initiation of anti-EGFR therapy. The authors therefore propose that the time to resistance is equivalent to the time required for a resistant subclone to repopulate a tumour lesion and to cause marked expansion [44].

Other mechanisms of acquired resistance include EGFR ectodomain mutations (S492R, G465R) or the overexpression of members of the EGFR familiy (HER1-4).

Decline of Resistance After Withdrawal of Anti-EGFR Directed Therapy

Resistance appears to be a dynamic process that is defined in its course not only by the genetic characteristics of the tumour, but also by treatment itself. It is therefore of interest to note that resistance to anti-EGFR agents via an outgrowth of

RAS-mutant subclones may specifically be induced by the selective pressure of anti-EGFR therapy. In fact, Siravegna and coworkers report that mutated KRAS clones, which emerge in blood during EGFR blockade, decline upon withdrawal of anti-EGFR agents [45]. Antibody withdrawal may therefore alter the clonal composition of the tumour to an extent that favours the expansion of KRAS wild-type cells and thus allows the tumour to regain sensitivity to anti-EGFR agents. This hypothesis is supported by a report from Santini and cowokers [46]. In their study, patients (n = 39) initially responding to anti-EGFR based therapy were given a window-therapy when first progression occurred. Re-exposure to anti-EGFR agents at further progression led to an unexpectedly high response rate of 54 % and a PFS of 6.6 months. These results suggest that a re-challenge to anti EGFR agents may be a successful strategy that needs to be further explored in prospective clinical studies.

BRAF Mutation

Activating mutations of the BRAF gene are mostly characterised as BRAF V600E mutations. BRAF mutations are observed in approximately 8–10 % of mCRC patients. Concurrent mutations of RAS- and BRAF genes are very rare (0.001 %). Gene mutations of RAS (KRAS and NRAS) and BRAF V600E may therefore be reagarded as virtually mutually exclusive [47].

More recently, also other BRAF mutations such as BRAF V598 and BRAF V599 have been described.

BRAF mutations are more frequently observed in right-sided colon cancer. There is an association with high-grade mucinous CRC, microsatellite instability, and methylator phenotype [47]. The mutation mostly relates to an aggressive course of disease with early onset of multiorgan metastasis including lymph node and peritoneal metastasis. The prognosis of patients with BRAF V600E mutant mCRC is almost invariably very poor.

Retrospective evidence based on small subgroups from randomised studies supports the view that BRAF mutation may not only have a prognostic importance, but may predict resistance to anti-EGFR agents. Pietrantonio and coworkers performed a meta-analysis of randomised studies comparing chemotherapy plus anti-EGFR agents to chemotherapy or best supportive care alone. From nine phase III trials and one phase II trial they included 463 RAS-wt/BRAF-mut mCRC patients. Overall, the addition of anti-EGFR agents did not significantly improve ORR (relative risk 1.31), PFS (HR 0.88) or OS (HR 0.91). Due to its prognostic and negative predictive relevance, the authors concluded that BRAF mutation assessment should be integrated into the initial diagnostic work-up before treatment start.

In a parallel publication, Rowland and coworkers published a further meta-analysis including seven randomised studies. They concluded that there was insufficient evidence to definitively state that RAS-wt/BRAF-mut individuals attain a different treatment benefit from anti-EGFR mAbs compared to RAS-wt/BRAF-wt individuals [48]. They finally state that the available data are insufficient to justify the exclusion of patients with RAS-wt/BRAF-mut mCRC from anti-EGFR mAb therapy.

Table 9.6 TRIBE study: efficacy results in RAS and BRAF subgroups [49]

	N	FOLFIRI + bev Median OS	FOLFOXIRI + bev Median OS	HR	P
ITT population	508	25.8	29.8	0.80	0.03
Extended RAS and BRAF population	357	24.9	28.6	0.84	0.16
RAS + BRAF wt	93	33.5	41.7	0.77	0.52
RAS mutant	236	23.9	27.3	0.88	ns
BRAF mutant	28	10.7	19.0	0.54	ns

While the two meta-analyses appear to come to different conclusions, they are both based on a limited and retrospective data base which simply does not allow a firm conclusion.

The question if cetuximab rather than bevacizumab-based first-line chemotherapy should be preferred in patients with BRAF mutant mCRC was addressed in a subgroup analysis of the FIRE-3 study. In 48 evaluable patients with RAS-wt/BRAF-mut mCRC, overall survival was short and comparable (12.3 vs 13.7 months) independent if cetuximab or bevacizumab had been added to FOLFIRI [49]. This analysis led to the conclusion that neither an anti-EGFR directed nor an anti-VEGF strategy were, by their own right, able to improve outcome when combined with standard chemotherapy.

Further evidence regarding more intensive chemotherapy is available from the TRIBE study (Table 9.6) [50]. Patients with BRAF mutant tumours treated with FOLFOXIRI plus bevacizumab (n = 16) had a markedly longer overall survival than patients in the control arm (n = 12) receiving FOLFIRI plus bevacizumab (19.0 vs 10.7; HR 0.54). Despite the very small patient number, these data are encouraging and are the basis for current treatment recommendations. Since bevacizumab was used in both treatment arms of the TRIBE study, there is no answer to the question if treatment intensification alone or the additional use of bevacizumab were responsible for the observed effect.

Efficacy of Cetuximab in Tumours with pG13D Mutation

RAS mutation, in general, is understood as a negative predictive factor that excludes patients from treatment with anti-EGFR agents. Nevertheless, there was a controversial discussion to which extent different RAS mutations were associated with different sensitivity to anti-EGFR agents [51]. In 2010, de Roock and coworkers reported a retrospective analysis of 579 patients with chemotherapy-refractory mCRC treated with cetuximab. The study included patients from several clinical trials such as CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL, or SALVAGE. In this analysis, the authors found that use of cetuximab was associated

with longer overall and progression-free survival in patients with p.G13D mutated tumours than with other KRAS-mutated tumours [52]. Analyses from several first-line studies including CRYSTAL, OPUS, and PRIME yielded divergent and lastly inconclusive results. More recently, an Australian group reported on the ICECREAM study [53]. This study was performed in p.G13D mutated mCRC and compared cetuximab to cetuximab plus irinotecan in 53 patients refractory to irinotecan. There was no statistically significant improvement of disease control at 6 months (primary end point) with either treatment. Time to tumour progression was short and comparable between both treatment arms (2.5 vs 2.6 months). The results of this study need to be interpreted in the context of the BOND study. This study was performed in an unselected population of irinotecan-refractory mCRC patients and demonstrated a TTP of 4.1 months in the cetuximab/irinotecan arm [15]. In view of these data, there is no clear evidence for activity of cetuximab in p.G13D mutated mCRC. Accordingly, RAS mutation, independent of subgroups, remains a clear exclusion criterion for use of anti-EGFR mABs.

HER2 Overexpression

HER2 is part of the EGFR tyrosine kinase receptor family also including EGFR, HER3 and HER4. Inhibition of HER2 by the monoclonal antibody trastuzumab and by the tyrosine kinase inhibitor lapatinib has been extensively studied in breast cancer where these agents have shown consistent clinical activity. Since then, overexpression and amplification of HER2 have also been observed in other cancers such as gastric cancer and colorectal cancer.

In mCRC, HER2 overexpression has been related to resistance against anti-EGFR-therapy. Due to its ability to form heterodimers with EGFR and HER3 it may contribute to activation of the MAPK and AKT pathways and may thus circumvent EGFR blockade [54–56]. It appears that HER2 overexpression is not a prognostic marker in CRC, but is strongly associated with RAS/RAF wild-type status [56]. In patient derived xenografts, HER2 overexpressing mCRC was shown to be sensitive to dual HER2 blockade with trastuzumab and lapatinib.

Strong overexpression of membranous HER2 is an infrequent event and was reported in only 1.3 % (25/1914) of stage II-III tumours and 2.2 % (29/1342) of stage IV tumours. The recently reported HERACLES trial focused on KRAS wild-type patients refractory to anti-EGFR therapy and found HER2 overexpression (IHC2+/3+) in 5.4 % (46/849) of patients [57]. In 23 patients evaluable for response an ORR of 35 % and a disease control rate of 78 % were observed. The study thus met its predefined end point reaching an ORR >30 %. Time to progression was 5.5 months in the whole patient cohort, while it was 7.3 months in HER2 IHC 3+ patients and 4.2 months in HER2 IHC 2+ patients. The authors of the HERACLES trial concluded that HER2-targeted therapy was a valuable treatment option for HER+ mCRC.

The Vascular Endothelial Growth Factor Pathway

The vascular endothelial growth factor (VEGF) family of ligands comprises VEGFA, VEGFB, VEGFC, and the placental growth factor (PlGF). These ligands bind to VEGFR1, VEGFR2, and VEGFR3.

VEGFA binds to VEGFR2 and appears to be the key driver of tumour angiogenesis [58]. VEGFR2 is responsible not only for proliferation, migration and survival of endothelial cells, it also increases vascular permeability and thus contributes to the regulation of interstitial pressure [59, 60]. Preclinical evidence suggests that blocking of the VEGFA-VEGFR2 interaction effectively inhibits tumour neoangiogenesis [61].

VEGFR1 activity is modulated by three ligands, VEGFA, VEGFB and PIGF. This receptor is mainly involved in monocyte chemotaxis, hematopoietic stem cell survival and inhibition of dendritic cell maturation [63, 64]. VEGFC and VEGFD bind to VEGFR3, which is thought to regulate lymphangiogenesis, but may also be involved in angiogenesis [58].

Mechanism of Action of VEGF Inhibitors

Inhibitors of the VEGF pathway may induce antitumour effects via different mechanisms of action, which may depend more on the characteristics of the tumoural microenvironment than of the tumour itself (Fig. 9.3). The most popular hypothesis is that VEGF inhibitors work by inhibition of tumour neoangiogenesis impacting on proliferation and metastasis of tumours [59]. On the other hand, there is also evidence that VEGF inhibitors may cause normalisation of tumour vessels and reduce interstitial pressure, which then may contribute to improved transport of antitumour agents to the neoplastic tissue. Another hypothesis developed in animal models relates to the observation that tumoural hypoxia caused by VEGF-inhibitors may induce a more invasive phenotype of the tumour and may thus increase its metastatic potential [65].

Based on the present knowledge, VEGF-inhibitors do not have a primary effect on tumours, but primarily impact on tumoural growth and metastasis via their effects on tumour vessels. Therefore, it is not surprising that biomarkers predicting the efficacy of antiangiogenic agents are essentially lacking and therefore have not found entrance into clinical decision making. Presently, three antiangiogenic agents, bevacizumab and aflibercept, and ramucirumab are registered for treatment of mCRC.

Bevacizumab

Bevacizumab is a recombinant humanised monoclonal IgG-1 antibody directed against VEGFA. The drug is registered for treatment of mCRC in combination with fluoropyrimidine-based chemotherapy independent of treatment line. A predictor of

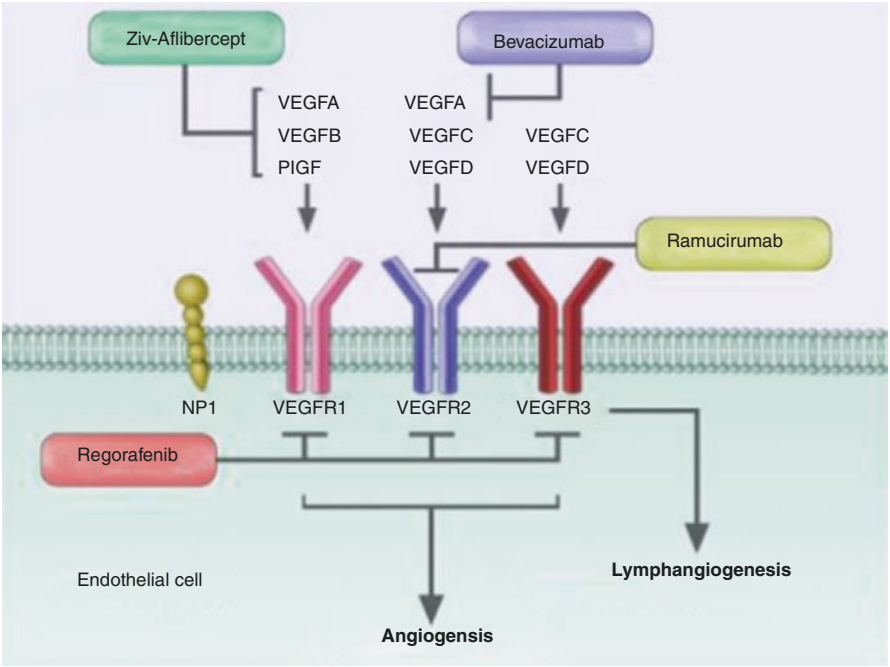

Fig. 9.3 Antiangiogenic agents

bevacizumab activity is not available. Therefore, the agent is used in an unselected patient population.

Bevacizumab-Based First-Line Therapy (Table 9.7)

The effect of bevacizumab on response rate is moderate and ranges from 0 to 10 % in most studies. Inclusion of bevacizumab into conversion therapy, where improvement of ORR is a goal, therefore cannot be its primary use. However, a common theme throughout most randomised studies is the observation that addition of bevacizumab to fluoropyrimidine-based chemotherapy causes a notable and significant prolongation of progression-free survival reaching hazard ratios in the range of 0.44–0.86 (Table 9.7).

By contrast, overall survival was significantly improved only in two randomised studies comparing the bolus 5-FU/irinotecan regimen (IFL) plus bevacizumab to IFL alone [81, 83], while a further phase III study investigating this combination was negative [82]. Due to its toxicity, IFL is, however, not a standard of care any more. The evidence from the aforementioned studies therefore hardly has an impact on daily clinical practice. The largest study comparing FOLFOX/XELOX plus bevacizumab to FOLFOX/XELOX alone (NO16966) failed to show a survival benefit [84]. Randomised studies evaluating the effect of bevacizumab in addition to

Table 9.7 Randomised first-line studies investigating the effect of bevacizumab

Study	Refs.	Regimen	No of patients	ORR (%)	OR (P)	PFS (mo)	HR PFS (P)	OS (mo)	HR OS (P)
Hurwitz (Phase III)	[81]	IFL + Bev IFL	402 411	44.8 34.8	(0.004)	10.6 6.2	0.54 (<0.001)	20.3 15.6	0.66 (<0.001)
Stathopoulos (Phase III)	[82]	IFL + Bev Bev	114 108	36.8 35.2	(n.s.)	NR	NR	22 25	1.05 (0.139)
ARTIST (Phase III)	[83]	mIFL + Bev mIFL	142 72	35.3 17.2	(0.013)	8.3 4.2	0.44 (<0.001)	18.7 13.4	0.62 (0.014)
NO16966 (Phase III)	[84]	FOLFOX/XELOX + Bev FOLFOX/XELOX	699 701	47 49	0.90 (0.31)	9.4 8.0	0.83 (0.0023)	21.3 19.9	0.89 (0.077)
ITACa (Phase III)	[85]	FOLFOX4/FOLFIRI + Bev FOLFOX4/FOLFIRI	176 194	50.6 50	(0.865)	9.6 8.4	0.86 (0.182)	20.8 21.3	1.13 (0.317)
Kabbinavar (Phase II) 2003	[86]	5-FU/LV 5-FU/LV + Bev 5 mg/kg 5-FU/LV + Bev 10 mg/kg	36 35 33	17 40 24		5.2 9.0 7.2		13.8 21.5 16.1	
Kabbinavar (Phase II) 2005	[87]	FU/LV + Bev FU/LV	104 105	26.0 15.2	(0.055)	9.2 5,5	0.50 (0.0002)	16.6 12.9	0.79 (0.16)
AVEX (Phase III)	[88]	Cape + Bev Cape	140 140	19 10	(0.04)	9.1 5.1	0.53 (<0.0001))	20.7 16.8	0.79 (0.18)
MAX (Phase III)	[89]	Cape + Bev Cape	157 156	38.1 30.3	NR (0.16)	8.5 5.7	0.62 (<0.001)	NR	0.88 (0.314)

Legend: *IFL* irinotecan, bolus fluorouracil, and leucovorin, *Bev* bevacizumab, *Cape* capecitabine

FOLFIRI as a chemotherapy backbone are simply missing. Therefore, it remains unclear if addition of bevacizumab to infusional-FU-based regimens has a relevant impact on OS (Table 9.7).

Antiangiogenic Second-Line Therapy (Table 9.8)

In contrast to the less homogeneous data obtained in first-line studies, the effect of antiangiogenic therapy in second line studies appears to be very consistent. All studies evaluated in Table 9.8 show a significant prolongation of PFS and OS achieved by the addition of bevacizumab to chemotherapy. Similar results were also observed for other antiangiogenic agents such as aflibercept or ramucirumab (Table 9.8). Based on this data, the evidence is strong to support application of antiangiogenic agents in second-line, while benefit from its addition to combination chemotherapy in first-line treatment remains controversial.

Bevacizumab-Based Maintenance Therapy

In most patients, reduction of treatment intensity becomes necessary after 4–6 months of first-line therapy. Maintenance therapy with less toxic regimens is then offered to stabilise disease dynamics and to maintain the so far achieved treatment effects. Controlled studies indicate that prolonged progression-free survival can be obtained with maintenance therapy compared to complete drug holidays or continued treatment. However, the impact of maintenance therapy on overall survival is less clear [66].

The MACRO study was the first phase III study which – after induction with six cycles XELOX plus bevacizumab – compared continuation of this treatment to maintenance with bevacizumab. This study could not show the noninferiority of the bevacizumab-alone arm [67]. The SAKK 41/06 study applied standard induction chemotherapy for 4–6 months whereafter nonprogressive patients were randomised to maintenance with bevacizumab or drug holiday. This study could not demonstrate inferiority of the treatment-holiday compared to continuation of bevacizumab [68].

In the CAIRO3 study, patients received six cycles of induction chemotherapy with CAPOX plus bevacizumab and were then randomised to maintenance treatment with capecitabine plus bevacizumab or observation. On first progression (defined as PFS1), patients in both groups were to receive the induction regimen of CAPOX-B until second progression (PFS2), which was the study's primary endpoint. PFS2 in the maintenance arm of CAIRO3 was significantly superior to observation (11.7 vs 8.5 months; HR 0.67, $p<0.0001$). This did, however, not translate into a statistical improvement of OS (HR 0.89, $p=0.22$) [69]. Comparable data favouring maintenance treatment with a fluoropyrimidine plus bevacizumab over bevacizumab or drug holiday alone were also obtained in the AIO 0207 study [70]. Also in this study, overall survival was not affected by the choice of maintenance therapy. This observation can be explained by the rather short duration of maintenance therapy as well as by the low re-induction rate [66].

Table 9.8 Randomised second-line studies investigating the effect of anti VEGF agents

Study	Refs.	Pretreatment	Regimen	No of patients	ORR (%)	OR (P)	PFS (mo)	HR PFS (P)	OS (mo)	HR OS (P)
E3200 (Phase III)	[90]	Fluoropyrimidne and irinotecan (0 % Bev)	FOLFOX4 + Bev FOLFOX4	286 291	22.7 8.6	(<0.0001)	7.3 4.7	0.61 (<0.0001)	12.9 10.8	0.75 (0.0011)
TML (Phase III)	[75]	Chemotherapy (100 % Bev)	Chemotherapy + Bev Chemotherapy	409 411	5 4	(n.s.)	5.7 4.1	0.68 (<0.0001)	11.2 9.8	0.81 (0.0062)
BEBYP (Phase III)	[91]	Chemotherapy (100 % Bev)	Chemotherapy + Bev Chemotherapy	92 92	21 17	(0.573)	6.8 5.0	0.70 (0.010)	15.5 14.1	0.77 (0.043)
Chinese (Phase II)	[92]	Oxaliplatin-based (0 % Bev)	FOLFIRI + Bev FOLFIRI	65 77	47.7 28.5	(<0.001)	8.5 5.1	NR	15.2 11.3	NR
VELOUR (Phase III)	[72]	Oxaliplatin-based (30.4 % Bev)	FOLFIRI + Aflibercept FOLFIRI + Placebo	612 614	19.8 11.1	(0.0001)	6.9 4.7	0.76 (<0.0001)	13.5 12.1	0.82 (0.0032)
RAISE (Phase III)	[74]	Fluoropyrimidine and oxaliplatin (100 % Bev)	FOLFIRI + Ramucirumab FOLFIRI + Placebo	536 536	13.4 12.5	(0.63)	5.7 4.5	0.79 (0.0005)	13.3 11.7	0.84 (0.0219)

Legend: *Bev* bevacizumab

Aflibercept

Aflibercept is a recombinant fusion protein containing VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2, fused to the Fc portion of human immunoglobulin G1 (IgG1) [71]. Aflibercept blocks the activity of VEGFA, VEGFB, and placental growth factor (PlGF) by acting as a high-affinity ligand trap to prevent these ligands from binding to their endogenous receptors [72]. The clinical benefit from additional trapping of VEGFB and PIGF still needs to be clarified. Aflibercept was tested in the randomised VELOUR trial comparing FOLFIRI plus aflibercept to FOLFIRI alone in patients whose disease was resistant to or had progressed following an oxaliplatin-based regimen. This placebo-controlled study could show a moderate, but statistically significant, survival benefit induced by the addition of aflibercept (HR 0.817; 95.34 % CI, 0.713–0.937; p = 0032) (Table 9.8) [72]. The comparability of the VELOUR trial with other randomised second-line trials such as TML or RAISE is limited in that only 30 % of the patients had received prior treatment with bevacizumab.

Ramucirumab

Ramucirumab is a fully human IgG-1 monoclonal antibody that targets the extracellular domain of VEGFR2. The binding affinity of ramucirumab to the receptor is high and exceeds that of VEGFA by about ninefold [73]. Ramucirumab blocks binding of all VEGF ligands to VEGFR2 and thus prevents its activation.

The randomised, placebo-controlled RAISE study tested the activity of ramucirumab in mCRC patients who had progressed after first-line treatment with a combination of a fluoropyrimidine, oxaliplatin and bevacizumab [74]. This phase III study showed that FOLFIRI plus ramucirumab significantly improved OS compared to FOLFIRI plus placebo (HR 0.844; 95 % CI, 0.730–0.976; log-rank p = 0.0219) (Table 9.8). Predictive biomarkers indicating the activity of ramucirumab in mCRC have not been identified so far.

Comparing VELOUR, TML and RAISE

The comparability of the three randomised second-line trials investigating antiangiogenic agents is limited [58]. Therefore, several issues have to be pointed out in this context:

1. The chemotherapy regimen in the VELOUR and RAISE study was standard FOLFIRI, while second-line chemotherapy in the TML-study was dependent on first-line treatment which had been left to the discretion of the treating physicians.
2. All of the patients in the VELOUR and RAISE study had received first-line chemotherapy with oxaliplatin, while only 41 %/42 % of patients in the TML study had received prior oxaliplatin-based chemotherapy.

3. The VELOUR and RAISE studies included patients with disease progression at various times after first-line treatment (including fast-growing tumours). By contrast, in the TML study patients were excluded if they had a first-line progression-free survival of less than 3 months, and if they were given less than 3 months (consecutive) of first-line bevacizumab. Rapid progression is clearly associated with poor-prognosis. The exclusion of these patients therefore impacts on outcome.
4. Also, the TML study excluded patients if they had a diagnosis of progressive disease for more than 3 months after the last bevacizumab administration.
5. RAISE and VELOUR were performed as world-wide studies of 24 and 28 countries respectively, while TML was mainly a European study performed in 15 countries [72, 74, 75].

Head-to-Head Comparisons of Anti-EGFR and Anti-VEGF Agents

Head-to-head comparisons of anti-EGFR directed agents and bevacizumab were performed in three randomised studies (FIRE-3, PEAK, CALGB 80405) performed in first-line treatment of mCRC. The FIRE-3 phase III trial compared FOLFIRI plus cetuximab to FOLFIRI plus bevacizumab and evaluated ORR as a primary end point [1]. Evaluation of the RAS wild-type population failed to show a significant difference in ORR and PFS, while OS was markedly superior in the cetuximab arm (HR 0.70, $p = 0.0059$) [33].

The PEAK study was performed as a randomised phase II study comparing FOLFOX plus panitumumab to FOLFOX plus bevacizumab exploring PFS as a primary end point. In the subgroup of RAS wild-type patients, this study showed a significant superiority of the panitumumab arm for PFS (HR 0.65, $p = 0.029$). Also overall survival was clearly longer in the panitumumab- compared to the bevacizumab-arm (41.3 months vs 28.9 months). However, due to the low event rate (<50%), the level of statistical significance was not reached (HR 0.63, 0.058) [77].

As a third trial, the CALGB/SWOG 80405 study performed a randomised comparison of chemotherapy plus cetuximab versus chemotherapy plus bevacizumab. The choice of the chemotherapy backbone, FOLFOX (76%) or FOLFIRI (24%) was left to the discretion of the participating centres. This study reported a significantly greater response rate in the cetuximab arm (69% vs 54%, $p < 0.01$), however no significant difference between treatment arms was observed with regard to PFS and OS [78].

A meta-analysis based on the published study reports performed a common evaluation of the three head-to-head comparisons including overall 2014 patients [79]. This meta-analysis supported the superiority of the anti-EGFR arm with regard to ORR (odds ratio 1.46, $p = 0.004$) and overall survival (HR 0.77, $p = 0.016$). As expected, PFS (HR 0.92, $p = 0.5$) was comparable between treatment arms [79]. The superior treatment results favouring anti-EGFR over anti-vascular treatment were obtained not only for KRAS wild-type, but also for RAS wild-type patients.

Optimal Sequence of Biological Agents

Anti EGFR- and anti-VEGF agents are characterised not only by distinctly different mechanisms of action, but also by different effects on response parameters. Wainberg and coworkers tried to integrate both classes of biological agents and developed a rationale for optimal sequencing [80].

Their work is firstly based on the observation that a number of clinical trials showed a survival benefit when anti-EGFR agents were applied to RAS wild-type patients in first-line treatment allowing the use of anti-VEGF agents in later lines of therapy. This positive effect on survival is explained by a more rapid treatment effect accompanied by a greater depth of response. Antivascular agents, by contrast, have a less pronounced effect on ORR and prolongation of overall survival is more likely to be achieved by prolongation of PFS.

The authors suggest that first-line treatment with EGFR inhibitors creates a unique biological condition that sensitises cells to the use of anti VEGF agents, while the reverse sequence is markedly less beneficial. In addition, the authors propose that cells resistant to anti-EGFR agents remain sensitive to VEGF inhibitors, while cells resistant to antivascular agents concurrently develop resistance to EGFR inhibitors [80]. Based on this biological rationale, the authors conclude that an optimal sequence for patients with RAS wild-type tumours would include first-line use of anti-EGFR agents followed by second-line application of VEGF inhibitors. Future trials specifically designed to test optimal drug sequencing need to verify this concept.

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