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CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Magnus Åbrink (167)
Department of Anatomy, Physiology and Biochemistry, The Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden

Magda Carneiro-Sampaio (51)
Department of Pediatrics, Children's Hospital, Faculdade de Medicina da Universidade de São Paulo, Av. Enéas Carvalho Aguiar 647, 05403-900 São Paulo, Brazil

Antonio Coutinho (51)
Instituto Gulbenkian de Ciência, Oeiras, Portugal

Jean-Pierre David (149)
Group Bone Cell Differentiation, Deutsches Rheuma-Forschungszentrum (DRFZ), Cahritéplatz 1, Berlin, Germany

Kenneth Dorshkind (1)
Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Thomas Hünig (111)
Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany

John G. Monroe (1)
Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Room 311, BRBII, 421 Curie Blvd., Philadelphia, PA 19104, USA

Michel C. Nussenzweig (83)
Howard Hughes Medical Institute, 1230 York Avenue, New York NY, USA

Gunnar Pejler (167)
Department of Anatomy, Physiology and Biochemistry, The Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden

Maria Ringvall (167)
Department of Anatomy, Physiology and Biochemistry, The Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden

Hedda Wardemann (83)
Max Planck Institute for Infection Biology, Campus Charite Mitte, Schumannstrasse 21/22, D-10117 Berlin, Germany

Sara Wernersson (167)
Department of Anatomy, Physiology and Biochemistry, The Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden

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CHAPTER 1

Fate Decisions Regulating Bone Marrow and Peripheral B Lymphocyte Development

John G. Monroe* and **Kenneth Dorshkind†**

Contents

* Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania
† Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California

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Abstract In adult mammals, bone marrow pluripotent hematopoietic stem cells generate B lymphoid-specified progeny that progress through a series of well-characterized stages before generating B-cell receptor expressing B lymphocytes. These functionally immature B lymphocytes then migrate to the spleen wherein they differentiate through transitional stages into follicular or marginal zone B lymphocytes capable of responding to T-dependent and -independent antigens, respectively. During the terminal stages of B lymphocyte development in the bone marrow, as well as immediately following egress into the peripheral compartments, B lymphocytes are counterselected to eliminate B lymphocytes with potentially dangerous self-reactivity. These developmental and selection events in the bone marrow and periphery are dependent on the integration of intrinsic genetic programs with extrinsic microenvironmental signals that drive progenitors toward increasing B lineage commitment and maturation. This chapter provides a comprehensive overview of the various stages of primary and secondary B lymphocyte development with an emphasis on the selection processes that affect decisions at critical checkpoints. Our intent is to stress the concept that at many steps in the developmental process leading to a mature immunocompetent B lymphocyte, B lineage cells are integrating multiple and different signaling inputs that are translated into specific and appropriate cell fate decisions.

1. INTRODUCTION

B lymphocytes are classically defined as the immune effectors responsible for humoral immunity. However, this simple definition belies the increasing awareness that two main populations of B lymphocytes, each of which is composed of distinct subpopulations defined by phenotype and function, exist.

A minor population of B-1 B lymphocytes, which accounts for around 5% of B lymphocytes, is localized to serous cavities. B-1 B lymphocytes, which are subdivided into B-1a and B-1b subpopulations, based on phenotype and function, are effectors of innate immunity and mainly respond to T-cell-independent immunogens that include carbohydrate antigens. Although the bone marrow retains the potential to produce B-1 B lymphocytes, they are most efficiently generated from progenitors that arise during embryogenesis. Several reviews that describe advances in our understanding of B-1 B lymphocyte development and function have been published and should be consulted for detailed information regarding these cells (Hardy, 2006a,b; Montecino-Rodriguez and Dorshkind, 2006, 2007).

The predominant population of B lymphocytes, referred to as B-2 B lymphocytes, resides in the spleen and lymph nodes. B-2 B cells are produced in the bone marrow during postnatal life and then migrate to the spleen where, after progressing through several transitional stages, they mature into a major population of follicular (FO) and a minor population of marginal zone (MZ) B lymphocytes. MZ B lymphocytes do not circulate and respond to blood-borne antigens while FO B lymphocytes respond to protein antigens and, in response to T-cell help, undergo class switching and affinity maturation into B-2 B lymphocytes.

This chapter will focus on how cell-extrinsic signals from the hematopoietic microenvironment are integrated with intrinsic gene expression and signaling pathways to regulate fate decisions at defined stages of primary and secondary B-2 (hereafter noted without the B-2 designation) B lymphocyte development. The initial sections will describe the stages of B lymphopoiesis in the bone marrow and the cell-intrinsic and -extrinsic signals which regulate that process. Subsequently, the factors that regulate the maturation of newly produced B lymphocytes into immunocompetent effectors following their migration to the spleen will be reviewed.

2. B LYMPHOCYTE DEVELOPMENT IN THE BONE MARROW

B lymphocytes, like all blood cells, are derived from hematopoietic stem cells (HSCs). Multiple stages of development between the HSC and newly produced surface IgM^{pos} ($sIgM^{pos}$) B lymphocytes have been defined, and progenitors must successfully navigate each of these in order to mature into functional B lymphocytes capable of participating in a productive immune response (Hardy and Hayakawa, 2001; Kondo *et al.*, 2003; Pelayo *et al.*, 2006). This section will review the stages of primary B lymphocyte development in the bone marrow, describe the environment in which these developmental processes occur, and discuss primary B lymphopoiesis in the context of a long-standing debate on how fate decisions within the hematopoietic system are regulated.

2.1. Stages of B lymphopoiesis

Advances in the identification of cell surface determinants expressed on hematopoietic cells, the production of antibodies to them, and the development of increasingly sophisticated flow cytometric programs have made it possible to identify and isolate cells at various stages of development between HSC and newly produced B lymphocytes. Figure 1 depicts some of the surface markers that are used to define intermediates in the B lymphocyte developmental pathway. As discussed below, these cell surface determinants provide convenient correlates for the early stages of immunoglobulin (Ig) heavy chain (HC) and light chain (LC) recombination status and for delineation of developmental stages subsequent to expression of the B-cell receptor (BCR) (Osmond *et al.*, 1998).

HSCs are defined by their lineage negative (Lin^{neg}) c-kit^{high} Sca-1^{pos} phenotype (Spangrude *et al.*, 1988). Lin^{neg} refers to the absence of cell surface determinants expressed by more mature lymphoid and myeloid

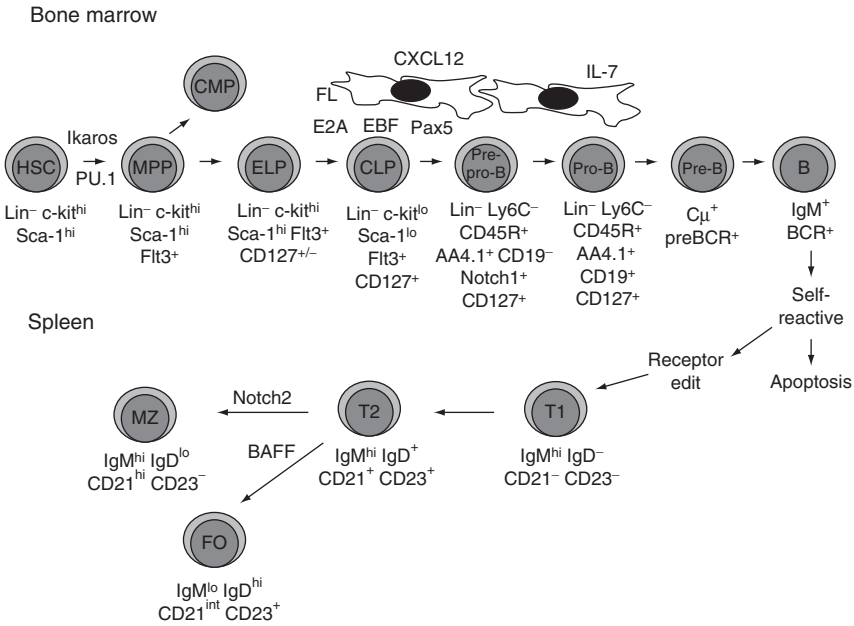

FIGURE 1 Stages of primary B lymphocyte development in the bone marrow and of transitional stages in the spleen. Some of the cell surface determinants used to define cells at the different stages of development as well as the environmental signals that affect the growth and/or differentiation of cells at various stages of development are indicated. FL, Flt3 ligand; FO, follicular B lymphocyte; MZ, marginal zone B lymphocyte; HSC, hematopoietic stem cell; MPP, multipotent progenitor; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor.

lineage cells. The HSC compartment is heterogeneous and includes cells, termed long-term HSCs, defined in transplantation studies by their ability to repopulate all hematopoietic cells for the life of the recipient, and others, termed short-term HSCs, which do so only transiently (Morrison and Weissman, 1994). The latter compartment includes an intermediate, termed the multipotent progenitor (MPP) (Adolfsson *et al*., 2001; Pelayo *et al*., 2006), from which the common myeloid progenitor (CMP) (Akashi *et al*., 2000) and the early lymphoid progenitor (ELP) (Tudor *et al*., 2000) derive. While HSC and MPP share a Lin^{neg} c-kit^{high} Sca-1^{pos} phenotype, MPPs also express the Flt3 receptor (Adolfsson *et al*., 2001). As implied by its name, the CMP is the intermediate from which erythroid and myeloid cells are generated while lymphoid cells are produced from Lin^{neg} c-kit^{high} Sca-1^{pos} $Flt3^{pos}$ IL-$7R^{neg}$ ELP.

Although ELPs are a highly enriched population of bone marrow, they are nevertheless heterogeneous and include cells with differing patterns of gene expression and potentiality. For example, some ELPs express the recombinase-activating genes (*RAG*) *RAG1* and *RAG2*, and have immunoglobulin D_H-J_H IgHC gene rearrangements (Igarashi *et al*., 2002), suggesting that they may already be on the path to becoming a B lymphocyte. Other ELPs may be destined to seed the thymus, and the presence of circulating cells with an ELP phenotype is consistent with this hypothesis (Schwarz and Bhandoola, 2004). Such cells are thus likely precursors of the intrathymic early T lymphocyte lineage progenitor (ETP) (Allman *et al*., 2003). Some degree of extrathymic commitment to the T lymphocyte lineage may be operative, as a subpopulation of ELPs that express CD62L and CD44 and have potent T and weak B lymphocyte potential (Perry *et al*., 2003, 2004), and circulating blood cells that are largely T lineage restricted have been described (Krueger and Von Boehmer, 2007). As reviewed, ''the relationship between T-cell progenitors in the bone marrow, blood, and thymus remains unresolved'' (Petrie, 2007) and further discussion of this issue is beyond the scope of this chapter.

The decreased expression of c-kit and Sca-1, the acquisition of additional cell surface determinants that include the interleukin-7 receptor (IL-7R) and AA4.1 (CD93), as well as the increased expression of *RAG1/2* are hallmarks of further maturation in the B lymphocyte lineage (Payne *et al*., 1999). These Lin^{neg} Sca-1^{lo} c-kit^{lo} AA4.1^{pos} CD127 (IL-7Rα^{pos}) $Flt3^{pos}$ cells are referred to as common lymphoid progenitors (CLPs) (Kondo *et al*., 1997). As shown in Fig. 1, CLP progeny include pre-pro-B, pro-B, pre-B, and finally, newly produced $sIgM^{pos}$ B lymphocytes. These newly produced $sIgM^{pos}$ lymphocytes then migrate from the marrow to the spleen wherein they undergo further maturation as will be discussed later.

Most laboratories use an immunophenotyping strategy initially defined by Hardy and colleagues to identify cells between the CLP and B lymphocyte stages of development (Hardy *et al*., 1991). Pre-pro-B lymphocytes were

originally characterized as CD45R^{pos} CD43^{pos} CD24^{neg} cells, and pro-B lymphocytes were defined based on their CD45R^{pos} CD43^{pos} CD24^{pos} phenotype. However, it is now appreciated that the CD45R^{pos} CD43^{pos} compartment is developmentally heterogeneous, and accurate resolution of cells within it necessitates that non-B lineage lymphocytes be depleted. Thus, pre-pro-B lymphocytes are now defined as Lin^{neg} CD45R^{pos} CD43^{pos} AA4.1^{pos} CD19^{neg} Ly6C^{neg} and pro-B lymphocytes as CD45R^{pos} CD19^{pos} CD43^{pos} AA4.1^{pos} (Li *et al.*, 1996; Tudor *et al.*, 2000). CD45R is a member of the CD45 leukocyte common antigen family that includes glycoproteins of multiple sizes generated by alternative splicing of *cd45* mRNA and subsequent glycosylation of CD45 protein (Johnson *et al.*, 1989). Two 220,000 molecular weight isoforms of CD45, CD45R and CD45RA, are distinguished by distinct patterns of glycosylation. The CD45R determinant is recognized by the RA3–6B2 antibody while another commonly used antibody, termed 14.8, recognizes the CD45RA determinant. The term ''B220'' has been used interchangeably to describe both molecules. However, because they are distinct and the RA3–6B2 antibody is frequently used, we will use the CD45R designation herein and avoid the B220 terminology.

The various stages of B lymphopoiesis defined by the above cell surface phenotypes also correlate with the status of Ig gene rearrangements (Hardy and Hayakawa, 2001; Pelayo *et al.*, 2005, 2006). Thus, IgHC, but not IgLC, gene rearrangements are actively occurring in CLP, pre-pro-B, and pro-B lymphocytes, and if successful, pro-B lymphocytes mature into pre-B cells, defined by the expression of IgμHC protein in their cytoplasm and the pre-BCR on the cell surface. The pre-BCR is composed of μHC protein associated with the surrogate LC (SLC) proteins VpreB and λ5 and the Igα (CD79a) and Igβ (CD79b) transmembrane proteins. Finally, following productive IgLC gene rearrangement, IgM^{pos} B lymphocytes are produced from pre-B cells (Hardy and Hayakawa, 1995, 2001; Rumfelt *et al.*, 2006).

2.2. Specification versus commitment during B lymphopoiesis

The linear model in Fig. 1 provides a convenient framework for discussing B lymphocyte development from progenitors. However, it is important to note several caveats. First, such diagrams imply that development-associated cell fate decisions occur at abrupt transition points, but this is almost certainly not the case. Instead, as noted for the ELP, cells within a phenotypically defined compartment are not necessarily at the same stage of development. Instead, the precise developmental status of an individual progenitor is determined by the activation of one or more signaling pathways, the concentration of multiple transcription factors, and the expression of various cell surface determinants such as cytokine receptors

(Medina and Singh, 2005), and it is unlikely that these events are synchronized even within a highly purified, phenotypically homogeneous population. A second point is that cells do not lose the potential to generate non-B lineage cells until relatively late in the scheme shown in Fig. 1. For example, while the ultimate fate of CLP is to generate B lymphocytes, non-B lineage developmental programs have not been completely extinguished and they retain the potential to differentiate into T (Allman *et al*., 2003; Kondo *et al*., 1997; Petrie, 2007), natural killer (NK), and dendritic cells (DCs) (Bjorck and Kincade, 1998; Kouro *et al*., 2002; Martin *et al*., 2003; Pelayo *et al*., 2006). Thus, CLPs are most appropriately referred to as B lineage *specified* rather than B lineage *committed* progenitors. Only when NK, DC, and T-cell developmental potential is lost at the pro-B lymphocyte stage, it is appropriate to consider cells as B lineage committed. A third point is that the split between lymphoid and myeloid developmental potential is not necessarily as dichotomous as implied in the diagram. For example, cells with the potential to generate lymphoid and myeloid but not megakaryocyte and erythroid cells have been described (Adolfsson *et al*., 2005). The CMP, despite its name, has been reported to retain residual B lymphocyte developmental potential (Kondo *et al*., 2003), and B lymphocyte-macrophage progenitors are present in the bone marrow (Montecino-Rodriguez *et al*., 2001). Thus, while it may be appropriate to consider the scheme in Fig. 1 as depicting the dominant pathway of blood cell development in the bone marrow, other rare intermediates that do not precisely fit into it exist. Finally, even cells that are committed to a particular lineage retain lineage plasticity as B lymphocytes have been converted to macrophages by expression of C/EBP-α and C/EBP-β transcription factors (Xie *et al*., 2004). While the conversion of a mature B lymphocyte to a macrophage may not normally occur, it could be a factor that underlies malignant transformation. With these points in mind, Fig. 1 provides a framework for describing the various developmental checkpoints as cells become specified and ultimately commit to the B-cell lineage.

2.3. Developmental checkpoints during B lymphopoiesis

The various intermediates between the HSC and newly produced $sIgM^{pos}$ B lymphocyte described above have been isolated and examined at the molecular level. Such studies have made it possible to determine when particular genes that encode signaling molecules, transcription factors, and various cell surface proteins are expressed. Many of these genes have in turn been mutated in genetically engineered mice, and together the information obtained from analysis of these strains has made it possible to define several developmental checkpoints. The picture that emerges from these studies is that the transition from one stage of development to the

next is dependent in part on integration of the activities of various transcription factors that are expressed at precise concentrations in a temporally regulated manner (Hagman and Lukin, 2006; Medina and Singh, 2005; Pelayo *et al*., 2005, 2006).

2.3.1. The HSC to MPP transition

The HSC to MPP transition is distinguished by the expression of the Flt3 cytokine receptor and a diminution of self-renewal potential (Adolfsson *et al*., 2001). These events are dependent on the expression of the Ikaros and PU.1 transcription factors. Ikaros is a member of a family of zinc finger transcription factors that is expressed in all hematopoietic lineages. In contrast to transcription factors that function by promoting gene expression, Ikaros acts to repress gene expression (Cobb and Smale, 2005; Georgopoulos, 2002). PU.1 is a member of the Ets transcription factor family and is expressed in HSC, MPP, and all differentiating cells except those in the erythroid, megakaryocytic, and T lineages (Scott *et al*., 1997). Ikaros (Nichogiannopoulou *et al*., 1999) and PU.1 (Dekoter *et al*., 2002) together regulate Flt3 expression.

Because Ikaros and PU.1 are involved in the development of lymphoid progenitors and each is expressed in HSC and MPP, the question arises as to how development of myeloid precursors such as the CMP occurs. One possibility is that not all HSCs/MPPs, in fact, express both of these factors and that those stem cells which do not enter the myeloid developmental pathway. Another possibility is that the intracellular levels at which these factors are expressed in turn influence the myeloid versus lymphoid fate decision. The latter possibility is suggested by retroviral transduction experiments in which low levels of PU.1 expression induce B lymphocyte development in PU.1$^{-/-}$ progenitors, while high levels suppress B lymphopoiesis and promote macrophage differentiation (Dekoter and Singh, 2000).

2.3.2. The MPP to ELP transition

MPP and ELP are phenotypically overlapping populations defined by a common Lin^{neg} c-kit^{high} Sca-1 Flt3^{pos} phenotype. However, lymphoid specification has clearly initiated in ELP because some of the cells in this population express the RAG genes, terminal deoxynucleotidyl transferase (TdT), an enzyme that inserts N-nucleotides into junctions between Ig variable region genes, and have initiated IgHC D-J$_H$ recombination (Igarashi *et al*., 2002; Medina *et al*., 2001). Also, as noted above, a CD62L^{pos} subset of ELP is T lineage specified.

2.3.3. The ELP to CLP transition

While few ELPs express the IL-7R, its sustained expression denotes the maturation of cells into CLPs. IL-7R expression is dependent on both PU.1 (Dekoter *et al*., 2002; Medina *et al*., 2004) and signaling through the Flt3 receptor (Borge *et al*., 1999). Cells at the CLP stage of development are actively undergoing D-J_H IgHC gene recombination and can be considered to be a B lineage specified population whose ultimate fate is to generate B lymphocytes.

2.3.4. The CLP to pro-B lymphocyte transition

CLPs are not B lymphocyte committed because they still possess T (Allman *et al*., 2003; Kondo *et al*., 1997), NK (Kouro *et al*., 2002), and DC potential (Karunsky *et al*., 2003; Shigematsu *et al*., 2004). This multipotentiality is not due to the presence of distinct lineage progenitors within the CLP population because clonal analyses have shown that these populations derive from single CLP (Kondo *et al*., 1997; Kouro *et al*., 2002). The development of NK cells from CLPs is dependent on expression of various DNA-binding proteins (Lian and Kumar, 2002) that include Ets (Barton *et al*., 1998) and Id2 (Yokota *et al*., 1999), while the maturation of CLP into pro-B lymphocytes is dependent on downregulation of Id proteins and upregulation of *E2A* gene expression (Bain *et al*., 1994; Murre *et al*., 1989; Sun and Baltimore, 1991). The *E2A* gene encodes the E12 and E47 transcription factors, and mice that cannot express E2A proteins have a block in B lymphocyte development at the CLP stage (Zhuang *et al*., 1994). In addition to binding to IgHC gene enhancer regions, E2A proteins regulate the expression of additional B lineage genes that include *mb-1* (which encodes Igα), λ5, *RAG1/2*, and *VpreB*.

E2A proteins, along with PU.1, and signaling through the IL-7R (Medina *et al*., 2004; Seet *et al*., 2004), regulate expression of an additional transcription factor, termed early B lymphocyte factor (EBF) (Hagman and Lukin, 2006). The expression of EBF ensures the adoption of a B lymphocyte fate decision, as EBF-deficient mice exhibit a block in B lymphocyte development (Lin and Grosschedl, 1995). Precisely, how EBF functions remains to be determined, but there is evidence that it promotes demethylation of critical B lineage genes, thus potentiating their expression (Maier *et al*., 2004). These could include Bcl11a (Liu *et al*., 2003) and *Pax5*.

The ultimate commitment of a progenitor to the B lineage is critically dependent on Pax5. Its essential role is illustrated by the fact that phenotypically identifiable pro-B lymphocytes from *Pax5*$^{-/-}$ mice retain the potential for multilineage differentiation. Pax5 functions by blocking expression of non-B lineage genes involved in cell signaling, cell

adhesion, cell migration, transcription, and cellular metabolism at the time of B lymphocyte commitment (Busslinger, 2004; Chiang and Monroe, 1999, 2001; Delogu *et al.*, 2006; Nutt *et al.*, 1999) as well as by activating expression of B lineage genes that include *mb-1* and *cd19*. In addition, Pax5 blocks *Notch1* gene expression, which, as discussed in the following section, blocks the potential for T-cell development.

2.3.5. The pro-B to pre-B lymphocyte transition

The pro-B to pre-B lymphocyte transition is dependent on the successful rearrangement and expression of one of the two IgHC gene alleles. IgHC gene recombination is an error-prone process, and it has been estimated that only one-third of pro-B cells undergoes productive IgHC recombination and mature into pre-B cells (Alt *et al.*, 1987). Pre-B cells are defined by the expression of IgμHC protein in their cytoplasm and the pre-BCR on the cell surface. The pre-BCR consists of μHC, the VpreB and λ5 SLC, and Igα and Igβ (Karasuyama *et al.*, 1996; Melchers *et al.*, 1993). As discussed in more detail in subsequent sections, successful generation of a pre-BCR complex is required for continued developmental progression.

2.3.6. The pre-B to immature B lymphocyte transition

The pre-B to immature B lymphocyte transition is the final developmental checkpoint during bone marrow B lymphopoiesis. While pro-B lymphocytes are cycling cells, the transition to the pre-B lymphocyte stage is associated with a loss of proliferation, cessation of SLC expression, reactivation of the recombinatorial machinery, and conventional IgLC gene rearrangement and expression. The fully assembled form of the BCR, composed of IgHC and IgLC proteins complexed with Igα and Igβ is then expressed on the surface of a newly produced B lymphocyte. At this point, B lymphocytes acquire the ability to recognize polymorphic antigen and become subject to both negative selection to rid the repertoire of potentially autoreactive specificities and positive selection events that may promote maturation into the long-lived peripheral B lymphocyte pool and influence the peripheral B lymphocyte repertoire.

2.4. Selection for functional BCR complexes

Inherent to Ig gene recombination is a high level of unsuccessful recombination attempts that ultimately favor diversity for antigen binding at the expense of generation of progenitors with nonproductive rearrangements or otherwise nonfunctional BCRs. In fact, progression through each of the developmental checkpoints defined above depends in part on the successful recombination of Ig genes, and progenitors that have nonproductive rearrangements are purged. In this section, we will discuss the intermediate BCR assemblies that are interrogated during these

checkpoints as well as outline current thoughts concerning the nature and mechanism of the generated signals that result in the appropriate fate decisions by developing B lymphocytes.

2.4.1. D-J_H recombination

As noted previously, IgHC D-J_H recombination is initiated in very early progenitors such as the ELPs. The observation that IgHC D-J_H recombination can be detected in thymocytes, as well as other hematopoietic cells, argues that D-J_H recombination is a relatively promiscuous event that in itself does not signify B lineage specification or commitment (Kurosawa *et al.*, 1981). Nevertheless, the initiation of an assembly of the BCR is clearly a pivotal event on the road to becoming a B lymphocyte. Interestingly, expression of BCR components, Igα and Igβ occurs early and before IgHC gene recombination is completed, and possibly even before it is initiated (Igarashi *et al.*, 2002). This expression may merely be the consequence of stepwise expression of each BCR component or, rather, may evidence a need for Igα/Igβ signaling at these early stages. It is currently controversial as to whether either signaling protein is expressed on the surface at this stage. However, some studies suggest that they may be brought to the surface in association with the ER protein calnexin (Nagata *et al.*, 1997) and that they are capable of generating signals necessary for developmental progression through the early stages marked by IgHC locus V_H-D-J_H recombination (Gong and Nussenzweig, 1996; Pelanda and Torres, 2006).

2.4.2. V_H-D-J_H recombination

V_H-D-J_H region recombination begins during the pro-B stage. Those progenitor B lymphocytes that successfully navigate the complex genetic processes associated with V_H-D-J_H recombination, resulting in an in-frame coding region, go on to express IgHC on the surface, in combination with the Igα/Igβ heterodimer and SLC proteins λ5 and VpreB (Melchers, 2005). Generation of the pre-BCR complex marks the latest pro-B stage, and signals generated through it trigger changes in gene expression that allow progression through the pro-B to pre-B developmental checkpoint (Schebesta *et al.*, 2002).

Increased responsiveness to IL-7 is associated with the pro-B to pre-B lymphocyte checkpoint in the mouse (Marshall *et al.*, 1998). Pre-BCR-expressing cells undergo multiple rounds of mitosis that are dependent on integration of signals generated through it and the IL-7R (CD127) (Marshall *et al.*, 1998; Rolink *et al.*, 2000). Importantly, proliferation at this stage does not occur with equal efficiency for all clones, as evidenced by a skewing toward preferential expansion of pre-BCR containing distal IgHC V_H regions (Ten Boekel *et al.*, 1997). It is thought that the preference for certain V_H region genes reflects better pairing with SLC and,

ultimately, more efficient assembly of the BCR (Martensson *et al.*, 2002). Therefore, one might predict that more stable associations between SLC and IgHC would result in a pre-BCR able to more effectively generate signals for continued developmental progression and survival. This interpretation in fact appears to be the case as mutations in the CDR3 that generate IgHC variants which pair poorly with SLC and show decreased expression on the late pro-B surface are poorly represented in the peripheral B lymphocyte pool (Wang and Clarke, 2007; Wang *et al.*, 2001). The observation that these IgHCs are compromised with regards to their ability to generate sufficient signals for developmental progression and survival argues for a hierarchy for SLC:IgHC pairing and the ability of pre-BCR to facilitate continued development.

2.4.3. Signaling through the pre-BCR

Data obtained using genetically manipulated mice have indicated a requirement for pre-BCR membrane expression and signaling for continued survival and development of pre-B cells. Loss of BCR expression or function results in abortive maturation and cell death at all stages where it has been assessed. In fact, once initiated, disruption in assembly, expression, or signaling capability results in developmental arrest and cell death (Gauld *et al.*, 2002; Kraus *et al.*, 2004; Lam *et al.*, 1997; Meffre and Nussenzweig, 2002; Meffre *et al.*, 2000).

As with the mature BCR, pre-BCR signaling is dependent on immunoreceptor tyrosine-based activation motifs (ITAMs) (Papavasiliou *et al.*, 1995). Function of the ITAMs as signaling motifs depends on phosphorylation of tyrosine residues located at strictly defined positions (Underhill and Goodridge, 2007). Although still somewhat controversial (Rolli *et al.*, 2002), the initial phosphorylation event is generally believed to be mediated by Src-family protein tyrosine kinases of which Fyn, Lyn, Blk, and in some cases Lck have been shown to be important (Dal Porto *et al.*, 2004; Gauld and Cambier, 2004). Relevant to pre-BCR signaling, single loss mutants of Fyn, Lyn, or Blk have minimal, if any, effect on pre-BCR-dependent development. However, triple $Fyn^{-/-}$, $Lyn^{-/-}$, $Blk^{-/-}$ B lymphocytes exhibit total developmental arrest at the pro-B to pre-B developmental checkpoint (Saijo *et al.*, 2003). While these results indicate a requirement for Src kinases in promoting B lymphocyte development, they do not directly establish that they are involved in pre-BCR signaling. However, gain-of-function experiments have shown that a constitutively active form of Blk can substitute for the pre-BCR when expressed in either $Rag2^{-/-}$ or μMT mice, initiating many of the processes normally triggered by pre-BCR signaling (Tretter *et al.*, 2003). Although these studies implicate Blk as the important kinase at this stage, it remains to be determined if constitutively active forms of Lyn, Fyn, Lck, Hck, or Fgr, each of which is expressed in B lymphocytes, can also substitute for the pre-BCR.

Following tyrosine phosphorylation, ITAMs serve as docking sites for the recruitment and activation of Syk-family protein tyrosine kinases (Futterer *et al.*, 1998; Rolli *et al.*, 2002). Studies have identified roles for both Syk and ZAP-70 family members in pre-BCR signaling. While Syk is the kinase most frequently and consistently associated with ITAM signaling in B lymphocytes, deletion of both *syk* and *zap-70* genes leads to a more profound block in development at the pro-B stage than does deletion of Syk alone (Schweighoffer *et al.*, 2003). Recruitment and activation of Syk is required for recruitment and phosphorylation of B lymphocyte adapter protein BLNK (SLP-65, BASH) (Ishiai *et al.*, 1999). Consistent with this linear pathway, B lymphocyte development in $blnk^{-/-}$ mice is impaired beginning at the pro-B to pre-B transition, although this block is leaky (Jumaa *et al.*, 1999, 2001). Interestingly, among the B lymphocytes that proceed through the block imposed by the BLNK deficiency, some develop into clonal B lymphoma (Flemming *et al.*, 2003).

The leakiness of the block in B-cell development in $blnk^{-/-}$ mice is thought to be due to the ability of other adapter proteins to mediate some of the signaling functions of the pre-BCR. In this regard, there appears to be specific roles for linker of activated T cells (LAT) (Su and Jumaa, 2003) and a scaffolding function of Bruton's tyrosine kinase (Btk) (Middendorp *et al.*, 2005), as loss of these proteins compounds the effects of BLNK deficiency. Therefore, while BLNK-deficient pro-B lymphocytes fail to downregulate RAG1/2 and λ5 expression and initiate IgLC recombination, they nevertheless do upregulate expression of CD25 (which is expressed between the pro-B-cell and pre-B-cell stages) and demonstrate normal allelic exclusion at the IgHC locus (Flemming *et al.*, 2003; Hayashi *et al.*, 2003).

Downstream of Src, Syk, BLNK, and Btk function, the limited available evidence points to the Ras-MAP kinase pathway as a critical regulator of B lymphocyte fate. Expression of dominant-negative Ras protein results in a reduction in the generation of $CD45^{pos}$ B lineage cells (Iritani *et al.*, 1997). However, characterization of these cells indicates a block at the pro-B to pre-B lymphocyte checkpoint that is associated with reduced life span and decreased Bcl-xL expression (Nagaoka *et al.*, 2000). On the other hand, expression of a constitutively active Ras protein in $RAG2^{-/-}$ or $JH^{-/-}$ B cells results in production of a population of B lymphocytes that expresses mature-stage markers CD23 and CD21 (Shaw *et al.*, 1999a,b).

Studies have identified the immediate-early gene-encoded transcription factor Egr-1 as a downstream target of the Ras/Erk pathway in B lymphocytes (Mcmahon and Monroe, 1995). The ability of Egr-1 to drive developmental progression of pro-B-arrested B lymphocytes, when overexpressed in transgenic mice on a RAG2-deficient background, further substantiates the importance of Ras signaling for pre-BCR-dependent development through the pro-B to pre-B transition (Dinkel *et al.*, 1998). However, the ability of Egr-1 alone to facilitate developmental progression

is limited as B lymphocytes in RAG2$^{-/-}$/Egr-1 transgenic mice developed to the CD45^{low} BP-1^{pos} stage (late pro-B), but not to the CD45^{low} BP-1^{pos} CD25^{pos} pre-B stage.

Other studies indicate additional roles for Erk at the pro/pre-B lymphocyte stage beyond that of BCR signaling. Specifically, Erk also appears to play a role in integrated signal transduction between the pre-BCR and IL-7R. A cooperative role for pre-BCR signaling for optimal IL-7 responsiveness of late pro-B lymphocytes is evidenced by the observed enhanced IL-7 induced proliferation by pre-BCR expression compared to non-expressing late pro-B lymphocytes. (Marshall *et al.*, 1998). Subsequent studies revealed that pre-BCR expression and IL-7R signaling led to enhanced Erk expression even at pM levels of IL-7 (Fleming and Paige, 2001). These studies argue that there is a threshold effect of ERK activation required for the proliferation of late pro-B lymphocytes and that signal integration between the pre-BCR and IL-7R may contribute to it. The manner by which these pre-BCR signals are initiated and maintained is at present unclear, but models exist that support both ligand-independent as well as ligand-dependent mechanisms (Fuentes-Panana *et al.*, 2005; Melchers, 2005; Monroe, 2006; Ohnishi and Melchers, 2003).

The ligand dependency of the pre-BCR for generating signals has been the subject of reviews and we refer the reader to these for more details (Burrows *et al.*, 2002; Geier and Schlissel, 2006; Monroe, 2006). Suffice it to note that the mechanism by which the pre-BCR, as well as other Igα/Igβ complexes that lack ability to engage conventional antigen, is able to initiate signals is an area of interest to a number of laboratories. One thought is that Igα/Igβ-containing complexes are able to mediate ''tonic signaling'' independent of antigen or other nonpolymorphic ligands (Fuentes-Panana *et al.*, 2005; Monroe, 2006). These signals have been shown to be sufficient to drive maturation of progenitors to the mature FO, but not MZ or B-2, stage of development (Fuentes-Panana *et al.*, 2006).

2.4.4. IgLC recombination

Progression to the late pre-B stage at which IgLC recombination is initiated is presumably regulated by signaling pathways in addition to Ras/Erk/Egr-1. In this regard, Muljo and Schlissel (2003) observed that pharmacological inhibition of Abelson protein tyrosine kinase (Abl) leads to RAG1/2 induction and the initiation of IgLC recombination. These data argue that regulation of Abl activity may play a role in continued development of B cells through the pre-B stage. These studies should be interpreted with some caution at this point, however, because the experimental design focused on the inhibition of v-Abl, which may act differently from c-Abl. Nevertheless, in these studies, DNA microarray analysis of Abl-transformed pre-B cell lines identified *Spi-B* and *IRF-4* as

Abl-suppressed target genes. These targets are provocative based on studies that show Spi-B and IRF-4 to be IgLC enhancer binding proteins (Brass *et al.*, 1999; Eisenbeis *et al.*, 1995; Escalante *et al.*, 2002).

Both Spi-B and IRF-4 appear to play a role in pre-BCR-dependent development, as *PU.1*$^{+/-}$, *Spi-B*$^{-/-}$ mice have significant reductions in the number of bone marrow immature B lymphocytes (Hu *et al.*, 2001), and *IRF-4,8*$^{-/-}$ mice exhibit developmental arrest at the cycling pre-B stage (Hardy Fraction C′) (Lu *et al.*, 2003). Perhaps of relevance is the observation that Spi-B is together with PU.1 necessary for regulating the expression of *c-rel* (Hu *et al.*, 2001). Reintroduction of c-Rel expression or forced expression of Bcl-2 phenocopied the *Spi-B*$^{-/-}$*/PU.1*$^{+/-}$ background. Specifically, both had restored normal B-cell numbers, suggesting that the importance of Spi-B and downstream c-Rel expression is for enhanced survival or early stage B cells.

The developmental block at the cycling pre-B stage observed in *IRF-4/8*$^{-/-}$ mice (Lu *et al.*, 2003) appears coincident with failure to downregulate pre-BCR expression and is associated with elevated levels of SLC and pre-BCR as well as impaired IgLC transcription and recombination. What may link Abl-inhibition and the *IRF-4/8*$^{-/-}$ phenotypes is that downregulation of Abl activity is necessary for release of suppression of *Spi-B* transcription. This in turn may then suppress SLC transcription and pre-BCR expression leading to RAG1,2 induction and the initiation of IgLC recombination and expression. The phenotypes of the *Spi-B*$^{-/-}$*/PU.1*$^{+/-}$ and *IRF-4/8*$^{-/-}$ mice together with the studies demonstrating increased Spi-B and IRF-4 expression and an associated increase in RAG1,2 expression and IgLC rearrangement suggest that downregulation of v-Abl effector pathways, such as JAK/Stat activity, is necessary to relieve the transcriptional repression of *Spi-B* and *IRF-4*. This in turn allows for RAG1,2 induction, the initiation of IgLC recombination, and the suppression of SLC transcription and consequently pre-BCR expression, respectively.

Signals generated by the pre-BCR shut down continued recombination at the alternative IgHC locus (allelic exclusion), facilitate positive selection past the pro-B → pre-B checkpoint, integrate with IL-7R signals to facilitate clonal expansion, and initiate recombination at the IgLC locus (Grawunder *et al.*, 1995; Kitamura and Rajewsky, 1992; Reth *et al.*, 1987). Like BCR-induced signals, signals generated by the pre-BCR may be ''tuned'' by association with positive and negative coreceptors. For example, ''tuning'' that modulates the strength and efficiency of pre-BCR function can be mediated by CD19, a well-established positive coreceptor for BCR signaling. Along these lines, Otero and Rickert (2003) have shown that pro/pre-B lymphocytes isolated from *CD19*$^{-/-}$ mice that were maintained *in vitro* in the presence of IL-7 showed reduced Btk activity and reduced pre-BCR/IL-7-dependent proliferation, compared to their CD19-sufficient counterparts.

2.5. The hematopoietic microenvironment

The above discussion has documented the cell-intrinsic patterns of gene expression necessary for the development of B lymphocytes in the bone marrow. To what degree patterns of gene expression are affected by signals from the hematopoietic microenvironment remains an issue. In order to set the stage for a discussion of this issue, the anatomical organization of the bone marrow and the mechanisms by which signals from it may impact B lymphocyte development are briefly presented.

Hematopoiesis takes place in the intersinusoidal spaces of the medullary cavity, and the anatomy of this region is best understood in the context of the circulatory supply to bone. Bone is a highly vascular tissue, and the arteries that supply blood to it ultimately form an anastomosis at its endosteal surface. From that point, multiple venous sinusoids course toward the center of the medullary cavity, wherein they join together to form a central vein whose tributaries ultimately exit the bone. HSCs are located at the endosteal surface in association with osteoblasts in what is referred to as the stem cell niche (Adams and Scadden, 2006). HSCs destined to generate B lymphocytes, or other blood cell lineages, ultimately exit from this niche and associate with a population of sessile stromal cells that form a three-dimensional network between the venous sinusoids (Dorshkind, 1990; Nagasawa, 2006).

One way in which stromal cells regulate blood cell development is through the secretion of various cytokines that affect progenitor cell growth, differentiation, and/or survival. For example, targeted disruption of the genes encoding CXCL12 or its receptor CXCR4 disrupts fetal B lymphocyte development, and *CXCR4*$^{-/-}$ fetal liver cells do not efficiently generate B lymphocytes, following transfer into normal recipients. The precise role played by CXCL12/CXCR4 in B lymphocyte development is not fully defined, but it may be involved in homing and retention of hematopoietic progenitors in the bone marrow (Egawa *et al*., 2001; Nagasawa *et al*., 1996; Zou *et al*., 1998). The binding of Flt3 ligand (FL) to the Flt3 receptor is critically important for normal B lymphopoiesis, as the number of pre-pro-B and pro-B lymphocytes is reduced in FL and Flt3-deficient mice (Mackarehtschian *et al*., 1995; Sitnicka *et al*., 2003). For example, *FL*$^{-/-}$ mice have a tenfold reduction in the frequency of CLP as compared to wild-type mice. However, Lin^{neg} c-kit^{low} Sca-1^{low} IL-7R^{neg} cells are present, albeit at reduced frequency compared to wild-type mice, and bone marrow cells from *FL*$^{-/-}$ donors can repopulate B lineage cells in the bone marrow and peripheral blood of wild-type recipient mice to the same extent as cells from wild-type donors. Finally, murine (but not human) B lymphocyte development is absolutely dependent on the presence of stromal cell-derived IL-7 and the expression of its receptor on developing B lineage cells (Peschon *et al*., 1994; Von Freeden-Jeffry *et al*., 1995).

In addition to potentiating growth and survival of progenitors, IL-7 plays an important role in IgHC gene rearrangements and the recombination of variable (V)-region genes with a rearranged D-J_H complex (Corcoran *et al.*, 1998).

A second general means by which stromal cells regulate B lymphopoiesis is through direct cell contact. For example, both murine and human pre-B lymphocytes express the VLA-4 integrin that interacts with a stromal cell ligand identified as vascular cell adhesion molecule-1 (VCAM-1). VLA-4 also promotes binding to fibronectin, an extracellular matrix protein. CD44 on developing B lineage cells also has been implicated in mediating stromal cell–lymphocyte interactions in the mouse through binding to stromal cell-derived hyaluronate (Dorshkind, 1990; Kincade *et al.*, 1989; Nagasawa, 2006).

Pre-pro-B and pro-B lymphocytes are uniformly distributed throughout the medullary cavity, indicating that there is no single B lineage microenvironment. Nevertheless, pre-pro-B lymphocytes tend to be associated with CXCL12^{pos} IL-7^{neg} stromal cells, while pro-B lymphocytes associated with CXCL12^{neg} IL-7^{pos} stroma. Together, these data suggest the existence of distinct cellular niches that regulate the growth, differentiation, and/or survival of developing B lineage cells (Tokoyoda *et al.*, 2004). The question, which has long been debated, is whether these niches provide permissive or deterministic environments.

2.6. Permissive versus deterministic models of B lymphopoiesis

There are two general ways of thinking about how signals from the hematopoietic microenvironment affect B lymphopoiesis. One is that they *determine* the fate choice that will be adopted by a progenitor. The other is that they merely provide signals that *permit* cell-intrinsic developmental programs to progress. Arguments have been made for both the deterministic and permissive regulation of hematopoiesis (Enver *et al.*, 1998; Metcalf, 1998). One reason why the debate over which mechanism is correct has lingered for several decades is that data can often be interpreted as supporting either model. For example, on injection of Lin^{neg} c-kit^{low} Sca-1^{low} IL-7R^{neg} cells from *FL*$^{-/-}$ mice into normal recipients, CLPs that express the IL-7R as well as their downstream progenitors are produced. One interpretation of these data is that FL in the recipient induced the expression of the IL-7R and cells were then able to further mature. In this case, FL would be acting as a deterministic factor. However, FL can promote CLP survival (Sitnicka *et al.*, 2002), thereby permitting progression through an intrinsically programmed scheme. Similarly, the actions of IL-7 can be interpreted as either allowing cells to survive and progress through a preprogrammed developmental program or as a deterministic factor. Thus, although it is difficult to conclude how

cell-extrinsic influences are acting, the burden of proof in this debate seems to fall on the side of providing evidence that the environment can function in a deterministic manner.

One study of relevance in this regard involved the transduction of CLP with a retrovirus containing a functional human granulocyte-macrophage receptor (GM-CSFR). Following culture of these cells in the presence of GM-CSF for 2 days, GM-containing colonies were generated from the transduced but not wild-type CLPs (Kondo *et al.*, 2000). Thus, an interpretation of this finding is that expression of the GM-CSFR directed the development of myeloid cells from a B lineage-specified progenitor.

The role played by the Notch1-signaling pathway arguably provides the strongest case that hematopoietic fate choices can be deterministic. An increasingly accepted model is that the decision of a precursor to generate T or B lineage progenitors is regulated by activation of the Notch1-signaling pathway (Busslinger *et al.*, 2000; Macdonald *et al.*, 2001). Notch1 is a transmembrane receptor, and following activation by ligand, its intracellular domain is cleaved and translocates to the nucleus, wherein it interacts with two other transcriptional regulators, Mastermind and RBJ-k, to regulate transcription (Artavanis-Tsakonas *et al.*, 1999; Weinmaster, 2000).

Two key studies are largely responsible for proposing Notch1 as a regulator of T versus B lymphocyte lineage commitment. Pui *et al.* (1999) transplanted bone marrow cells transduced with a constitutively active form of Notch1 into recipient mice and demonstrated that the donor cells could generate T, but not B, lymphocytes. These results are consistent with findings of Radtke *et al.* (1999) who demonstrated that conditional inactivation of Notch1 results in an early block in T, but not B, lymphocyte development. It is difficult to interpret these data in any manner other than the environment determining B versus T lymphocyte cell fate.

Notch1 is expressed by pre-pro-B, but not pro-B, lymphocytes. Its absence in pro-B lymphocytes is consistent with the observation that Pax5 blocks Notch1 expression (Hofliner *et al.*, 2004). However, the question is how a pre-pro-B lymphocyte could ever become a pro-B cell, given that it is $Notch1^{pos}$ and Notch ligands are expressed by stromal cells. One possibility is that not all pre-pro-B lymphocytes express Notch1, and these are the populations from which B lymphocytes are generated. Even if Notch1receptors are expressed in lymphoid precursors, they may not be activated due to the coexpression of Notch-modulating factors such as Fringe (Irvine, 1999; Pourquie, 1999; Wu and Rao, 1999) or Numb (Campos-Ortega, 1996). While these possibilities cannot be excluded, immunohistochemical evidence supports a model in which pre-pro-B lymphocytes are associated with stromal cells that do not express the Notch ligands Delta-1 and Jagged-1 (Tokoyoda *et al.*, 2004), and as a result, they do not receive T lineage-potentiating signals and instead adopt a B lineage fate.

The discussion of how hematopoiesis is regulated will likely continue. Further complicating the issue is that paradigms which may hold true for the most immature HSCs and progenitors might not be applicable to more developmentally restricted progenitor cells. Thus, a middle ground position is that stochastic mechanisms may be operative in the most primitive hematopoietic precursors while actual lineage commitment of progenitors is a regulated phenomenon. In such a ''have it both ways'' model, one could envision of pool of primitive HSCs in which gene activation or repression occurs randomly. Such cells would be primed to develop along a particular lineage. Their subsequent maturation would then be ''determined'' by their localization to a specific microenvironmental niche that provides the signals necessary for development in that lineage. In the absence of exposure to those signals, maturation of the ''lineage-primed'' HSC would be blocked.

2.7. Maintenance of early B lineage fate

In addition to the dependence of B lymphopoiesis on the presence of the various transcription factors described previously, their sustained expression is needed to maintain the cell fate decision. For example, studies in which Pax5 expression has been conditionally inactivated in pro-B lymphocytes demonstrated that cells reacquired macrophage and T lineage potential (Mikkola *et al.*, 2002) and that the continued repression of non-B lineage genes requires continuous Pax5 activity (Delogu *et al.*, 2006). Taken together, these observations indicate that maintenance of lineage commitment is not a passive process.

2.8. Perturbations affecting primary B lymphopoiesis

The developmental scheme described in the previous sections applies to B lymphopoiesis in young, healthy individuals. However, this pattern of B lymphocyte development is disrupted during aging and following infection with pathogens.

2.8.1. Effects of aging on B lymphocyte development

It is now well recognized that B lymphocyte development in old mice does not occur at the same level as in younger animals (Linton and Dorshkind, 2004). For example, both the frequency and absolute number of CLP, pre-pro-B, pro-B, and pre-B lymphocytes are significantly reduced in 18-month-old mice (Miller and Allman, 2003; Min *et al.*, 2006). This diminution of B-cell production has been associated with multiple factors.

There is evidence that the hematopoietic microenvironment does not efficiently support B lymphocyte development (Labrie *et al.*, 2005), in part

because of defects in IL-7 production (Stephan *et al.*, 1998). Cell autonomous changes that affect developing B lineage cells have also been documented. In addition to the finding that CLP, pre-pro-B, and pro-B lymphocytes from old mice do not proliferate as efficiently as their young counterparts (Miller and Allman, 2003; Min *et al.*, 2006), the ability of these cells to mature and transition between stages is also compromised by aging. For example, CLPs from old mice do not efficiently mature into pre-pro-B lymphocytes (Min *et al.*, 2006). The precise molecular basis for these age-related defects has not been defined, but there are reports that the levels of E2A protein are decreased in pro-B lymphocyes of old mice (Frasca *et al.*, 2003). Given the pivotal role of this transcription factor during B lymphopoiesis, the lower levels of this factor may explain why B lymphocyte development is diminished, but not blocked, during senescence.

While there is no doubt that fewer B lineage cells are produced in old mice and that this is due in part to cell-intrinsic changes, whether aging affects progenitor fate decisions is less clear. The analysis of the HSC to ELP transition in old mice is illustrative of this point. Allman and colleagues have proposed that the frequency of ELP is reduced in old mice (Miller and Allman, 2005). One explanation for this decline is that ELPs are produced with the same efficiency in young and old animals, but that reduction in their number results from age-related defects that accumulate in ELP following their generation. Another nonmutually exclusive alternative is that with increasing age the potential for HSC to generate lymphoid-specified progeny declines. Whole genome microarray analysis on purified HSC from young and old mice provides support for this latter hypothesis. This study revealed that genes involved in lymphoid specification, which were surprisingly expressed in young HSC, were significantly downregulated in HSCs from old animals (Rossi *et al.*, 2005). In this case, an argument can be made that aging affects B lineage fate decisions at the earliest stages of hematopoietic development.

2.8.2. Infection and primary B lymphopoiesis

A second perturbation that alters the tempo of B lymphopoiesis is exposure to pathogens (Ueda *et al.*, 2004, 2005). The bone marrow, like the thymus, is a primary lymphoid organ, and the traditional assumption has been that, by definition, cell production in it proceeds at a constant rate that is not influenced by extramedullary influences. However, studies indicate that bone marrow B lymphopoiesis is suppressed while myelopoiesis is increased by inflammation (Ueda *et al.*, 2004, 2005). This effect is mediated via the binding of various inflammatory mediators to Toll-like receptors (TLRs) that are expressed on HSCs and progenitor cells. The TLRs comprise a family of cell surface and cytoplasmic receptors that recognize conserved bacterial and viral determinants. Recent work has

demonstrated that HSCs express TLRs that include TLR2 and TLR4-MD-2 and that their activation promotes myelopoiesis. TLR-mediated signals also affect the developmental potential of B lineage-specified progenitors such as the CLPs, which are induced to generate DCs at the expense of B lymphocytes on ligand binding (Nagai *et al*., 2006).

There are two nonmutually exclusive means by which the effects of TLR signaling may be mediated. As noted by Nagai *et al*. (2006), binding of ligand to TLRs on HSC or CLP could suppress lymphoid developmental programs. Alternatively, they may activate granulocyte, macrophage, and DC developmental programs. At the very least, these data necessitate a reevaluation of classical models indicating that hematopoiesis is impervious to external influences. In addition, these observations suggest that exogenous agents that bind to TLRs are important fate-determining factors that stimulate the ''real-time'' generation of specific types of pathogen-responsive effectors (Holl and Kelsoe, 2006).

3. DEVELOPMENT OF IMMATURE B CELLS IN THE BONE MARROW AND PERIPHERY

Following productive IgHC and IgLC gene rearrangements, the Ig molecule is assembled and the BCR is expressed on the surface of newly produced B cells. Strikingly, of the $\sim 2 \times 10^7$ BCR^{pos} B cells that develop daily in mouse bone marrow, only ~10% exit to the periphery and only a third of these progress to the mature B lymphocyte pool (Osmond, 1993). The dramatic loss of B lymphocytes following production is due in part (together with competition) to negative selection of self-reactive B lymphocytes that directs them toward an apoptotic cell fate decision (Norvell and Monroe, 1996; Norvell *et al*., 1995).

This sensitivity to apoptosis, along with distinctive phenotypic characteristics, is characteristic of these newly produced B lymphocytes which have not yet acquired the functional potential of mature splenic B lymphocytes. Allman *et al*. (1992, 1993) first noted that the recent B lymphocyte emigrants from the bone marrow into the spleen exhibited similar functional and phenotypic characteristics, and termed these peripheral, immature B lymphocytes ''transitional B lymphocytes'' to indicate their position in the maturational progression linking bone marrow immature to splenic mature B lymphocytes.

Several groups have subdivided transitional B lymphocytes into two separate subpopulations termed transitional 1 (T1) and transitional 2 (T2) cells based on surface phenotype and functional characteristics (Allman *et al*., 2001; Chung *et al*., 2002; Loder *et al*., 1999; Su and Rawlings, 2002) (Fig. 1). T1 B lymphocytes can be distinguished by their IgM^{high} IgD^{neg} $CD21^{neg}$ $CD23^{neg}$ phenotype and appear to be excluded from the splenic

FO region (Chung *et al*., 2002; Loder *et al*., 1999; Schiemann *et al*., 2001). T2 B cells retain high levels of sIgM but are also IgD^{pos} $CD21^{pos}$ $CD23^{pos}$. Unlike T1 cells, T2 B lymphocytes have migrated into the splenic B lymphocyte follicles (Chung *et al*., 2002; Loder *et al*., 1999; Schiemann *et al*., 2001). All immature and transitional B lymphocytes can be distinguished from the mature compartment by their relatively higher expression of CD93 that is generally monitored using the AA4.1 or 493 antibodies.

There is more or less general agreement concerning the markers used to separate the subpopulations though some differences exist in the number of subgroups and in the functional characteristics of the T2 population (King and Monroe, 2000; Sater *et al*., 1998; Su *et al*., 2004). For example, some studies indicate that, like bone marrow immature and T1 B lymphocytes, T2 B lymphocytes do not proliferate in response to BCR engagement *in vitro* and are instead directed toward an apoptotic fate (Allman *et al*., 2001; Chung *et al*., 2002). However, other studies suggest that T2 B lymphocytes are able to proliferate and are resistant to apoptosis (Petro *et al*., 2002; Su and Rawlings, 2002). In the latter case, upregulation of prosurvival proteins in the T2 population was documented (Su and Rawlings, 2002). The reason for these differences remains enigmatic and could reflect the fact that the T2 population consists of distinct subsets of cells. In this regard, studies identified a T2-like B lymphocyte as an FO mature-derived precursor to an MZ B lymphocyte (Srivastava *et al*., 2005). Thus, the BCR-responsive T2 B lymphocytes that undergo proliferation may represent an intermediate between FO mature and MZ B lymphocytes.

It has been generally accepted that T1 and T2 populations represent requisite precursors to the FO mature B lymphocyte subset in the spleen and that they are present only in the periphery. However, recent studies suggest these clear relationships and necessary localization to peripheral compartments may be more fuzzy. Although it is likely that the T1 → T2 → mature FO relationship is probably the most common progression, some B lymphocytes may reach the T1 and T2 stages while still in the bone marrow (Lindsley *et al*., 2007), and mathematical modeling based on the kinetics of movement through each compartment suggests that, in some cases, the T1 stage may be bypassed (Shahaf *et al*., 2004). Regardless, the opportunity to sample bone marrow and peripheral self-antigens and thereby identify self-reactive B lymphocytes during the immature stage would still exist in all models.

Allman *et al*. (2001) described a potential third nonproliferating transitional subset, termed T3, which resembles T2 cells with the exception of a lower level of sIgM. Its assignment to the transitional compartment and positioning within a stepwise maturation toward the mature compartment remains to be determined. The lower IgM levels on the putative T3 population also suggest that it could represent a population resulting

from exposure to antigen and, therefore, represent T2 cells that are being positively selected into the mature pool or, alternatively, on their way to deletion or anergy. Studies of Merrell *et al*. (2006) documented that the T3 subset of splenic BCR transgenic monoclonal B lymphocytes is maintained by the continued presence of antigen and represent an apparently antigen-induced unresponsive population. Of relevance, these authors also observed that, in non-BCR transgenic mice, this population is typified by a high frequency of self-reactive B lymphocytes.

Although less well studied than their murine counterparts, transitional B lymphocytes have also been defined in the human (Carsetti *et al*., 2004; Ghia *et al*., 1996; Sims *et al*., 2005). Like murine transitional immature B lymphocytes, responses of human transitional B lymphocytes to BCR stimulation are characterized by decreased proliferative capacity and decreased survival.

4. BCR-DEPENDENT SIGNALING AND FATE DECISIONS BY BONE MARROW AND PERIPHERAL IMMATURE B LYMPHOCYTES

Transitional B lymphocytes retain functional characteristics of their bone marrow precursors. Both populations are short lived (Allman *et al*., 1993) and respond to BCR signaling *in vitro* by apoptosis (Norvell *et al*., 1995; Sandel and Monroe, 1999). Both *in vivo* and *in vitro* studies have established that it is within the developmental windows comprising the immature and transitional B lymphocyte that negative selection of self-reactive B lymphocytes occurs (see the following in-depth reviews of this topic: Nemazee, 2006; Pelanda and Torres, 2006) (Fig. 2).

4.1. Biochemistry of BCR-induced fate decisions during negative selection

The organization of the signaling complex (signalosome) organized as a consequence of antigen-induced aggregation of the BCR on mature B lymphocytes is illustrated in Fig. 3. Transitional and mature B lymphocytes exhibit a differential response to BCR signaling, and some of these differences are thought to be the consequence of differences in the organization or stability of this signalosome. Some of these differences are outlined in the following sections.

4.1.1. The PIP_2/PKCβ pathway

Mature B lymphocytes respond to BCR cross-linking by increasing phosphatidylinositol 4,5 bisphosphate (PIP_2) hydrolysis and elevating intracellular calcium levels. Activation of this pathway appears to be more

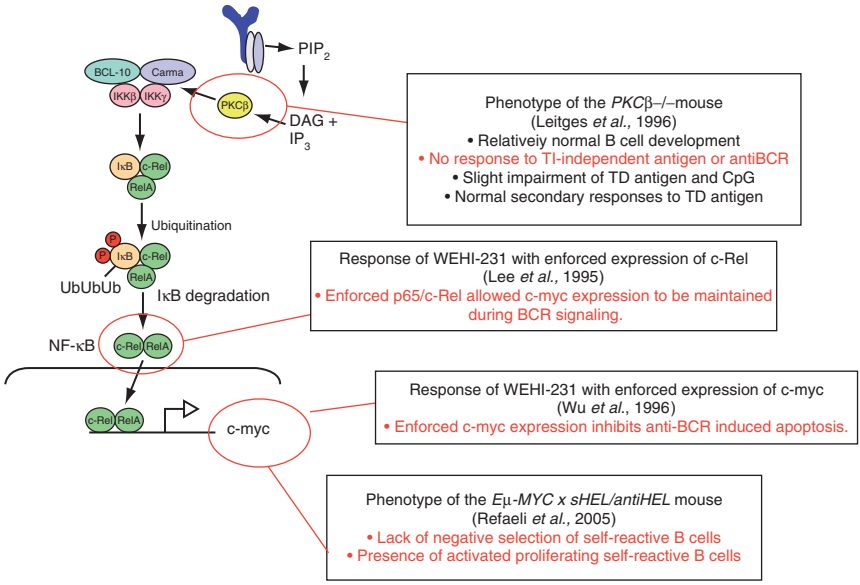

FIGURE 2 Proposed pathway establishing the fate decisions associated with the differential antigen responsiveness of mature and immature B lymphocyte subsets. Highlighted are points where genetic studies have directly linked a component of the pathway to responsiveness and/or tolerance sensitivity.

transient in immature B lymphocytes (Benschop *et al.*, 2001; Karnell *et al.*, 2005; Yellen *et al.*, 1991), and it has been postulated that the relative inability of transitional cells to activate diacylglycerol (DAG)-responsive protein kinase C (PKC) isoenyzmes may be responsible for BCR apoptosis.

PIP_2 hydrolysis and activation of PKCβ in BCR-stimulated B lymphocytes appears to be associated with a linear pathway involving the canonical NF-κB pathway and induction of *c-myc* transcription (Fig. 2). Several lines of evidence directly link this pathway to the regulation of BCR-induced B lymphocyte fate decisions at the immature/transitional immature stage. First, while B lymphocyte development in $PKC\beta^{-/-}$ mice is relatively normal, splenic B lymphocytes in these mice are unresponsive to thymus-independent antigens and to *ex vivo* anti-BCR stimulation (Leitges *et al.*, 1996). Since responses to either stimuli depend on sustained BCR signaling, these results suggest that activation of PKCβ is required for sustained signaling and supports the idea that the relative inability to do so accounts for the signaling phenotypes observed in immature and transitional immature B lymphocyte. Accordingly, pharmacological inhibition of PKCβ *in vitro* redirects anti-BCR-triggered responses toward apoptosis in mature FO splenic B lymphocytes, and sustained activation of PKCβ in transitional immature B lymphocytes blocks BCR-induced

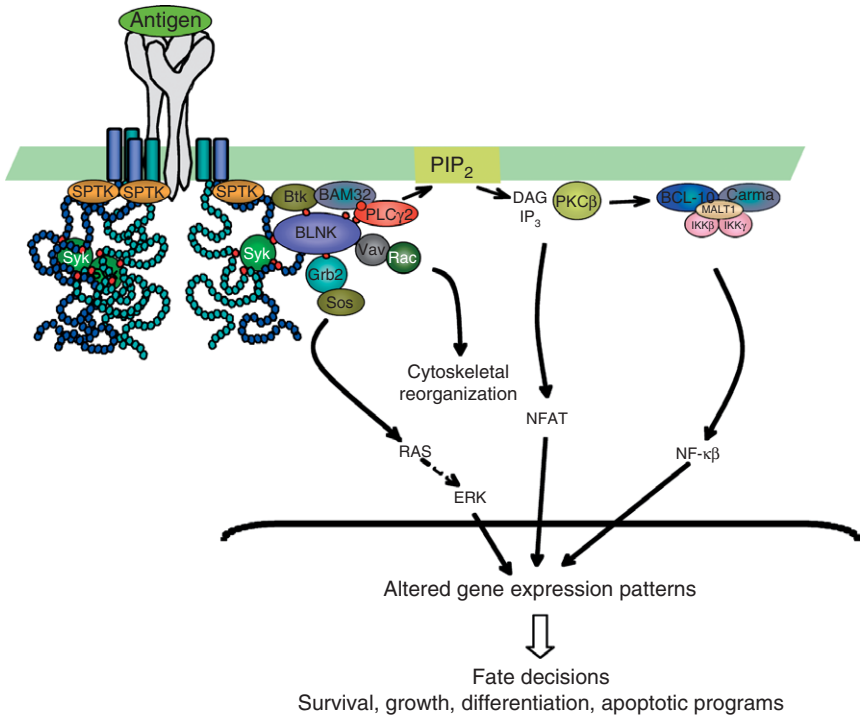

FIGURE 3 Diagram of the composition of the BCR signalosome following antigen-induced BCR aggregation and organization into lipid rafts compartment in mature B lymphocytes. As discussed in the text, differences in organization and/or stability of the signalosome are predicted by available data.

apoptosis (King *et al.*, 1999). Together, these genetic and biochemical approaches indicate that the fate of BCR-stimulated B lymphocytes can be redirected by manipulation of PKCβ activity, indicating that this kinase is pivotal in determining the fate of the B lymphocyte to antigen stimulation.

PKCβ has been shown to participate in the activation and degradation of IκB kinase (IKK) and generation of nuclear c-Rel/RelA complexes (Saijo *et al.*, 2002; Shinohara *et al.*, 2005; Sommer *et al.*, 2005) that in turn have been shown to be critical for B lymphocyte homeostasis and survival (reviewed in Gerondakis and Strasser, 2003). B lymphocytes from *c-rel*$^{-/-}$ mice respond in a manner similar to transitional B lymphocytes in that they fail to proliferate to anti-BCR and BCR signaling, which in turn leads to apoptosis. As with transitional B lymphocytes (Sater *et al.*, 1998), CD40 signaling restores BCR-induced survival and proliferation in *c-rel*$^{-/-}$ B lymphocyte. The immature B lymphocyte line WEHI-231 responds to BCR signaling

by cell cycle arrest and apoptosis coincident with downregulation of *c-myc* expression (Fischer *et al.*, 1994). Enforced expression of c-Rel/p65 maintains *c-myc* expression during anti-BCR stimulation (Lee *et al.*, 1995), and enforced expression of *c-myc* blocks anti-BCR-induced apoptosis (Wu *et al.*, 1996). Refaeli *et al.* (2005) generated mice constitutively expressing *c-myc* in B lymphocytes under the control of the Eμ promoter. When these animals were crossed onto mice transgenic for anti-HEL BCR and soluble HEL antigen, the investigators noted the presence of activated, autoreactive B lymphocytes that were normally absent due to anergy or deletion in mice in which *c-myc* was not expressed constitutively.

Taken together, these studies argue that there is a more or less linear pathway initiated by BCR-induced PIP_2 hydrolysis and DAG-activated PKCβ, leading to NF-κB activation and sustained *c-myc* induction, thereby directing BCR signaling toward a prosurvival and proliferative fate decision. By extension, failure to sustain signaling through this axis is expected to have negative effects on the ability of BCR signals to promote survival and proliferative responses consistent with the response phenotype of immature B lymphocytes.

4.1.2. Plasma membrane cholesterol and lipid rafts

The relative inability of transitional immature B lymphocytes to sustain PIP_2 hydrolysis may reflect a difference in the organization of supramolecular-signaling complexes that form in response to antigen receptor ligation. Mature B lymphocytes copolarize the BCR with lipid rafts following BCR aggregation, whereas in transitional B lymphocytes and immature B lymphocyte lines, anti-BCR treatment leads to receptor aggregation without colocalization with lipid rafts (Chung *et al.*, 2001; Sproul *et al.*, 2000). PIP_2 is preferentially located within lipid rafts, suggesting that the lack of spatial access to PLCγ substrates may mediate the observed differences in PIP_2 hydrolysis.

The requirement for recruitment and stable organization of signal transduction effectors such as the Src-family protein tyrosine kinases Lck, Lyn, and Fyn (Casey, 1995), the Zap-70-family protein tyrosine kinase Syk (Stauffer and Meyer, 1997), and even lipids such as PIP_2 (Pike and Casey, 1996) into lipid rafts, after receptor ligation to promote their activation and subsequent downstream-signaling events (Cheng *et al.*, 1999; Stauffer and Meyer, 1997), argues that lipid rafts provide another level of organization for these related signaling proteins. The findings that transitional B lymphocytes do not stably compartmentalize the BCR within lipid rafts following BCR-mediated stimulation and that they display deficits in PIP_2 hydrolysis indicate that the proper assembly and spatial organization of the BCR signalosome is an acquired phenotype associated with B lymphocyte maturity and may be a required characteristic for selection into the long-lived mature B lymphocyte pool.

Recent studies have identified differences in plasma membrane cholesterol levels between transitional and mature B lymphocytes. Furthermore, studies in which cholesterol levels were augmented in transitional B lymphocytes to levels comparable to those in mature B lymphocytes suggest that these differences are responsible for both the differential ability to stably organize BCR into lipid rafts and relative ability to maintain signaling pathways associated with PI-hydrolysis and PKCβ activation (Karnell *et al.*, 2005; Monroe, 2005). The mechanisms regulating the developmental difference in cholesterol levels are unknown but could reflect variations in cholesterol uptake and/or biosynthesis by B lymphocytes. The implications of these results are profound, for they indicate that developmental-related differences in plasma membrane cholesterol levels affect the ability to organize lipid rafts and point to potential risk factors for B lymphocyte autoimmune diseases. For example, genetic or metabolic defects that alter serum cholesterol levels, cellular uptake, or cholesterol biosynthesis could lead to elevations in the cholesterol content of the plasma membrane of immature or transitional B lymphocytes. If membrane cholesterol did increase, immature B lymphocytes might then be able to organize lipid rafts, imparting on them a more mature B lymphocyte-signaling phenotype that could compromise their sensitivity to negative selection, thereby affecting the elimination of self-reactive B lymphocytes in the bone marrow and/or periphery.

4.2. Alternative BCR-induced fate decisions

4.2.1. Receptor editing

In vitro studies using purified isolates of bone marrow immature or splenic transitional B lymphocytes and anti-BCR as surrogate antigen indicate that the hardwired response of immature B lymphocytes to BCR signaling is apoptotis (King and Monroe, 2000). These results suggest that in the absence of B lymphocyte extrinsic influences, the default fate decision of immature B lymphocytes to antigen (at least to strong antigenic signaling) would be deletion. However, studies by several laboratories have suggested that high-avidity interactions with antigen by immature B lymphocytes in the bone marrow do not necessarily lead to deletion, but instead may result in replacement of the BCR by a process termed receptor editing (reviewed in Nemazee, 2006; Pelanda and Torres, 2006) or in some cases, activation with the potential of recruitment into an ongoing immune response.

During receptor editing, the expression of RAG1/2 is extended and the time spent at the pre-B → immature window is prolonged, thereby facilitating continued IgLC gene rearrangement (Casellas *et al.*, 2001). In some instances, secondary IgHC gene rearrangements can occur, although IgLC rearrangement appears more common. LCs produced during the

secondary rearrangement can replace the original LC in an attempt to eliminate antigen reactivity. Since the majority of BCRs generated at this stage appear to be self-reactive (Wardemann *et al.*, 2003), the rationale for receptor replacement in this case is presumed to be an attempt to eliminate self-reactivity. Studies in non-Ig transgenic mice have placed estimates of the extent of receptor editing to as high as 25% of the peripheral mature B lymphocyte pool (Casellas *et al.*, 2001), indicating this to be a fairly common response to BCR signaling in the bone marrow.

While receptor editing could potentially play a role in maintaining self-tolerance, it is difficult to reconcile how a BCR-induced signaling event can lead to receptor editing in cells that have presumably also received a signal to undergo BCR-induced apoptosis. In particular, *in vitro* studies indicate that processes directing the cell toward an apoptotic fate are initiated within 20 min of BCR engagement (Sater *et al.*, 1998), arguing that the available time to replace the self-reactive receptor and thereby terminate signaling would be extremely brief and likely insufficient to generate a nonreactive BCR.

Work of Fang *et al.* (1998) indicated that transgenic overexpression of Bcl-xL in BCR transgenic mice protected self-reactive B lymphocytes from deletion and redirected the responses toward anergy and receptor editing. These observations support the idea that if immature-stage B lymphocytes in the bone marrow can be protected from BCR-induced apoptosis, they can be open to alternative fate decisions that alter the mechanism of tolerance induction. Studies by Sandel and Monroe (1999; Sandel *et al.*, 2001) extended this idea by identifying a cellular constituent in the bone marrow that could *in vitro* protect transitional B lymphocytes from anti-BCR-induced apoptosis. In this case, the immature B lymphocyte apoptotic response to BCR engagement was redirected toward continued LC recombination in the presence of unfractionated whole bone marrow. This effect was shown to be bone marrow intrinsic, as the relevant cellular entity could not be detected in peripheral compartments (Sandel and Monroe, 1999) including germinal centers (Sandel, P. and Monroe, J. G., unpublished data). This second observation fits with earlier studies of Carsetti *et al.* (1995) who showed that hapten-reactive immature B lymphocytes are deleted only in the periphery, despite expression of antigen in the bone marrow, suggesting that the fate decision between deletion and receptor editing might be influenced by the location at which antigen encounter occurs.

4.2.2. Interactions between transitional B lymphocytes and CD4 T helper cells

The previous studies argue that the BCR-induced intrinsic program of the immature-stage B lymphocytes can be altered by extrinsic factors so that autoreactive B lymphocytes have the opportunity to avoid clonal deletion

and instead adapt by altering the specificity of their BCR by receptor editing. However, once leaving the protective microenvironment of the bone marrow, an immature B cell that continues to express a BCR reactive to endogenous proteins would be expected to be very vulnerable to induced cell death. A prediction made by this simplified model is that newly emigrating immature B cells exposed to foreign antigens in the periphery would be deleted. One could envision, however, that this response might not always be advantageous to the host, as pathogen-specific peripheral immature B cells might also be eliminated. In such cases, processes may have evolved to selectively rescue clones from deletion that might be of use in an ongoing immune responses. This appears to be the case, as BCR-induced apoptosis of T1 and T2 B cells can be redirected toward survival and proliferation in the presence of T-cell help. However, the sensitivity of T1 and T2 cells to T-cell signals are not equivalent, as T2 B cells proliferate more vigorously and are rescued from BCR-induced apoptosis to a greater degree than are T1 cells (Chung *et al.*, 2002). Therefore, the potential to respond to helper T-cell-derived signals, such as IL-4, or other stimuli, such as activating anti-CD40 antibodies, is acquired late and is associated with immature/transitional B cells that have gained access to peripheral compartments enriched in antigen and activated T cells.

The above studies suggest that antigen-induced deletion can be abrogated in the periphery, although there appears to be a temporal window in that the B cell needs to interact with activated T cells within 8 h of antigenic encounter (Sandel and Monroe, 1999; Sater *et al.*, 1998). Protection, however, would likely be contingent on the ability of the immature B cell to process and present antigen to the antigen-reactive T cell. The ability of immature B cells to internalize, process, and present antigen has been evaluated for fetal liver-derived developing B cells and immature B cell lines (Brines and Klaus, 1993; Morris *et al.*, 1992; Scott *et al.*, 1987). In these cases, it was concluded that immature B cells can function as antigen-presenting cells to T-cell lines.

Part of the activation response of mature B cells to BCR signaling is upregulation of CD86 (B7.2) expression. This costimulatory molecule is important in the productive interaction between antigen-reactive B and T cells. Interestingly, immature and transitional B cells do not upregulate the costimulatory molecule CD86 in response to anti-BCR stimulation (Chung *et al.*, 2002; Marshall-Clarke *et al.*, 2000). TCR binding of class II: peptide complexes on the antigen-presenting cell (signal 1), without concurrent ligation of CD28 with CD80 or CD86 (signal 2), has been shown to lead to anergy in the responding naive T cells (Schwartz, 2003). On the other hand, activated T lymphocytes are not as dependent on costimulation by the antigen-presenting cell, in this case, the B lymphocyte. Therefore, while interaction between antigen-reactive transitional B lymphocytes and naive class II-restricted T lymphocytes may be

unproductive or even tolerogenic, activated T lymphocytes might be able to recruit transitional immature B lymphocytes (at least T2) into an already triggered immune response.

Until recently, the ability of the bone marrow-derived transitional B lymphocyte to process and productively present antigen to class II-restricted resting and activated T lymphocytes was untested. Chung *et al*. (2003) demonstrated that T1 and T2 subsets were both able to process and present peptide in the context of class II to peptide:class II-restricted TCR transgenic CD4 T lymphocytes. In this case, T lymphocyte proliferation and optimal IL-2 production, as well as BCR-dependent transitional B lymphocyte proliferation, was dependent on an exogenous source of CD28 ligand. Thus, while T lymphocytes responded well to BCR-stimulated, peptide-presenting FO mature B lymphocytes, addition of agonist anti-CD28 antibodies was required for comparable responses using transitional B lymphocytes.

5. BCR SIGNALING DURING THE TRANSITIONAL TO MATURE B-LYMPHOCYTE TRANSITION

Like the pre-BCR at the pro-B-cell to pre-B-cell checkpoint, genetic disruption of certain signaling proteins leads to blocks in various stages of transitional B-cell development. The fact that many of these signal transduction proteins can be linked to BCR signaling suggests that tonic or ligand-induced BCR-initiated signals are required for survival of transitional B lymphocytes and/or continued developmental progression. This conclusion is further supported by the original studies by Lam *et al*. (1997) who showed that inducible ablation of BCR expression negatively affected survival of peripheral immature and mature B lymphocytes. These studies were confirmed in another inducible BCR loss model in which Igβ protein expression was gradually ablated as B cells transited the bone marrow pre-B and immature B-cell compartments (Meffre and Nussenzweig, 2002). Studies have more precisely linked these effects to loss of BCR-signaling function (Kraus *et al*., 2004). Using a model that facilitated inducible Cre-mediated excision of the DNA encoding the ITAM-containing portion of the cytoplasmic tail of Igα at the peripheral immature stage, the signaling capacity of Igα, but not BCR surface assembly, was ablated at this developmental stage. An inducible loss in immature and mature B cells was observed coincident with loss of BCR signaling.

As with the pre-BCR, the precise signaling pathways that are relevant to transitional and mature B lymphocyte survival and continued development are just beginning to be defined. Again, genetic loss-of-function

studies and their effect on peripheral B lymphocyte subsets have been informative. The precise localization of the arrest lends insights into the signals necessary for continued B-cell development and/or survival.

5.1. Igα/β and ITAMs

ITAM-associated tyrosine phosphorylation of the BCR Igα/β complex is initiated by activated Src kinases, particularly Lyn (Niiro and Clark, 2002). Evidence argues for a necessary role for Lyn expression for normal maintenance of peripheral B lymphocytes. Allman *et al.* (2001) found in $lyn^{-/-}$ mice that there was a marked effect on the transitional B-cell populations. Importantly, the ratio between transitional subsets (T1:T2:T3) was not changed, but the absolute number of each subset was decreased, suggesting that the intrinsic survivability of each developmental stage was affected by loss of Lyn. The equivalent effects of Lyn depletion on T1- and T2-cells contrast with what has been observed in mice lacking expression *Syk* (Turner *et al.*, 1997) where a developmental block occurs at the T1 to T2 B-cell stages.

5.2. B-cell linker protein

B-cell linker protein (BLNK/BASH/SLP-65) links Igα/β recruitment and activation of Syk to downstream elements such as Grb2, PLCγ, Vav, and Nck (Fu *et al.*, 1998). BLNK-deficient mice exhibit a significant but incomplete block at the pro-B to pre-B checkpoint. In addition, the few cells that progress beyond this checkpoint are then arrested at the T2 to mature stage transition (Pappu *et al.*, 1999; Xu *et al.*, 2000).

Recruitment of guanine nucleotide exchange factor protein Vav to BLNK is associated with the activation of Rac, which is a member of the RhoA family of small GTPases. Vav1/Vav2 null mice and Rac1/Rac2 null mice exhibit similar defects in B-cell development. Each is characterized by an impairment in the ability to progress beyond transitional immature to FO mature B cells (Doody *et al.*, 2001; Tedford *et al.*, 2001; Walmsley *et al.*, 2003). *In vitro*, anti-BCR-induced proliferation and survival are compromised in B lymphocytes isolated from these null mice. Additionally, the adaptor protein Bam32 is required for efficient GTP-loading of Rac1. B lymphocytes lacking Bam32 are deficient in Rac1-GTP, do not undergo membrane ruffling, and have a transient signaling phenotype characterized by susceptibility to anti-BCR-induced cell death (Allam *et al.*, 2004; Niiro *et al.*, 2002). Together, these studies identify the Vav/Rac1 pathway as important in the normal developmental progression of peripheral B lymphocytes.

5.3. Bruton's tyrosine kinase

A loss-of-function point mutation in the Btk pleckstrin homology domain in mice causes a mild x-linked immunodeficiency (xid) phenotype (Rawlings and Witte, 1994) in which peripheral B lymphocytes are skewed toward the immature phenotype with increased IgM^{high} IgD^{low} and no IgM^{low} IgD^{high} mature FO B lymphocytes (Rawlings and Witte, 1994). A closer examination of xid transitional B lymphocyte subsets indicated the block to be in the T2 to mature B lymphocyte stages (Loder *et al.*, 1999; Su and Rawlings, 2002). Similarly, Allman *et al.* (2001) confirmed a block at the T2 stage in BALB/c-xid congenic mice.

Deficits in PLCγ (Wang *et al.*, 2000), p85α-subunit of PI3K (Fruman *et al.*, 1999; Suzuki *et al.*, 1999), Vav (Doody *et al.*, 2001), and B-cell adapter for phosphoinositide-3 kinase (BCAP) (Yamazaki *et al.*, 2002) all demonstrate a developmental block similar to those found in Btk-deficient mice. The similarity of the phenotypes argues for the proposed concept of a signalosome with PLCγ as a ''central response regulator'' with inputs from many independent signaling proteins. Deficits in one of the related inputs will therefore lead to a common general defect.

5.4. Involvement of other BCR-signaling proteins

Other murine knockout models involving proteins in the BCR-signaling pathway demonstrated developmental blocks at the later transitional B lymphocyte stages. BCR-coupled activation of NF-κB, in particular c-Rel and RelA, are implicated in the survival and development of peripheral B lymphocytes. *Rag-1*$^{-/-}$ mice reconstituted with *c-rel*$^{-/-}$*rela*$^{-/-}$ fetal liver HSCs showed arrested B lymphocytes at the T1 stage (Grossmann *et al.*, 2000), indicating involvement of NF-κB signaling in developmental progression beyond this stage. Also, these cells showed reduced levels of Bcl-2 and enhanced apoptosis suggesting that the importance of NF-κB may relate to its ability to mediate survival signals to the developing B lymphocytes. In support of this interpretation, enforced expression of a bcl-2 transgene blocked the elevated apoptosis and promoted further differentiation of *c-rel*$^{-/-}$*rela*$^{-/-}$ B lymphocytes.

$CD45^{-/-}$ mice have been shown to accumulate IgM^{high} IgD^{low} cells, indicative of a block at the T2 to mature FO transition (Kishihara *et al.*, 1993). Crossing of $CD45^{-/-}$ and $SHP\text{-}1^{-/-}$ me^{v}/me^{v} mice led to the rescue of mature B lymphocytes suggesting a positive and negative role, respectively, for CD45 and SHP-1, in the latter stages of peripheral B lymphocyte development (Pani *et al.*, 1997).

Since both developmental arrest and decreased survival of particular subsets would each manifest themselves as similar phenotypes,

it is at present unknown whether particular signaling pathways affect BCR-induced programs directing continued developmental progression or, alternatively, survival and turnover. This is an important distinction for mapping and coupling the biochemical and genetic programming directing specific cell fate decisions at checkpoints in B lymphocyte development. The significance of this checkpoint lies with the concept that these stages of development are influenced by selection processes to eliminate self-reactive B lymphocytes as well as decisions to diverge down developmental pathways that lead to phenotypically and functionally distinct mature B lymphocyte subsets.

6. PERIPHERAL B LYMPHOCYTE SURVIVAL: SYNERGY BETWEEN BCR AND BAFF-R SIGNALING

Although the above studies implicate BCR-initiated signals for survival and continued development of transitional B lymphocytes, other signals are increasingly appreciated to play a critical, if not determining, role in these processes. In particular, the TNF-family protein BAFF has been shown to influence the fate of B lymphocytes as they transit through the T1, T2, and FO mature compartments.

BAFF (BLys) is a monocyte/macrophage-, DC-derived cytokine that binds to either the TAC1 or the BR3 receptor. Only cells that express the BCR and CD23 (T2 and mature B lymphocytes) bind to BAFF (Hsu *et al.*, 2002), and as cells mature through the various splenic transitional stages, BAFF binding increases (Miller *et al.*, 2006). While it may have once been thought that BCR signaling was itself necessary and sufficient for peripheral B lymphocyte survival, we know that this is incorrect or at least an oversimplification. Spontaneous or induced mutations leading to impaired BAFF-R signaling or expression leads to nearly complete loss of T2 and mature B lymphocyte compartments, despite expression of signaling-competent BCR (Harless *et al.*, 2001; Lentz *et al.*, 1996). BAFF appears to function as a survival factor based on the finding that signaling via BR3 increases the expression of Bcl-2, Bcl-xL (Amanna *et al.*, 2003), but it may also act to upregulate expression of CD21 and CD23 (Gorelik *et al.*, 2004; Rolink *et al.*, 2002).

It is clear that expression of BAFF-R on peripheral late transitional immature and FO mature B lymphocytes, as well as BAFF production by an as yet unknown source, is critical for B lymphocyte survival (Gross *et al.*, 2000, 2001; Sasaki *et al.*, 2004; Schiemann *et al.*, 2001; Thompson *et al.*, 2001; Yan *et al.*, 2001). Nevertheless, studies already described clearly document a critical role for BCR signaling for the survival of peripheral B lymphocytes.

How then does one integrate these two requirements? Recent studies may provide some insight. One study indicates that BAFF-R signaling initiates prosurvival programs in the B lymphocyte via an Akt-dependent mechanism that is postulated to promote glycolysis and nutrient uptake (Patke *et al.*, 2006). By analogy to growth factor cytokines, the increased metabolic activity and energy production is proposed to function in order to facilitate survival, activation, and differentiation programs that have been initiated by BCR signaling.

Moreover, studies by Smith and Cancro (2003) have shown that anti-BCR stimulation of FO mature and T2 B lymphocytes leads to upregulation of BAFF-R (BR3) levels. Although the requirement for BCR signaling for maintaining homeostatic levels of BAFF-R has not yet been ascertained, the available studies indicate a connection between BCR signaling and BAFF-R expression. Of even more interest are two recent studies that may identify an unexpected synergy between BCR and BAFF-R signaling for B-cell survival.

Using constitutively active IKK2 to selectively engage the canonical NF-κB pathway, which is engaged by both BCR and BAFF-R signaling in mice lacking the BAFF-R, Sasaki *et al*. (2006) made the surprising finding that most BAFF-dependent processes, such as survival and normal B lymphocyte development, were restored by expression of IKK2. Nevertheless, this effect was found to be dependent on some level of BCR signaling. The latter conclusion was supported by the finding that these mice failed to develop or maintain an MZ compartment in the absence of the BCR positive coreceptor CD19.

BAFF-R engages not only the canonical NF-κB pathway that involves degradation of inhibitory IκB to generate active NF-κB complexes but also the alternative pathway that generates active p52 via processing of a precursor protein p100 (Dejardin, 2006; Enzler *et al*., 2006). Enzler *et al*. (2006) reported that BAFF-mediated *in vitro* survival is linked to the NF-κB1 canonical pathway, whereas the alternative NF-κB2 pathway also influences survival and, moreover, is absolutely necessary for maintaining expression of prosurvival kinase Pim-2 and integrin expression levels. The latter likely influences retention of B lymphocytes in the spleen, particularly the MZ compartment.

An analysis of these two studies by Stadanlick and Cancro (2006) suggests a model for integration of BAFF-R and BCR signaling. These authors suggest that NF-κB1 activation via the BCR and BAFF-R canonical pathways generates increased p100, which is the intermediate for the alternative pathway engaged selectively by BAFF-R signaling (Sasaki *et al*., 2006). Thus, a synergy is predicted whereby BCR signaling provides optimal p100 levels for BAFF-R-triggered processing in order to yield NF-κB necessary for optimal survival and metabolic fitness (Patke *et al*., 2006).

7. DEVELOPMENT OF FO AND MZ MATURE B LYMPHOCYTES

As noted previously, FO B cells constitute the majority of splenic B lymphocytes and reside in primary follicles, wherein they are poised to respond to T-dependent antigens. Following contact with antigen and in response to T helper cell-derived signals, these cells generate secondary lymphoid follicles. A second, minor population of MZ B lymphocytes is localized to the outer limit of the splenic white pulp. These MZ B lymphocytes generally respond to blood-borne, T-independent antigens. The development of FO and MZ B lymphocytes from transitional precursors is dependent on the integration of multiple signals.

The strength of signaling through the BCR is one critical determinant of peripheral B lymphocyte maturation. There is evidence that weak signaling through the BCR results in the adoption of an MZ fate and strong signaling the development of FO B lymphocytes. Support for this model is based on analysis of mice that lack intracellular mediators that affect BCR-signaling thresholds. For example, mice that do not express the Ikaros family member Aiolos have enhanced BCR signaling and generate FO, but not MZ, B lymphocytes (Cariappa *et al.*, 2001). Mice that do not express CD21 have attenuated BCR signaling and an increase in MZ B lymphocyte number (Cariappa *et al.*, 2001), and mice that lack negative regulators of BCR signaling have an MZ B lymphocyte deficiency (Samardzic *et al.*, 2002). However, other studies support a model whereby strong BCR signals direct B lymphocytes into the MZ compartment (Lopes-Carvalho and Kearney, 2004). At present, it is difficult to reconcile these opposed points of view. This is an important issue for further study, given the increasing awareness of the importance of the MZ compartment for the rapid ''innate-like'' B lymphocyte response to certain pathogens.

More definitive are studies linking MZ differentiation to Notch signaling. As discussed previously, Notch1 plays a critical role in B versus T lineage fate decisions in primary lymphoid organs. In the periphery, however, Notch2 and its ligand Delta1 have a critical role in MZ B lymphocyte development. That Notch2 signaling is required for MZ B lymphocyte development is based on observations that mice that lack the key Notch mediator RBP-J_k lack MZ B lymphocytes (Tanigaki *et al.*, 2002), mice with either a deletion of Notch2 (Saito *et al.*, 2003) or its ligand Delta1 (Hozumi *et al.*, 2004) are MZ B lymphocyte deficient, and mice with a mutation in the negative Notch regulator Msx2-interacting nuclear target protein (MINT) have an increased number of MZ B lymphocytes (Kuroda *et al.*, 2003). Finally, inhibition of the activity of Mastermind, a potent Notch coactivator, also results in diminished MZ B lymphocyte development (Maillard *et al.*, 2004).

If transitional B lymphocytes express Notch2, and Delta1 is present on the splenic stroma, how does a transitional B lymphocyte ever become an

FO B lymphocyte? One possibility is that Delta1 is expressed in distinct splenic niches, and a transitional cell will only enter the MZ B lymphocyte pathway if it lodges in one of these and Notch2 signaling ensues. The precedent for distinct niches in which Notch ligands are differentially expressed was demonstrated above in the case of the bone marrow wherein Delta1 and Jagged-1 were selectively expressed on stromal cells (Section 2.6). Another possibility, as discussed by Pillai *et al*. (2005), is that the first step in the adoption of an FO versus MZ fate is BCR signaling. If the signaling strength is sufficient to commit the cell to an FO fate, then subsequent signals that may be delivered by Notch2 are ignored. If BCR signals are weak, then the transitional cell is receptive to Notch2-mediated signals.

8. CONCLUDING REMARKS

It was our intent when selecting and organizing the literature for this chapter that we highlighted the enormous and until fairly recently, unappreciated plasticity in cell fate decisions at nearly all positions in the development and antigen-triggered response of the B lymphocyte. As many studies document, early B lymphocyte specification is guided by integrated intrinsic programming and B lymphocyte extrinsic cues. Many, but likely not all, of these have been identified. However, we still lack much of a mechanistic understanding of how each is integrated so as to collectively ensure the complex genetic reprogramming of MPPs toward increasing levels of commitment to the B lineage. More work is needed in this area not only to flesh out our understanding of stem cell biology and hematopoietic cell lineage commitment, but also to provide insights into targets for intervention in cases where B lymphocyte output is weakened, such as during aging or immunodeficiency diseases, or augmented such as with autoimmunity.

These translational goals will be dependent on extending studies primarily performed in mouse models to the human. Many parallels will likely exist between mouse and man, and principles developed in the former species will apply to the latter. However, differences in primary and secondary developments will also be observed. In the latter case, while IL-7 is an obligate B lymphopoietic factor in mice, it is not required for human B lymphopoiesis. Also, the anatomical organization of the spleen in mouse and man also differs (Brendolan *et al*., 2007). Thus, it will be no surprise that differences between species will exist.

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CHAPTER 2

Tolerance and Autoimmunity: Lessons at the Bedside of Primary Immunodeficiencies

Magda Carneiro-Sampaio* and **Antonio Coutinho‡**

Contents

* Department of Pediatrics, Children's Hospital, Faculdade de Medicina da Universidade de São Paulo, Brazil
‡ Instituto Gulbenkian de Ciência, Oeiras, Portugal

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Abstract

The recent progress in the genetic characterization of many primary immunodeficiencies (PIDs) allows for a better understanding of immune molecular and cellular mechanisms. The present chapter discusses associations between PIDs and autoimmune diseases (AIDs) in this new light. PIDs are classified according to the frequency of association with AIDs, defining four groups of conditions: systematic (more than 80% of all patients), strong (10–80%), mild (less than 10%), and absent (no available descriptions). Several general conclusions could be drawn: (1) pathological autoimmune (AI) manifestations are very frequently associated with PIDs, indicating that, contrary to conventional notions, antimicrobial protection and natural tolerance to body tissues share many basic mechanisms; (2) in some gene defects, association is so strong that one could speak of "monogenic" AIDs; (3) basic types of PIDs are selectively associated with AID of a particular set of target tissues; (4) while for some gene defects, current theory satisfactorily explains pathogenesis of the corresponding AID, other situations suggest extensive gaps in the present understanding of natural tolerance; and (5) not exceptionally, observations on the AI phenotype for the same gene defect in mouse and man are not concordant, perhaps owing to the limited genetic diversity of mouse models, often limited to a single mouse strain. Overall, clinical observations on PID support the new paradigm of "dominant" tolerance to self-components, in which AID owes to deficits in immune responses (i.e., in regulatory mechanisms), rather than from excessive reactivity.

1. INTRODUCTION

Acquisition of natural tolerance remains the central question of immunology. Over the last few years, however, a marked progress in the understanding of the mechanisms regulating autoimmunity (AI) has been achieved. Thus, beyond the purging of autoreactive repertoires ensured by negative selection that had been demonstrated in the 80's (von Boehmer *et al.*, 1989), much evidence has been accumulated for the critical role of regulatory T cells (Tregs) in the control of pathogenic reactivities (Coutinho *et al.*, 2005). In other words, current understanding has moved from a predominant notion of natural tolerance based on ''recessive'' mechanisms to that of a ''dominant'' process owing to the activity of a special class of autoreactive cells. Some go as far as speaking of a paradigmatic shift, for the new perspective turns problems and solutions around. Thus, while AI disease (AID) was previously viewed as an excess of autoreactivity and was treated by immunosuppression, it may now be expected to represent one of the manifestations of immunodeficiency,

the therapeutics of which await novel drugs to selectively stimulate regulatory mechanisms.

The ''experiments of nature'' that are afforded by genetic diseases have always provided useful insights into physiology. While targeted gene inactivation in mice has provided a powerful means in such analysis, the forward genetics of human diseases offers a broader view and a wider range of phenomenology, notably by including allelic series and partial defects in gene function, all in richly heterogeneous ''genetic backgrounds.'' The recent and extraordinary progress in the genetic characterization of primary immunodeficiency diseases (PIDs) provides for novel possibilities of analysis (Cunningham-Rundles and Ponda, 2005; Notarangelo *et al*., 2006). We felt it timely, therefore, to revisit gene defects leading to PID, in the light of their associations with AIDs. This seemed all the more appropriate as the study of ''regulatory'' mechanisms ensuring natural tolerance revealed a series of evolutionary ''solutions'' that are based on novel and quite unexpected genetic, molecular, and cellular mechanisms. Other than unraveling putative mechanisms of tolerance, the analysis of AI manifestations in PIDs offers another advantage. All major AIDs that have been studied to date are of multifactorial origin, with complex genetics involving a number of susceptibility loci (Concannon *et al*., 2005; Krishnan *et al*., 2006; Morahan and Morel, 2002; Nath *et al*., 2004; Tsao, 2004; Wandstrat and Wakeland, 2001; Wicker *et al*., 2005). In contrast, PIDs are simple monogenic disorders, such that their specific association with AI illustrates the importance of a given gene (molecular or cellular mechanism) in tolerance.

Arthritis was one of the clinical manifestations in Bruton's first description of a PID (Bruton, 1952; Stiehm and Johnstone, 2005). Reports of AI phenomena in PIDs have become frequent, as improved medical management of PID patients greatly prolongs their survival and allows time for the clinical expression of AI phenomena (Arkwright *et al*., 2002; Etzioni, 2003; O'Shea *et al*., 2003; Rioux and Abbas, 2005; Ulmanen *et al*., 2005; Wulfraat *et al*., 2005). In some PIDs, such as immunodysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX syndrome); AI polyendocrinopathy, candidiasis, and ectodermal dystrophy (APECED); Omenn syndrome, AI lymphoproliferative syndrome (ALPS); and C1q deficiency, AI is the central disease component and is present in nearly all cases. In many other PIDs, AI manifestations occur also frequently [e.g., Wiskott–Aldrich syndrome (WAS), other deficiencies of early components of the classical complement pathway, IgA deficiency, common variable immunodeficiency (CVID), X-linked Hyper-IgM syndrome], and very few actually exist where no AI has been described (Table 1). This contrasts with ~5% prevalence of AID in the general population (Davidson and Diamond, 2001), knowing that AIDs typically appear in young adults while most PID patients are detected in early childhood. Yet, the current understanding of

TABLE 1 Association between primary immunodeficiencies and autoimmunity

Systematically associated (>80%)
IPEX (100%)
APECED (almost 100%)
Omenn syndrome (100%)
ALPS (more than 80%)
C1q deficiency (93%)
Strongly associated (<80%>10%)
C4 (75%) C1r/C1s (65%) and C2 (10–25%) deficiencies
Selective IgA deficiency (7–38%)
CVID (26%)
XL-Agammaglobulinemia (11–15%)
Hyper-IgM type 2 (AID deficiency) (21–25%)
XL-Hyper-IgM (CD40L def.) −20%
Wiskott-Aldrich syndrome (40–72%)
NEMO deficiencies (XL-EDA-ID) (23%)
Mildly associated (<10%)
C3 and C5–9 deficiencies
DiGeorge syndrome (5–10%)

Chronic granulomatous disease	
Neutropenias	
Hyper-IgE syndrome	
MHC class I deficiency	
MHC class II deficiency	
FcγRIIIb deficiency	
No descriptions found	
Asplenia	
Factor D deficiency	
IL-12/IL-23–IFN-γ axis deficiencies	
Ataxia telangiectasia syndrome	
IRAK-4 deficiency[a]	
WHIM syndrome[b]	

[a] Ku *et al.*, 2005.
[b] Gulino, 2003; Gulino *et al.*, 2004; Diaz, 2005.
The references for the other diseases are in the text and/or in Tables 2, 3, and 4.

PID does not systematically include AID as a major characteristic of such conditions. In light of the present chapter, however, AID and PID seem to be manifestations of the same basic processes, such that they could be aggregated within a single nosologic category. This conclusion gives credit to the new paradigm on ''dominant tolerance,'' and provides a better understanding of genotype–phenotype relationships, disease mechanisms, and respective clinical syndromes.

Our review of clinical observations of AI manifestations in PID patients is summarized in Tables 2–4, relating each AID to the PIDs in which it has been described. In Table 1, however, this presentation is reversed, classifying all PIDs, according to the frequency of clinical descriptions of any AI manifestations. We define association of PID with AI disorders as ''systematic,'' when AI manifestations are described in most cases (>80%), and as ''strong,'' when 10–80% of the cases present AI phenomena. Table 1 also lists PIDs in which a few cases of AI manifestations were already described (mildly associated) in spite of their recent identification, as well as PIDs where we could not find association with AID.

2. PIDs SYSTEMATICALLY ASSOCIATED WITH CLINICAL AI

Five groups of PIDs [IPEX, APECED, Omenn syndrome (OS), ALPS, and C1q deficiency] are systematically associated with clinical AI, such that the corresponding genes must encode critical steps in the process of establishing tolerance and will score as AID-susceptible genes [*FOXP3*, *AIRE*, *Fas/Fas Ligand* (*FasL*), *C1q*, and genes involved in the somatic recombination of T- and B-cell receptors]. These PIDs may be grouped as ''diseases of immunoregulation.''

2.1. Immunodysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX)

IPEX syndrome is due to mutations in the gene *FOXP3*, encoding a transcription factor that is essential for the development of Tregs. Incidentally, among over 60 cases (20 families) described, one family (with 2 male and 1 female probands) had no detected *FOXP3* mutations, suggesting genetic heterogeneity of the IPEX syndrome, the possibility of an unidentified autosomal locus (Owen *et al.*, 2003), which would also critically determine Treg development. An autosomal recessive IPEX-like syndrome was described in an 11-year-old boy with heterologous mutations in CD25 (Filipovich *et al.*, 2006). IPEX syndrome is usually a lethal condition, and certainly the earliest and the most severe manifestation of autoimmunity, together with Omenn syndrome (Bakke *et al.*, 2004; Bennett and Ochs, 2001; Gambineri *et al.*, 2003; Myers *et al.*, 2006;

TABLE 2 Connective tissue disorders in PIDs

AI disease/ manifestation	PID	Defective gene(s)
SLE	C1q deficiency (93%[a]) (Manderson *et al.*, 2004; Pickering *et al.*, 2001; Sullivan, 2004)	*C1q*
	C4 deficiency (75%) (Manderson *et al.*, 2004; Pickering *et al.*, 2001; Sullivan, 2004)	*C4*
	C1r/C1s deficiency (65%) (Manderson *et al.*, 2004; Pickering *et al.*, 2001; Sullivan, 2004)	*C1r/C1s*
	C2 deficiency (10–25%) (Manderson *et al.*, 2004; Sjöholm *et al.*, 2006; Sullivan, 2004)	*C2*
	C3 deficiency (about 10%) (Manderson *et al.*, 2004; Sullivan, 2004)	*C3*
	C5–9 deficiency (Manderson *et al.*, 2004; Sullivan, 2004)	*C5–9*
	IgA deficiency (Cunningham-Rundles, 2004; Fahl *et al.*, 2006; Koskinen, 1996)	Undetermined
Vasculitis	WAS (13–22%) (Dupuis-Girod *et al.*, 2003; Sullivan *et al.*, 1994)	*WASP*
	C4 deficiency (Sullivan, 2004)	*C4*
	C2 deficiency (Sullivan, 2004)	*C2*
	C3 deficiency (Sullivan, 2004)	*C3*
Henoch–Schönlein purpura	WAS (Duzova *et al.*, 2001)	*WASP*
Glomerulonephritis	C2 deficiency (Manderson *et al.*, 2004; Sullivan, 2004)	*C2*
	C1r/C1s deficiency (Manderson *et al.*, 2004; Sullivan, 2004)	*C1r/C1s*
	C3 deficiency (Manderson *et al.*, 2004; Sullivan, 2004)	*C3*
	ALPS (3%) (Oliveira and Fleisher, 2004)	*TNFRSF6, TNFSF6, CASP10*

(*continued*)

TABLE 2 (*continued*)

AI disease/ manifestation	PID	Defective gene(s)
Arthritis/ arthralgias/RA/ JRA	WAS (10–29%) (Dupuis-Girod *et al.*, 2003; Sullivan *et al.*, 1994)	*WASP*
	X-linked agammaglobulinemia (15%) (Howard *et al.*, 2006)	*BTK*
	X-linked Hyper-IgM syndrome (10%) (Levy *et al.*, 1997)	*CD40L*
	CVID (4%) (Cunningham-Rundles and Bodian, 1999)	Undetermined
	IgA deficiency (Cunningham-Rundles, 2004; Fahl *et al.*, 2006; Koskinen, 1996)	Undetermined
	DiGeorge syndrome (Jawad *et al.*, 2001)	Possibly *TBX1*
DM	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined
	WAS (Dupuis-Girod *et al.*, 2003)	*WASP*

[a] % of PID patients with the AI disorder.

TABLE 3 Hematologic AI disorders and PIDs

AI manifestation	PID	Defective gene(s)
Hemolytic anemia	WAS (36%[a]) (Dupuis-Girod *et al.*, 2003)	*WASP*
	ALPS (29%) (Oliveira and Fleisher, 2004)	*TNFRSF6, TNFSF6, CASP10*
	IPEX (about 20%) (Wildin *et al.*, 2002)	*FOXP3*
	CVID (5%) (Wang and Cunningham-Rundles, 2005)	Undetermined
	SIgAD (Cunningham-Rundles, 2004)	Undetermined
	Hyper-IgM due to AID (Quartier *et al.*, 2004)	*AID*
	X-linked Hyper-IgM (Levy *et al.*, 1997)	*CD40L*
	HLA class II deficiency (Klein *et al.*, 1993)	*CIITA, RFX5, RFXAP, RFXNK*
Thrombocytopenia	ALPS (23%) (Oliveira and Fleisher, 2004)	*TNFRSF6, TNFSF6, CASP10*
	IPEX (about 15%) (Wildin *et al.*, 2002)	*FOXP3*
	CVID (6%) (Cunningham-Rundles and Bodian, 1999)	Undetermined
	DiGeorge syndrome (4%) (Sullivan, 2004)	Possibly *TBX1*
	X-linked Hyper-IgM (Levy *et al.*, 1997)	*CD40L*
	SIgAD (Cunningham-Rundles, 2004)	Undetermined
	Hyper-IgM due to AID (Quartier *et al.*, 2004)	*AID*
Neutropenia	WAS (25%) (Dupuis-Girod *et al.*, 2003)	*WASP*
	ALPS (19%) (Oliveira and Fleisher, 2004)	*TNFRSF6, TNFSF6, CASP10*
	X-linked Hyper-IgM (>65%)[b] (Levy *et al.*, 1997)	*CD40L*
	X-linked agammaglobulinemia[b] (Conley and Howard, 2002)	*BTK*

[a] % of PID patients with the AI disorder.
[b] The AI etiology of neutropenia is controversial.

TABLE 4 Endocrine and epithelial AIDs in PIDs

AI disease/manifestation	PID	Defective gene
Thyroiditis	IPEX (20–50%[a]) (Gambineri *et al.*, 2003; Wildin *et al.*, 2002)	*FOXP3*
	APECED (10%) (Betterle *et al.*, 1998)	*AIRE*
	IgA deficiency (Cunningham-Rundles, 2004; Koskinen, 1996)	Undetermined
	TACI deficiency (Salzer *et al.*, 2005)	*TACI*
	FcγRIIIIb deficiency (about 10%) (de Haas *et al.*, 1995)	
Hypoparathyroidism	APECED (79–93%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998)	*AIRE*
Addison's disease	APECED (72–73%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998)	*AIRE*
	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined
Type 1 diabetes mellitus	IPEX (>90%) (Gambineri *et al.*, 2003; Wildin *et al.*, 2002)	FOXP3
	APECED (12%) (Ahonen *et al.*, 1990)	AIRE
Ovarian failure	APECED (43–50%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998)	*AIRE*
Testicular failure	APECED (14%) (Ahonen *et al.*, 1990)	*AIRE*
Hypophysitis	APECED (7%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998)	*AIRE*
Alopecia	APECED (29–37%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998)	*AIRE*
Vitiligo	APECED (15%) (Betterle *et al.*, 1998)	*AIRE*
	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined

Enteropathy	IPEX (almost 100%) (Gambineri *et al*. 2003; Ochs *et al*., 2005; Wildin *et al*., 2002)	*FOXP3*
	NEMO defects (IBD in about 20%)[b]	*NEMO (or IKKG)*
	WAS (IBD in 9%) (Dupuis-Girod *et al*., 2003)	*WASP*
	CVID (6%) (Cunningham-Rundles and Bodian, 1999)	Undetermined
	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined
Atrophic gastritis	APECED (15%) (Betterle *et al*., 1998)	*AIRE*
Pernicious anemia	APECED (15%) (Betterle *et al*., 1998)	*AIRE*
	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined
Celiac disease	APECED (5%) (Betterle *et al*., 1998)	*AIRE*
	IgA deficiency (Cunningham-Rundles, 2004)	Undetermined
Hepatitis	APECED (20%) (Betterle *et al*., 1998)	*AIRE*

[a] % of PID patients with the AI disorder.
[b] J. Orange (personal communication, 2006).

Ochs *et al.*, 2005; Powell *et al.*, 1982; Wildin *et al.*, 2002). Systemic and organ-specific AI disorders, apparently mediated by both antibodies and T cells, are typically observed from the first months of life, and some cases respond to bone marrow transplantation. Inflammatory enteropathy (100% of the cases), insulin-dependent diabetes mellitus (92%), thyroiditis, Coombs' positive hemolytic anemia, and thrombocytopenia are frequent; but neutropenia, lymphadenopathies, AI hepatitis, nephritis, and vasculitis have also been described, accompanied by a variety of autoantibodies to multiple targets (Ochs *et al.*, 2005; Wildin *et al.*, 2002). Interestingly, and as expected from a Treg deficit, almost all patients present concomitant allergic manifestations, notably, disseminated eczematoid dermatitis with eosinophilia and increased serum IgE levels. The levels of other Ig classes are usually within normal limits for age, and the patients are able to make antibody responses to immunization, which have been described to precipitate clinical crises (Bakke *et al.*, 2004; Ochs *et al.*, 2005). In many patients, circulating leukocytes and lymphocyte subsets (including $CD4^+$ $CD25^+$ cells) are in normal ranges (Bakke *et al.*, 2004).

The severity and breath of AI in IPEX patients, just like in the spontaneous mouse mutant *scurfy*, provides the most dramatic demonstration of the critical relevance of Tregs in physiology. All clinical manifestations of AI and allergy in IPEX patients can be explained by a complete Treg defect, demonstrating that *FOXP3* is indeed critical for Treg development, and that both IgE production and allergy (Lafaille and Lafaille, 2002), as well as natural tolerance to most body tissues and organs (Modigliani *et al.*, 1996), are ''dominantly'' ensured by Treg activity.

2.2. Autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy (APECED)

APECED, or AI polyglandular syndrome (APS) type 1, was associated to defects in the AI regulator (*AIRE*) gene (Eisenbarth and Gottlieb, 2004; Notarangelo *et al.*, 2004; Ulmanen *et al.*, 2005), a transcription factor which promotes ectopic expression of ''tissue-specific'' genes in thymic epithelial cells (Anderson *et al.*, 2002; Fischer, 2004; Gotter *et al.*, 2004; Peterson *et al.*, 2004; Sillanpää *et al.*, 2004). Characteristically, APECED patients present the triad of mucocutaneous candidiasis (83–100% of the cases), hypoparathyroidism (79–93%), and hypoadrenalism (72–73%) (Ahonen *et al.*, 1990; Betterle *et al.*, 1998). Candidiasis is usually manifested first, in the first years of life, while endocrinopathies appear later in childhood or adolescence, or even in adulthood (Perheentupä, 2006). Other organ-specific AI manifestations, such as ovarian (43–50%) or testicular failure, hypothyroidism (10%), insulin-dependent diabetes mellitus (2–12%), AI chronic hepatitis (20%), alopecia (29–37%), vitiligo (15%), as well as hypophysitis (7%) have also been observed (Ahonen *et al.*, 1990; Betterle *et al.*, 1998).

Strikingly, these patients do not present systemic AIDs, such as systemic lupus erythematosus (SLE), arthritis, dermatomyositis (DM), vasculitis, hemolytic anemia, immune thrombocytopenic purpura (ITP), inflammatory bowl disease (IBD), and glomerulonephritis (Tables 2 and 3).

AIRE represents the most surprising genetic mechanism to ensure a kind of a ''thymic shadow of self'' (Anderson *et al.*, 2002), that is, the representation of many ''tissue-specific'' antigens in the thymus, along T-cell development and selection. Much debate is still ongoing as to the cellular mechanisms involved: while indications exist for the AIRE-dependent negative selection of differentiating T cells, others have argued for the relevance of self-antigen expression by thymic epithelial cells (Derbinski *et al.*, 2005) in the selection and commitment of Tregs (Coutinho *et al.*, 2005). In either alternative, APECED is a relatively benign condition, as compared to IPEX, indicating that either negative selection is not a paramount mechanism in tolerance, or that Tregs are generated by mechanisms other than AIRE-dependent antigen expression. The selectivity of AI aggression to glandular and epithelial tissues is striking and reminiscent of those described by Sakaguchi in neonatally thymectomized mice (Sakaguchi and Sakaguchi, 1990), resulting from Treg deficits. Together with the absence of systemic AID, the clinical ''targets'' suggest that the ''AIRE mechanism'' is only relevant for antigens expressed in such tissues.

Interestingly, defective AIRE expression in thymic cells was demonstrated in two patients with OS (Cavadini *et al.*, 2005). As discussed in Section 2.3, however, this is probably secondary to the hypomorphic development of the thymic T-cell compartment. Thus, AIRE expression in thymus seems to depend upon normal organogenesis and T-cell development as well, the latter being required for a convenient maturation of thymic stroma (van Ewijk *et al.*, 2000).

2.3. Omenn syndrome (OS)

We consider OS as a PID systematically associated with AID because at least its most characteristic skin disorder is a consequence of AI mechanisms (Hönig and Schwarz, 2006) and responds well to cyclosporine (Ege *et al.*, 2005; Giliani *et al.*, 2006; Roifman *et al.*, 2006). Clearly, however, the diagnosis of OS is still based on clinical criteria, and the associated AID remains poorly characterized.

OS often manifests in the first weeks of life and is characterized by squamous erythrodermia (exfoliative in some patients), hepatosplenomegaly, lymphadenopathy, alopecia, and protracted diarrhea, accompanied by severe combined immunodeficiency with broad susceptibility to infections (Gennery *et al.*, 2005; Roifman *et al.*, 2006; Villa *et al.*, 2001). The hallmark of OS is the severely restricted TCR repertoire (Gennery *et al.*, 2005; Villa *et al.*, 2001), with oligoclonal B cells as well, whenever

these are present (Gennery *et al.*, 2005). Typically, T-cell lymphocytosis and low or absent B cells, eosinophilia, and elevated IgE levels are present in most cases (Ege *et al.*, 2005; Gennery *et al.*, 2005; Giliani *et al.*, 2006). The OS pathology has been attributed to autologous T cells, which are predominantly activated ($CD25^+$ $HLA\text{-}DR^+$) memory ($CD45RO^+$) cells, both in blood and in the tissues (Hönig and Schwarz, 2006; Villa *et al.*, 2001; Wada *et al.*, 2005). In OS, TCR repertoire is characteristically skewed with large oligoclonal expansions of the ''leaky'' TCR specificities. Skin biopsies reveal diffuse dermal infiltrates of activated autologous T cells with rare eosinophils, destruction of epidermal–dermal junction (Wada *et al.*, 2005), or perivascular lymphocytic infiltration, resembling in some cases graft versus host disease (GvHD) (Gennery *et al.*, 2005). Similar infiltrates can be seen in the gut and other organs.

OS is currently recognized as a genetically heterogeneous condition, the majority of patients bearing hypomorphic mutations of various genes involved in the process of somatic V-D-J recombination (Hönig and Schwarz, 2006). RAG-1 or RAG-2 is most frequently involved (Villa *et al.*, 2001), but mutations of the nonhomologous end-joining factor Artemis have been detected (Ege *et al.*, 2005), as well as mutations in the RNA component of the RNase mitochondrial RNA processing (RMRP RNA) gene (Roifman *et al.*, 2006). In almost half the patients, the genetic underlying defect could not be identified (Gennery *et al.*, 2005; Roifman *et al.*, 2006). Mutations of OS patients are characteristically ''leaky'' and maintain a residual recombination activity that allows for limited TCR (and BCR) rearrangements. Null homozygous mutations of those genes result in the complete absence of T and B cells, with an SCID, rather than an OS condition (Moshous *et al.*, 2001; Villa *et al.*, 2001). In a report, an *IL7RA* mutation was identified in a typical OS (Giliani *et al.*, 2006).

The severe restriction in TCR repertoires would suggest that the probability to generate Tregs is close to inexistent, explaining the pathogenesis of the OS according to current understanding of the mechanisms ensuring self-tolerance. OS is different from the similarly severe condition IPEX, in which Tregs are also absent, for the obvious reason that IPEX patients display the full repertoire of effector, autoreactive T cells, while OS may only generate a few TCR reactivities. For this interpretation, OS would essentially represent an IPEX with no effector cells to organs other than skin and intestine. Similarly, while IPEX is immunocompetent to most infections, OS is not, rather resembling an SCID in this respect. It becomes interesting, therefore, to try and understand why skin (and intestinal) lesions are paramount and systematically present, in spite of the expected ''randomness'' in the sporadic, ''leaky'' generation of TCR reactivities. In addition, as already mentioned, disturbance in central tolerance due to defective AIRE expression can also contribute to the AI pathology of OS (Cavadini *et al.*, 2005). The few T-cell clones that develop may thus escape

negative selection, and clones of autoreactive T cells are released into periphery. The finding of clinical OS at birth in some patients would seem to exclude that ''AI'' is secondary to bacterial colonization (Gennery *et al.*, 2005).

Interestingly, along the same line of interpretation, SCID infants with engraftment of maternal T cells following transplacental transfusion—obviously in numbers that necessarily imply their oligoclonality and severe TCR repertoire restriction—often present with a clinical phenotype mimicking OS (Villa *et al.*, 2001).

2.4. Autoimmune lymphoproliferative syndrome (ALPS)

ALPS, or Canale–Smith syndrome, is characterized as a defect of lymphocyte apoptosis, resulting in lymphadenopathy, splenomegaly, high numbers of TCR αβ+ $CD4^-$ $CD8^-$ (double negative) T cells, polyclonal hypergammaglobulinemia, and AI manifestations, the targets of which are essentially limited to blood cells (Bidère *et al.*, 2006; Bleessing *et al.*, 2000; Campagnoli *et al.*, 2006; Drappa *et al.*, 1996; Kanegane *et al.*, 2003; Oliveira and Fleisher, 2004; Rieux-Laucat *et al.*, 1999; Sneller *et al.*, 2003). Most ALPS are due to mutations of Fas, but mutations of FasL or of the downstream cysteine proteases (caspases 10 and 8) have also been described with a similar phenotype (Chun *et al.*, 2002; Worth *et al.*, 2006; Zhu *et al.*, 2006). At least one AI manifestation develops in nearly all ALPS patients, and a combination of AI features appears in many patients. Most frequently (up to 80%), they present clinical or laboratory manifestations of blood cell-directed AI disorders: AI hemolytic anemia (AIHA) (29%), thrombocytopenic purpura (23%), neutropenia (19%), and Evans' syndrome (combination of AIHA and ITP) (Sneller *et al.*, 2003). Other manifestations such as glomerulonephritis, uveitis, Guillain–Barré syndrome, AI hepatitis, and skin vasculitis were rarely described. Positive direct Coombs (40%), as well as anticardiolipin (51%), antinuclear (25%), and antineutrophil (46%), and anti-FcγRIIIb (CD16) antibodies have been described (Kwon *et al.*, 2003). Diagnosis of SLE has not been established in ALPS patients (Oliveira and Fleisher, 2004).

This selectivity of ALPS AI to blood cells remains unexplained (Tables 2–4) (Bidère *et al.*, 2006; Oliveira and Fleisher, 2004). While the loss of self-tolerance in ALPS is thought to represent the failure of negative selection due to apoptosis defects, it is not satisfactory to explain the predominant blood cell-directed AI in these patients, as that mechanism would command equally frequent AI to other tissues. Again, a putative reason could be found in the biology of Tregs. Thus, it has been established that thymic epithelium selection of Tregs, while ensuring tolerance to many peripheral tissues (Ohki *et al.*, 1987), is incapable of inducing full tolerance to hematopoietic cells (Thomas-Vaslin *et al.*, 1991). It could be

hypothesized, therefore, that blood cells represent the exceptional case, in which tolerance owes to negative selection, as in the classical Medawar experiments. If tolerance to all other body components is explained by Tregs, and since Treg production and operation is expected to proceed normally in ALPS patients, the ''phenotype'' predicted by this explanation would precisely be the selective loss of tolerance to blood cells. Interestingly, homozygous mutations of Fas^{-} (*lpr*) and $FasL^{-}$ (*gld*) in mice lead to features resembling SLE, but this is not at all the case in ALPS patients, who do not show either ''classical'' organ-specific endocrinopathies, even if they survive long.

It is interesting that patients with perforin defects [familial hemophagocytic lymphohistiocytosis (FHL)] also suffer severe lymphoproliferation, but do not present AI manifestations, in spite of overwhelming activation and proliferation of T cells, activation of macrophages, and high blood levels of IL-1, TNF-α, INF-γ, IL-6 (Feldmann *et al.*, 2002; Henter *et al.*, 1991; Jordan *et al.*, 2004; Ménasché *et al.*, 2005; Saint Basile and Fischer, 2003; Stepp *et al.*, 1999; Sullivan *et al.*, 1998). It may be argued that disease severity kills the patients before they develop AI clinical manifestations; but the clinical presentation of AI in IPEX patients, which is even earlier, weakens the argument. To be confirmed, the absence of AI associations with FHL would demonstrate that perforin is not critical in any other mechanism of tolerance, the operation of Tregs in particular. Rather, it would be an excellent indication for the robustness and specificity of the process, ensuring natural tolerance.

2.5. Complement deficiencies

Complement deficiencies also impart a high risk of developing AID, mainly SLE and glomerulonephritis. Patients affected by defects in early components of the classical pathway (C1q, C1r, C1s, C4, C2) frequently develop AI disorders, in addition to be very prone to severe infections with encapsulated bacteria (*Streptococcus pneumoniae, Haemophilus influenzae*) (Figueroa and Densen, 1991; Frank, 2000; Manderson *et al.*, 2004; Sjöholm *et al.*, 2006; Sullivan and Winkelstein, 2004). Defects in C1-complex proteins and in C4 represent the strongest susceptibility factors for the development of SLE so far identified in humans: SLE or SLE-like syndromes develop in 93% of homozygous C1q-deficient, in two-thirds of C1r/C1s-deficient, and in 75% of C4-deficient patients, while the association is weaker in C2-deficient individuals (10–25%) (Botto and Walport, 2002; Manderson *et al.*, 2004; Pickering *et al.*, 2001; Sjöholm *et al.*, 2006). Interestingly, lupus-like disease susceptibility drops to around or below 10% in patients with deficiencies of C3 (Pickering *et al.*, 2001) or of ''late'' components of the membrane-attack complex (C5–9) (Manderson *et al.*, 2004; Sullivan and Winkelstein, 2004). AID association is less clear for

partial deficiencies, as well as for mannose-binding lectin (MBL) (Pickering *et al.*, 2001).

Complete C1q deficiency thus represents the strongest risk for development of SLE, at ages as early as 12 months, with no female predominance, and in all ethnic/genetic backgrounds so far studied (Botto and Walport, 2002; Krishnan *et al.*, 2006; Manderson *et al.*, 2004; Nath *et al.*, 2004; Navratil *et al.*, 1999; Sullivan and Winkelstein, 2004; Tsao, 2004; Wulfraat *et al.*, 2005). These differential characteristics support the notion that no other genetic factors are involved and that this is a ''monogenic AID.'' Again, the immunopathogenesis of complement deficiency-related SLE is not fully understood, current hypotheses invoking compromises in the scavenging and clearance of apoptotic cells (Korb and Ahearn, 1997), in the clearance of immune complexes (by maintaining their solubility and by promoting their ingestion by phagocytic cells, via Fcγ and complement receptors), or even the activation of Tregs (Kemper *et al.*, 2003). The most striking feature, however, is the AID selectivity, as complement deficiencies do not impart susceptibility to any disease other than lupus (Tables 2–4).

3. PIDs STRONGLY ASSOCIATED WITH CLINICAL AI

This group includes the most common antibody deficiencies (Table 1) and is characterized by frequent, although variable (Tables 2–4), association with AID (10–80% of all patients). Typically, AID manifestations in this group are very broad as to target tissues and clinical presentation, missing the ''restricted patterns of susceptibility'' that are so characteristic of the previous group. This rule does not apply to the complement deficiencies, which as always, impart susceptibility to SLE.

Persistent bacterial or viral infections must be considered in the pathogenesis of AI manifestations of all PID patients, particularly in antibody and combined deficiencies as well as in phagocyte defects (Etzioni, 2003; O'Shea, 2003). Thus, chronic and recurrent infections may contribute to AI in CVID, agammaglobulinemia, Hyper-IgM syndrome, WAS, and other entities discussed in the following sections.

3.1. Selective IgA deficiency

Selective IgA deficiency (SIgAD) represents the most common PID, with a prevalence that ranges from 1:223 to 1:1000 in community studies, and from 1:400 to 1:3000 among healthy blood donors (Cunningham-Rundles, 2001; Schaffer *et al.*, 1991). Although some SIgAD individuals may present recurrent respiratory and gastrointestinal infections in early childhood, AI disorders represent the most important clinical manifestation in these

patients (Cunningham-Rundles, 2001, 2004; Liblau and Bach, 1992; Plebani *et al.*, 1986; Schaffer *et al.*, 1991). Both systemic and organ-specific AIDs have been associated with SIgAD: SLE, rheumatoid arthritis, juvenile rheumatoid arthritis, ITP, AIHA, pernicious anemia, Sjögren syndrome, DM, thyroiditis, IBD, celiac disease, type 1 diabetes mellitus, hepatitis, and vitiligo are the most common (Cunningham-Rundles, 2001, 2004). In long-term follow-ups of initially healthy donors or those identified as SIgAD in childhood, 23–38% developed AIDs (Fahl, 2007; Koshinen, 1996), both systemic and organ-specific. High frequency of autoantibodies (antithyroglobulin, red blood cells, nuclear proteins, basement membrane, cardiolipin, collagen, and adrenal cells), in addition to anti-IgA antibodies, has also been described in SIgAD, even in the absence of AID (Cunningham-Rundles, 2001).

It is unclear why SIgAD is associated to AI phenomena. Both the patterns of inheritance and the difficulty to identify the genetic basis of this condition would suggest that SIgAD is either a ''complex disease'' or else a heterogeneous group of genetic conditions. It follows that a variety of the molecular and cellular mechanisms may be responsible for association with AID. Although IgA represents the most abundant Ig produced in the organism and the second most abundant isotype in serum, it is remarkable that the biological activities and physiological functions of serum IgA are very poorly understood. It should be kept in mind that AID association with SIgAD may merely reflect either common manifestations of a single underlying defect (e.g., deficits in TGF-β), or linkage disequilibrium with yet unidentified loci of disease susceptibility. Interestingly, the increased frequency of the HLA-A1, B8, DR3 haplotype is shared by SIgAD and numerous AIDs (Gerbase-de-Lima *et al.*, 1998).

3.2. Common variable immunodeficiency

CVID designates another heterogeneous group of PIDs characterized by reduced serum levels of the major Ig classes, with variable T-cell numbers and function. Genetic heterogeneity has become clear in the last few years, with the identification of four genetic defects underlying CVID: ICOS, BAFF-R, TACI, and CD19 (Castigli *et al.*, 2005; Goldacker and Warnatz, 2005). It is interesting that CVID and SIgAD have been described in members of the same families, suggesting yet unknown genetic relationships between these immune defects (Hammarström *et al.*, 2000), as also implied in the finding that 9.6% of CVID women had one or more IgA-deficient children (Cunningham-Rundles and Bodian, 1999). AIDs have been observed in roughly 25% of both adults and children with CVID (Conley *et al.*, 1986; Cunningham-Rundles, 2002; Cunningham-Rundles and Bodian, 1999; Salzer *et al.*, 2004). It is interesting, though not understood, that CVID-associated AI shows a limited ''target range,'' mainly ITP

and AIHA, but also RA and JRA (Conley *et al.*, 1986; Cunningham-Rundles, 2002; Salzer *et al.*, 2004; Wang and Cunningham-Rundles, 2005), although TACI-deficient patients also present thyroiditis, vitiligo, and pernicious anemia (Salzer *et al.*, 2004).

Again, it is not clear how CVID gives rise to AI disorders. Again, AID appears associated with defects (e.g., BAFF-R) that diminish, rather than augment, immune responses, as expected if natural tolerance is indeed dominant. The relationships with SIgAD could invoke similar mechanisms, both remaining an interesting field of research.

3.3. Agammaglobulinemia

Among patients with X-linked agammaglobulinemia, manifestations of arthritis (15%), DM, AIHA, scleroderma, and alopecia have been often observed (Howard *et al.*, 2006; Ochs *et al.*, 2004). The AI nature of the frequent neutropenia in agammaglobulinemic children is not established (Conley and Howard, 2002).

Agammaglobulinemia is not an exception to our ignorance of the physiopathologic basis of the findings of AID susceptibility in PIDs. Ig-dependent selection of T-cell repertoires has long been described (Pereira *et al.*, 1989) and was revisited (Cascalho and Platt, 2006). Old phenomenology on the differential ability of IgG versus IgM to suppress or augment, respectively, immune responses (Henry and Jerne, 1968) may also be invoked in this context, as well as the role of antibodies in the determination of the class of helper T-cell responses (Parish, 1972). Clearly, however, the question is totally open and a very interesting one, indeed.

3.4. Hyper-IgM syndrome

X-linked Hyper-IgM syndrome, associated with CD40L defects, is frequently associated with episodic or chronic neutropenia (>65%) (Levy *et al.*, 1997; Winkelstein *et al.*, 2003), although its AI etiology is unclear, and circulating anti-neutrophil antibodies were found in only few patients. While some other AI manifestations were described, the paucity of AID in these patients is surprising, given the relevance of CD40/CD40L interactions in T-cell development, activation, and effector functions. This may indicate, however, that Tregs dispense of such receptors, both in their generation and operation.

Hyper-IgM syndrome due to activation-induced cytidine deaminase mutations is also associated with AIDs in a sizeable fraction (25%) of patients (Durandy *et al.*, 2005, Quartier *et al.*, 2004), who suffered from various T-cell-mediated inflammatory conditions, and showed autoantibodies to multiple targets. To be confirmed [Minegishi *et al.* (2000) did not find cytopenias or other AIDs in a series of 18 patients], this is most

interesting, as these patients are unable to generate somatic hypermutation of their antibodies. It follows that ''germ line'' antibodies may well be pathogenically autoreactive, while previous views would defend a strict requirement of affinity maturation in the pathogenicity of antibodies. Furthermore, AID mutations do not directly affect T cells, such that AI resulting from this defect can only be ascribed to either the loss of ''regulatory'' capacity for autoreactive T cells in a limited antibody repertoire, or else, to a deficit in antibody-dependent selection of T-cell repertoires (Pereira *et al.*, 1989). Interestingly, both alternatives invoke a ''network'' participation in the acquisition of natural tolerance, through repertoire selection and regulation of autoreactivities.

3.5. Wiskott–Aldrich Syndrome

WAS is a rare X-linked immunodeficiency, characterized by eczema, microthrombocytopenia, and variable immunodeficiency, which is due to mutations of a WAS protein (WASP), involved in cellular signaling to the actin cytoskeleton (Imai *et al.*, 2003; Ochs, 2002; Perry *et al.*, 1980). In addition to infections and bleedings, as well as the high risk of developing neoplasias (13%) (Perry *et al.*, 1980; Sullivan *et al.*, 1994), WAS patients frequently develop allergy and AI disorders (40–72% in different series, with 25% of the patients showing multiple disorders). A major characteristic of WAS AID is the wide spread of its ''targets'' that include hemolytic anemia (14–36%), neutropenia (25%), arthritis (10–29%), skin and cerebral vasculitis (13–22%), renal disease (3–12%), and inflammatory bowel disease (9%) (Dupuis-Girod *et al.*, 2003; Duzova *et al.*, 2001; Sullivan *et al.*, 1994), some patients showing association of IBD, uveitis, and AI neutropenia. Most interestingly, SLE and endocrinopathies were never found in WAS series (Dupuis-Girod *et al.*, 2003).

The immunopathogenic mechanisms in WAS are not understood, but claims were made for delayed clearance of apoptotic cells (Leverrier *et al.*, 2001) and reduced production of IL-2 (Schurman and Candotti, 2003).

3.6. NF-κB essential modulator defects

Although patients with NF-κB essential modulator (NEMO) defects associated with X-linked anhydrotic or hypohydrotic ectodermal dysplasia (XL-EDA-ID) have been described only recently, some AI manifestations were already reported (Orange *et al.*, 2004, 2005; Uzel, 2005). Among 56 patients reviewed by Jordan Orange, 10 have developed IBD, 2 arthritis, and 1 AIHA (J. Orange, personal communication, 2006). Interestingly, some of these patients present hyper-IgM (Orange *et al.*, 2005), raising interesting possibilities concerning the origin of the AID.

4. PIDs THAT ARE MILDLY ASSOCIATED WITH CLINICAL AI

This is a very heterogeneous group of conditions, most of which are quite rare, with limited cohorts of patients, some only recently described (Table 1). Owing, as well, to the expected great variety of AI manifestations, it is difficult, if at all appropriate, to engage in physiopathological considerations. Current findings are simply summarized in the following description.

Chromosome 22q11.2 deletion (DiGeorge) syndrome is one of the most common chromosomal disorders known, with an estimated prevalence of 1:4000 to 1:6000 (Botto *et al.*, 2003; Öskarsdóttir *et al.*, 2004). Variable ID is characteristic of this syndrome (Perez and Sullivan, 2002; Ryan *et al.*, 1997), but a profound T-cell dysfunction represents less than 1% of all the patients (Ryan *et al.*, 1997; Sullivan, 2004). AID, while not paramount, is associated in 5–10% of the patients (Jawad *et al.*, 2001), ITP and JRA being the most reported manifestations, occurring with a much higher prevalence than in the general population. Poor T-cell development in an abnormal thymus might explain the appearance of AID (Etzioni, 2003; Jawad *et al.*, 2001), but again, the primary T-cell-function deficiency would generally support the physiological role of Tregs in ''dominant tolerance.''

A variety of inflammatory and/or rheumatic conditions have been observed in some patients with chronic granulomatous disease (CGD), due to defects in the NADPH oxidase pathway. These primarily consist of obstructive lesions of oesophagus, gastrointestinal, and urinary tracts due to granuloma formation; but IBD, discoid lupus, SLE, ITP, and Behçet syndrome have also been reported (Marciano *et al.*, 2004; Segal, 2000; Winkelstein *et al.*, 2000). It is noteworthy that, in the large CGD series described by (Winkelstein *et al.*, 2000), 10% of X-linked recessive kindreds and 3% of autosomal recessive kindreds had family members with discoid lupus. Chorioretinitis has also been described in boys with X-linked granulocyte colony-stimulating factor (CGD) as well as in the carrier mothers (Goldblatt *et al.*, 1999). Vasculitis was observed in 4.1% and glomerulonephritis in 2.9% of the 853 patients in treatment with granulocyte-colony stimulating factor (G-CSF) in the Severe Chronic Neutropenia International Registry (Dale *et al.*, 2003).

Few patients with Hyper-IgE syndrome, characterized by the triad of high serum IgE levels (>2000IU/ml), recurrent staphylococcal skin abscesses, and pneumonias, of unknown physiopathology (Buckley, 1972; Grimbacher *et al.*, 1999) present AIHA and ITP, and one case of SLE has been described (Grimbacher *et al.*, 2005; Renner *et al.*, 2004).

Patients with TAP1–2 (transporter associated with antigen processing 1 and 2) deficiencies characteristically present severe bronchiectases with extracellular bacteria and necrotizing granulomatous skin lesions

(de la Salle *et al.*, 1994; Donato *et al.*, 1995; Fischer and Notarangelo, 2004; Moins-Teisserenc *et al.*, 1999). Histopathological examination shows inflammation and infiltration by blood vessels, resembling Wegener's granulomatosis. Since no pathogens could be cultured from such lesions, which are unresponsive to antibiotics and to immunossupressive therapy, their AI origin has been proposed (Gadola *et al.*, 2000; Moins-Teisserenc *et al.*, 1999; Zimmer *et al.*, 2005). It is speculated that exaggerated NK-cell activation, possibly stimulated by infection, cannot be counteracted, due to the genetic defect, by overexpression of HLA class I antigen.

Among 30 patients with MHC class II deficiency, who present a severe deficiency of cellular and humoral responses to foreign antigens, 2 patients present Coombs' positive hemolytic anemia and others various types of autoantibodies, including anti-nuclear ones (Klein *et al.*, 1993).

Among 21 patients derived from 14 unrelated families who did not express FcγRIIIb, either in neutrophils or NK cells, only 2 presented AI thyroiditis (de Haas *et al.*, 1995). Interestingly, while polymorphisms in this gene have been associated with susceptibility to SLE (Krishnan *et al.*, 2006), none of the patients described presented with lupus-like disease.

5. PIDs THAT ARE NOT ASSOCIATED WITH AID

We failed to find in the current literature descriptions of AID association with the PIDs listed in Table 1. While this may be due to incomplete clinical description, as these are rare and/or recently characterized diseases, it is surprising that AIDs have not been described in the few hundred patients with deficits in the IL-12/IL-23-IFN-γ axis (Dorman *et al.*, 2004; Fieschi *et al.*, 2003; Picard *et al.*, 2002; Rosenzweig and Holland, 2005). Thus, it could be expected that such a condition would favor (auto) antibody formation via the well-known Th1/Th2 balance. In a Japanese study, individuals heterozygous for IFN-γR1 Val14 Met and homozygous for IFN-γR2 Gln64 presented an increased risk of SLE (Nakashima, 1999; Tanaka, 1999). Asplenic patients, on the other hand, have long been described, and their resistance to AID in the face of heightened susceptibility to severe capsulated bacteria infections (Gilbert *et al.*, 2002; Schultze *et al.*, 2002) indicates, as the corresponding mouse mutant, that infections do not indiscriminately trigger AID, provided that the normal mechanisms ensuring natural tolerance are in place. Differently from other patients with antibody deficiencies, AIDs have not been described among the large number of cases with ataxia telangiectasia syndrome (ATS) already described (Nowak-Wegrzyn *et al.*, 2004; R. Gatti, personal communication, 2006).

6. CONCLUDING REMARKS

The analysis of clinical associations of AID with PID in patients who are currently very well characterized immunologically, but also as to the genetic and molecular basis of the original defects, offers the opportunity to understand the development and maintenance of self-tolerance following inactivation of a particular mechanism. It is to be expected that a better understanding of etiology and pathogenesis will ultimately lead to rational therapies of AID. While all major AIDs are of multifactorial origin, with complex genetics involving a number of susceptibility loci, most PIDs are simple, monogenic disorders such that their specific association with AID can elucidate the role of a given gene (molecule or cell type) in tolerance. PID patients are natural ''knockouts,'' and if analyzed together with the corresponding mouse models, may also clarify the relevance of mouse models to understand immune phenomena in humans. The study of PID patients with AID adds a series of ''new'' candidates to the list of AI-susceptible genes, such as those included in Tables 2–4. In addition, the analysis of genetically characterized PID/AID phenotypes in human populations allows for a much broader range of ''genetic backgrounds'' and putative ''modifier genes,'' as compared to the study of the corresponding ''knockouts'' in a few inbred mouse strains. Such genetic heterogeneity of human patients may explain why, not exceptionally, observations on the AI phenotype for the same gene defect in mouse and man are not concordant. For these very reasons, however, occasional discrepancies in the ''mouse and human phenotypes'' should not justify the conclusion that murine and human immune systems are radically different.

The present analysis of AID susceptibility in PID patients highlights the critical relevance of some immunological mechanisms for the establishment and maintenance of tolerance. This is particularly clear for the role of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs. Thus, IPEX patients, suffering from the earliest and the most severe AID so far described, teach us that Tregs are likely to be the most crucial players in natural tolerance. APECED patients, on the other hand, demonstrate the relevance of *AIRE* in the process of tolerance to tissue-restricted (endocrine and epithelial) antigens. The extreme susceptibility to SLE of C1 complex-, C4-, and C2-deficient patients show that we continue to pay too little attention to the role of the complement system in the maintenance of self-tolerance. This is all the more surprising, as this has been known for years, and an increasing number of groups dedicate great efforts to investigate the molecular and cellular bases of SLE. A similarly surprising lack of knowledge concerns the physiopathological roles of serum IgA, as well as the basis for the presence of AID in over 30% of all SIgAD patients. In general,

this analysis underlines the exquisite ''target'' specificity of AID, and it suggests that a variety of immune mechanisms are involved in its establishment. AID associated with ALPS is a prime example of this contention. In other words, while for some gene defects, current theory satisfactorily explains pathogenesis of the corresponding AID, other situations suggest extensive gaps in the present understanding of natural tolerance.

For the clinicians' point of view, the fact that pathological AI manifestations are very frequently associated with PID would suggest the good practice of investigating an underlying PID, whenever a patient with a diagnostic of AID (1) suffers recurrent, severe, and/or opportunistic infections, (2) presents multiple AI manifestations, (3) is a very young and/or male child, and (4) has a familial history of PID. The reverse also applies, namely that it seems appropriate to investigate AI in PID patients. Obviously, molecular or cellular substitutive therapies, which correct the immunodeficiency, are expected to correct as well the associated AI. Yet, the usefulness for the patients of all what discussed here will have to await rational and curative therapies for AID.

Overall, clinical observations on PID support the new paradigm of ''dominant'' tolerance to self-components, in which AID owes to deficits in immune responses (i.e., in regulatory mechanisms), rather than from excessive reactivity.

ADDENDUM IN PROOF

After the acceptation of this manuscript, a study by Kekalainen *et al*. (A defect of regulatory T cells in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J. Immunol.* **78,** 1208–1215, 2007) provided a solid demonstration for a Treg cell deficit in APECED patients.

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CHAPTER 3

B-Cell Self-Tolerance in Humans

Hedda Wardemann* and **Michel C. Nussenzweig†**

Contents

Abstract

Two mechanisms account for generation of the human antibody repertoire; V(D)J recombination during the early stages of B-cell development in the bone marrow and somatic mutation of immunoglobulin genes in mature B cells responding to antigen in the periphery. V(D)J recombination produces diversity by random joining of

* Max Planck Institute for Infection Biology, Campus Charite Mitte, Schumannstrasse 21/22, D-10117 Berlin, Germany
† Howard Hughes Medical Institute, 1230 York Avenue, New York, NY, USA

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gene segments and somatic mutation by introducing random point mutations. Both are required to attain the degree of antigen receptor diversification that is necessary for immune protection: defects in either mechanism are associated with increased susceptibility to infection. However, the downside of producing enormous random diversity in the antibody repertoire is the generation of autoantibodies. To prevent autoimmunity B cells expressing autoantibodies are regulated by strict mechanisms that either modify the specificity of autoantibodies or the fate of cells expressing such antibodies. Abnormalities in B-cell self-tolerance are associated with a large number of autoimmune diseases, but the precise nature of the defects is less well defined. Here we summarize recent data on the self-reactive B-cell repertoire in healthy humans and in patients with autoimmunity.

1. ANTIBODY DIVERSITY

Throughout life, B cells are generated from hematopoietic stem cells in the bone marrow where the nascent B-cell repertoire is generated by somatic recombination of immunoglobulin (Ig) heavy- and light-chain gene segments (Brack *et al.*, 1978; Tonegawa, 1983; Weigert *et al.*, 1978). Recombination starts at the heavy-chain locus at the pro B-cell stage and is associated with expression of terminal deoxynucleotidyl transferase (TdT) which adds a random number of nucleotides (N-nucleotides) to the Ig heavy-chain gene DNA ends created during V(D)J recombination (Alt and Baltimore, 1982). Successfully rearranged Ig heavy-chain genes are expressed on the cell surface together with the surrogate light-chain components VpreB and λ5 as part of the pre-B-cell receptor (BCR) complex, which induces proliferation and rearrangement of Ig light-chain genes. In humans, around 60% of B cells express Igκ light chains while 40% express Igλ, as opposed to a 20:1 Igκ:Igλ ratio in mice (Ghia *et al.*, 1996). Rearrangement at the Igκ locus is thought to precede Igλ rearrangements because the Igκ genes in Igλ-expressing cells are usually recombined and have frequently deleted the Igκ constant region (Brauninger *et al.*, 2001; Hieter *et al.*, 1981; Siminovitch *et al.*, 1985). The combination of random gene segment reassortment, imprecise joining, and addition of random nucleotides makes V(D)J recombination a powerful mechanism to diversify the antibody repertoire, and it is essential to establish and maintain a functional humoral immune system.

The second mechanism that diversifies the antibody repertoire is somatic mutation of Ig genes (McKean *et al.*, 1984). This process is initiated predominantly in B cells responding to antigen during T-cell-dependent immune responses (Berek *et al.*, 1991; Jacob *et al.*, 1991). Activation of B cells and costimulation by $CD4^+$ T helper cells leads to the formation of germinal centers (GCs) in secondary lymphoid organs, where Ig gene somatic

mutations are introduced and Ig class switching to downstream isotypes begins (MacLennan, 1994; Pape *et al.*, 2003). Both of these processes are initiated by a single enzyme, activation-induced cytidine deaminase (AID), which is expressed in activated B cells (Muramatsu *et al.*, 1999, 2000; Revy *et al.*, 2000). Although AID expression is most prevalent in GCs, B cells undergoing T-independent immune responses outside of GCs or activated by Toll-like receptor (TLR) ligands can also express AID and undergo somatic mutation or class switch recombination (Gourzi *et al.*, 2007; He *et al.*, 2004; Luther *et al.*, 1997; Ueda *et al.*, 2007). The largely random nature of V(D)J recombination and Ig gene somatic mutations generates an antibody repertoire that has been estimated to be more restricted by the number of B cells than by the number of possible antibody specificities that can be produced (Winter and Milstein, 1991).

An additional level of diversity in the antibody system is obtained by isotype class switching. Each constant-region isotype displays distinct functional properties mediated by interactions of the different constant regions with specific Ig Fc receptors or soluble molecules such as complement (Carroll, 2004; Ravetch and Bolland, 2001). Antibodies of different isotypes, but with similar or equal specificity, can modulate immune responses in opposite ways by interaction with activating rather than inhibitory Fc receptors or vice versa (Ravetch and Bolland, 2001).

2. ANTIBODIES AND SELF-TOLERANCE

Paul Ehrlich was the first to conclude that the immune system can distinguish between self- and nonself-components and thus avoid what he called ''horror autotoxicus'' (Silverstein, 2001). The number and diversity of antigenic epitopes, which are part of our own body (autoantigens), is probably as high as the number of antigenic epitopes expressed by the world of pathogens. To prevent the development of autoimmune diseases, the immune system has to discriminate between potentially harmful autoantibodies and protective antibodies directed against invading pathogens (Burnett, 1959; Lederberg, 1959). However, the extent to which diversity has to be limited to exclude the development of autoimmunity has not been clearly defined. In fact, the finding that polyreactive antibodies lacking specificity for individual antigens represent a substantial part of the normal immune repertoire suggests that there may be significant overlap between the autoreactive and protective antibodies (Coutinho *et al.*, 1995; Wardemann *et al.*, 2003). Furthermore, a beneficial role in immunity for low-affinity polyreactive autoantibodies in the initial response to invading pathogens may outweigh the low risk of autoimmunity associated with the weak interaction of such antibodies with self.

Nevertheless, the importance of establishing and maintaining self-tolerance in the antibody system is emphasized by the finding that a large number of autoimmune diseases are associated with high titers of serum autoantibodies (Tan, 1997). The best example of an antibody-mediated systemic autoimmune disorder may be systemic lupus erythematosus (SLE), but other diseases such as rheumatoid arthritis (RA), multiple sclerosis, myasthenia gravis, and Graves' disease are all associated with autoantibodies. The high titers of autoantibodies in serum of these patients indicate a breakdown of self-tolerance mechanisms that regulate autoreactive B cells (Melchers and Rolink, 2006; Rajewsky, 1996).

A large number of different cell types, soluble factors, and molecules have been shown to play an important role in the development of autoimmune diseases. Recent data from research in mouse and humans now helps to bring the pieces together to form a broader and more general picture of how the humoral immune system becomes autoaggressive and underlines the importance of self-tolerance checkpoints.

2.1. B-cell development and self-tolerance in the bone marrow

B-cell development starts in the bone marrow where Ig gene V, D, and J segments are recombined to produce membrane-bound Igs that are expressed on the surface of immature B cells. Membrane Ig associates with Igα- and Igβ-signaling subunits to form the BCR complex, which drives B-cell development forward (Nussenzweig *et al*., 1987; Reth and Wienands, 1997). Experiments with mice carrying preassembled Ig transgenes revealed three possible outcomes of self-reactivity in developing B cells. BCR cross-linking by self-antigen interrupts B-cell development and arrests cells in a stage where they undergo persistent rearrangement of Ig light-chain genes, thereby replacing self-reactive by nonself-reactive receptors (Gay *et al*., 1993; Tiegs *et al*., 1993). This process is referred to as receptor editing, and experiments in mice and humans have suggested that 20–50% of all developing B cells undergo receptor editing and, therefore, that same number of early B cells may express potentially harmful autoantibodies (Casellas *et al*., 2001; Oberdoerffer *et al*., 2003; Retter and Nemazee, 1998; Wardemann *et al*., 2004). A second mechanism that removes self-reactive antibodies from the repertoire involves deletion of the cells that express autoantibodies, a mechanism that was initially proposed by Lederberg (1959; Nemazee and Burki, 1989). However, deletion appears to be a secondary line of defense against anti-self-antibodies and is limited to B cells that fail to edit their receptors. The third mechanism to silence autoreactive B cells is referred to as anergy and involves making such cells unresponsive to antigen (Goodnow *et al*., 1988; Nossal and Pike, 1980). Experiments in a number of different BCR transgenic mouse models showed that low levels of continuous interaction of self-reactive B cells and

their cognate antigen can result in loss of responsiveness to BCR-mediated signals. These cells are prone to apoptosis, suggesting that most anergic cells are rapidly lost from the repertoire. Although receptor editing appears to be an important mechanism in silencing autoreactive B cells (Casellas *et al.*, 2001; Oberdoerffer *et al.*, 2003; Retter and Nemazee, 1998; Wardemann *et al.*, 2004), the extent to which deletion and anergy contribute to B-cell tolerance has not yet been determined. Whether failure to delete or edit autoreactive immature B cells in the bone marrow can directly cause autoimmune diseases is also unknown, but alterations in Ig light-chain gene usage suggestive of aberrant receptor editing have been associated with autoimmunity in a number of mouse models and human diseases (Dorner and Lipsky, 2005; Fields and Erikson, 2003; Verkoczy *et al.*, 2004).

To determine the number of human B cells that initially produce autoantibodies and to examine how these cells are regulated, we cloned antibodies from single B cells at different stages during their development (Fig. 1; Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). We found that the majority of nascent human B cells express self-reactive antibodies including antinuclear antibodies (ANAs) (Fig. 1A; Wardemann *et al.*, 2003). Surprisingly, a large fraction of the autoantibodies expressed by newly generated early immature B cells in human bone marrow are polyreactive with a number of structurally diverse foreign and self-antigens such as DNA, lipopolysaccharides (LPS), or protein, for example, insulin (Fig. 1B). The earliest committed precursor from which we were able to isolate Ig heavy and light chains were early immature B cells which lacked detectable surface IgM expression (Wardemann *et al.*, 2003). This compartment contains cells that are about to express surface Ig for the first time in development and may also contain B cells that are trapped due to interaction with self-antigen. Consequently, the early immature B-cell compartment may be enriched for self-reactive B cells actively undergoing secondary Ig light-chain gene recombination. However, if B-cell trapping in this compartment occurs, it is likely to be transient since the majority of the antibodies produced by these cells were readily silenced by Ig light-chain replacement *in vitro* (Casellas *et al.*, 2001; Wardemann *et al.*, 2004). In conclusion, human studies have shown that early developing B cells are highly self-reactive and that they express both polyreactive antibodies and ANAs (Fig. 1; Wardemann *et al.*, 2003).

The transition from the early immature B cell to the immature B-cell stage is accompanied by a significant decrease in the number of self-reactive antibodies (75–40%, Fig. 1A; Wardemann *et al.*, 2003) including a major loss of polyreactive antibodies (55–7%; Fig. 1B; Wardemann *et al.*, 2003) and ANAs (Wardemann *et al.*, 2003). Although the precise nature of the selection mechanisms has not been defined in humans, it is likely that Ig receptor editing plays a major role in shaping the antibody repertoire at this first bone marrow checkpoint (Dorner and Lipsky, 2005;

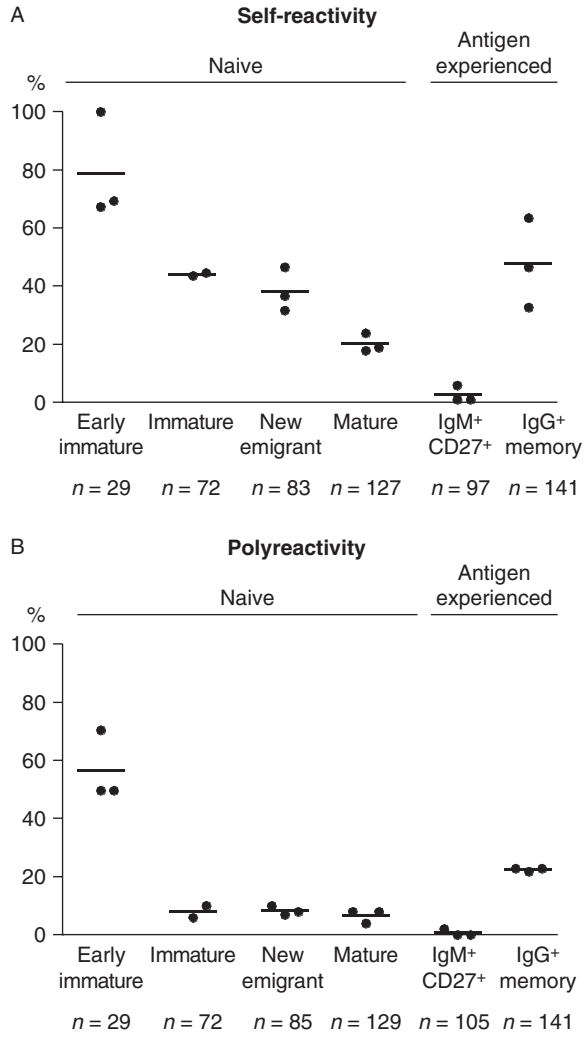

FIGURE 1 The frequency of self-reactive and polyreactive antibodies at different stages during B-cell development and differentiation in healthy humans. Recombinant monoclonal antibodies cloned from single human B cells were tested for (A) self-reactivity by ELISA with human HEp-2 cell line lysates and indirect immunofluorescence on HEp-2 cell slides and for (B) polyreactivity by ELISA with diverse antigens such as DNA, LPS, and insulin (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). The frequencies of self-reactive (A) and polyreactive (B) antibodies expressed by bone marrow-derived early immature and immature B cells and circulating peripheral new bone marrow emigrants, mature naive B cells, IgM^+ $CD27^+$and IgG^+ memory B cells in individual donors are summarized (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). Dots represent individual donors and horizontal lines indicate averages. The number of antibodies tested in each B-cell population is indicated below the graphs.

Oberdoerffer *et al.*, 2003; Wardemann *et al.*, 2003). Studies in mice show that a large proportion of the antibody repertoire (25–50%) can be attributed to editing, and *in vitro* studies have shown that the self-reactive antibodies in the early immature B-cell compartment in humans are readily silenced by random Ig light-chain replacement (Casellas *et al.*, 2001; Retter and Nemazee, 1998; Wardemann *et al.*, 2004).

2.2. Transitional B cells and peripheral selection of naive cells

B cells are released from the bone marrow in an immature state and must complete maturation in the periphery (Carsetti *et al.*, 2004; Monroe *et al.*, 2003; Thomas *et al.*, 2006). These transitional B cells display distinct responses to BCR cross-linking and must undergo further ill-defined maturation and selection steps in the periphery before they become mature, naive immunocompetent B cells ready to participate in immune responses (Carsetti *et al.*, 1995; Chung *et al.*, 2003; Lindsley *et al.*, 2007; Loder *et al.*, 1999; Sater *et al.*, 1998). It has been estimated that only about 10–20% of immature B cells produced in the bone marrow each day reach the spleen and only 5–10% will enter the mature naive B-cell pool (Allman *et al.*, 1993; Forster and Rajewsky, 1990; Rolink *et al.*, 1998). We found that differentiation into the mature naive B-cell compartment in humans is accompanied by passage through a second self-tolerance checkpoint that counterselects against expression of antibodies that display reactivity with self-antigens (Fig. 1; Wardemann *et al.*, 2003). The number of self-reactive B cells drops by half in this transition, as measured by self-reactivity ELISAs using human HEp-2 cell line lysates (Fig. 1A; Wardemann *et al.*, 2003). However, a significant fraction (20%) of mature naive B cells retain low levels of self-reactivity, predominantly with cytoplasmic antigens (Fig. 1A; Wardemann *et al.*, 2003). Consistent with our findings in humans, unfractionated mouse B cells and Epstein-Barr virus-immortalized human cell lines showed a significant frequency of low-affinity self-reactive and polyreactive antibodies (Dighiero *et al.*, 1983; Seigneurin *et al.*, 1988). How this second checkpoint is regulated is not well understood, and positive as well as negative selection mechanisms have been discussed (Cancro, 2004; Gu *et al.*, 1991).

An important regulator of the transition from immature to mature B cells in the spleen is the TNF-family member B-cell activating factor (BAFF) (Batten *et al.*, 2000; Moore *et al.*, 1999; Schneider *et al.*, 1999). BAFF acts as a survival factor for peripheral B lymphocytes. It is expressed by lymphoid stromal cells and induces NF-κB-mediated Bcl-2 expression on interaction with the BAFF receptor (BAFF-R), expressed on peripheral naive B cells. Mice deficient for BAFF or BAFF-R show a specific block in peripheral B-cell development at the transition to the mature naive B-cell compartment, while overexpression of BAFF in transgenic mice raises

the number of B cells that enter the mature naive pool (Mackay *et al.*, 1999; Sasaki *et al.*, 2004; Schiemann *et al.*, 2001; Shulga-Morskaya *et al.*, 2004). Interestingly, deregulated BAFF expression also results in autoimmunity and an SLE-like syndrome in mice (Mackay *et al.*, 1999). Self-reactive anergic B cells show decreased levels of surface BAFF-R expression, and under physiological conditions, BAFF expression is limited, resulting in a competitive advantage of nonself-reactive B cells over B cells expressing self-reactive BCRs (Lesley *et al.*, 2004). This model is supported by the finding that self-reactive B cells show increased survival in the absence of nonself-reactive competitors (Lesley *et al.*, 2004; Thien *et al.*, 2004). In addition to altered BAFF-R expression, there also appears to be a B-cell intrinsic decrease in BAFF responsiveness in self-reactive B cells (Lesley *et al.*, 2004; Thien *et al.*, 2004). Thus, BAFF regulates self-reactive B-cell survival at the transition to mature naive B cells in the periphery, and increased BAFF expression is associated with autoimmunity (Lesley *et al.*, 2004; Mackay *et al.*, 1999; Thien *et al.*, 2004). In humans, altered BAFF levels have been associated with a number of autoimmune diseases such as Sjögren's syndrome, SLE, and RA (Groom *et al.*, 2002; Pers *et al.*, 2005; Tangye *et al.*, 2006).

2.3. Defective early B-cell tolerance checkpoints and autoimmunity

SLE is characterized by production of high-affinity IgG autoantibodies predominantly directed against nuclear antigens (Tan, 1989), and systemic disease in lupus is associated with severe tissue damage in multiple organs due to the deposition of immune complexes that mediate inflammatory immune responses by engaging Fcγ receptors (FcγR) on innate immune cells such as macrophages (Nimmerjahn and Ravetch, 2006; Ravetch and Bolland, 2001). When treated by immunosuppressive therapy, SLE follows a remitting and relapsing course, but there is currently no cure for the disease (Davidson and Diamond, 2001). Therapy is based on nonspecific immunosuppression with steroids and cytotoxic agents, and ongoing clinical trials are designed to address the efficacy of bone marrow transplantation and anti-CD20-mediated B-cell depletion (Merrill *et al.*, 2004). The diagnosis of SLE is based on the fulfillment of 4 out of 11 criteria including serological markers, such as ANAs, present in the vast majority of patients (95%) and a variety of diverse clinical findings including renal insufficiency, proteinuria, urinary casts, seizures, psychosis, anemia, leukopenia, lymphopenia, or thrombocytopenia (Hochberg, 1997). The diversity of clinical symptoms is likely to reflect the diversity of genetic abnormalities associated with SLE or at least a predisposition toward the development of disease symptoms (Liu and Mohan, 2006; Wakeland *et al.*, 2001). The appearance of autoantibodies in the absence

of lupus symptoms frequently precedes disease onset, suggesting an initial early defect in humoral self-tolerance (Arbuckle *et al.*, 2003). It is important to note, however, that high autoantibody titers are not sufficient to predict the development of autoimmunity and are also occasionally observed in otherwise healthy individuals.

Serum antibodies are secreted by plasma cells, and therefore, B-cell self-tolerance in SLE must be broken sometime before this late stage in B-cell differentiation. Our observation that the vast majority of newly generated B cells in the bone marrow of healthy donors express self-reactive antibodies suggested that any failure to regulate naive B cells at the first or second checkpoint for self-tolerance would lead to increased numbers of circulating self-reactive naive B cells and could therefore predispose to the development of autoimmunity (Fig. 1; Wardemann *et al.*, 2003). To determine if SLE is indeed associated with loss of self-tolerance in early stages of the B-cell pathway, we cloned antibodies from single, isolated peripheral blood cells from three untreated, newly diagnosed patients with active disease and tested them for self-reactivity and polyreactivity (Yurasov *et al.*, 2005). Recombinant monoclonal antibodies were generated from two peripheral blood populations: new (bone marrow) emigrant B cells and mature naive B cells (Fig. 2). Based on our work on the bone marrow and blood B-cell antibodies of healthy controls, we knew that the antibody repertoire of new emigrant B cells resembles that of immature B cells in the bone marrow (Wardemann *et al.*, 2003). Therefore, any abnormalities in the new emigrant B-cell pool would directly reflect abnormalities in the first checkpoint for self-reactivity between the early immature and immature B-cell compartments in the bone marrow. We found that the frequency of self-reactive and polyreactive antibodies in the new emigrant B-cell fraction varied among the patients (Fig. 2; Yurasov *et al.*, 2005). However, significantly increased numbers of self-reactive and polyreactive antibodies were found in the mature naive B cells in all three patients (Fig. 2; Yurasov *et al.*, 2005). Abnormal levels of self-reactive mature naive B cells persist in the majority of patients in clinical remission (SLE-CR), suggesting that altered early B-cell tolerance checkpoints are a feature of SLE and may predispose to the development of initial disease symptoms and disease flares (Fig. 2; Yurasov *et al.*, 2006). Similar results were obtained by the Meffre laboratory, which used the same approach to analyze antibodies from new emigrant and mature naive B cells cloned from six patients with active RA (Fig. 2; Samuels *et al.*, 2005). Again, some patients showed increased levels of self-reactive antibodies in the new emigrant B-cell pool, but all patients showed significantly higher numbers of anti-self-antibodies in mature naive B cells. The diversity among the patients in terms of autoantibody frequencies and alterations in regulation of the two B-cell checkpoints likely reflects the enormous genetic and clinical variation in these

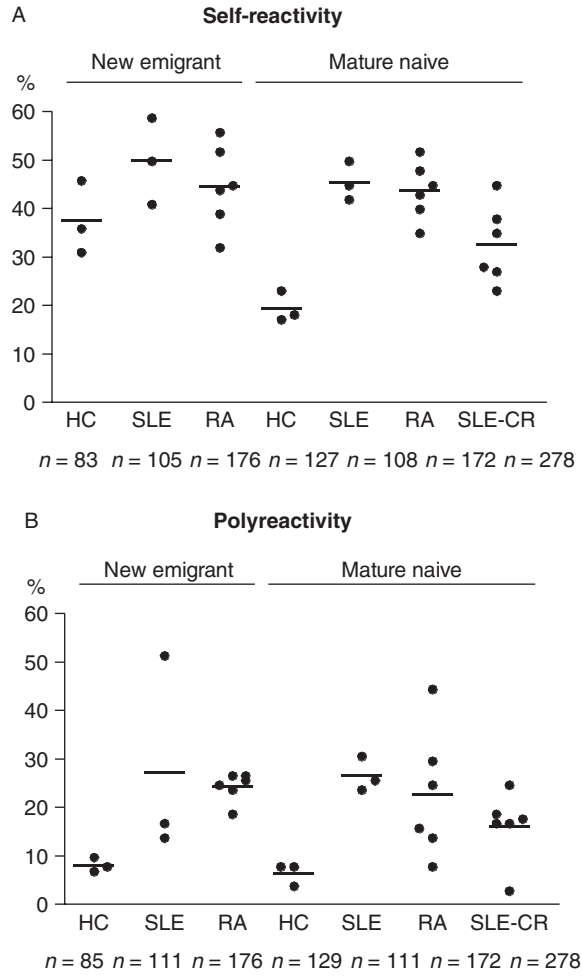

FIGURE 2 The frequency of self-reactive and polyreactive antibodies in new emigrant and mature naive B cells of healthy humans and patients with autoimmunity. Recombinant monoclonal antibodies cloned from single human new-emigrant and mature naive B cells of healthy controls (HC), patients with active untreated SLE, active rheumatoid arthritis (RA) and SLE patients in clinical remission (SLE-CR) were tested for (A) self-reactivity by ELISA with human HEp-2 cell line lysates and indirect immunofluorescence on HEp-2 cell slides and for (B) polyreactivity by ELISA with diverse antigens such as DNA, LPS, and insulin (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). The frequency of self-reactive (A) and polyreactive (B) antibodies in individual donors are summarized (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). Dots represent individual donors and horizontal lines indicate averages. The number of new emigrant and mature naive B-cell antibodies tested in HC and autoimmune patients is indicated below the graphs.

systemic autoimmune diseases, but the consistent finding that self-reactive mature naive B cells are enriched in these patients suggests that this may predispose to the development of serum autoantibodies and disease. However, the self-reactive antibodies that we find in the naive B-cell compartment are not the pathogenic high-affinity autoantibodies linked to disease, and whether the self-reactive naive B cells in SLE and RA are precursors of high-affinity autoantibody-producing plasma cells remains to be determined. Nevertheless, these cells are likely to capture and be stimulated by immune complexes containing TLR ligands, thereby perpetuating autoimmunity (Leadbetter *et al.*, 2002).

2.4. Molecular defects associated with altered self-tolerance in autoimmunity

Receptor editing by Ig light-chain replacement is one of the three known mechanisms that regulate developing self-reactive B cells in the bone marrow (Gay *et al.*, 1993; Tiegs *et al.*, 1993). On a molecular level, downstream J_L genes recombined with upstream V_L genes are a signature of secondary recombination events in the Ig light-chain locus, while increased usage of upstream J_L genes associated with downstream V_L genes is a sign of reduced or defective Ig light-chain editing (Dorner and Lipsky, 2005; Prak and Weigert, 1995). Analysis of J_L and V_L gene usage in active SLE and RA patients shows abnormalities that could be interpreted as due to altered secondary Ig light-chain recombination (Bensimon *et al.*, 1994; Samuels *et al.*, 2005; Yurasov *et al.*, 2006). Since editing strongly depends on BCR signaling, alterations in the function or expression level of BCR-associated signaling molecules are likely to contribute to the selection of self-reactive B cells (Hertz and Nemazee, 1997; Liossis *et al.*, 1996; Meffre *et al.*, 2000). Consistent with this idea, antibodies from X-linked agammaglobulinemia (XLA) patients with defective BCR signals exhibit signs of extensive secondary Ig light-chain recombination and are frequently self-reactive (Ng *et al.*, 2004). Furthermore, a polymorphic allele of the signaling molecule PTPN22 is associated with functional alterations and segregates with SLE and RA patients (Begovich *et al.*, 2004). Alternatively, early onset of Ig light-chain recombination at the Igκ locus in TdT-expressing pro-B cells may lead to the generation of unusually long Igκ CDR3 regions in autoreactive antibodies frequently expressed by B cells in the joints of RA patients (Bridges *et al.*, 1993).

Little is known about the second checkpoint for self-tolerance in the periphery in terms of molecular mechanisms, but elevated levels of BAFF have been found in SLE and other autoimmune patients and are correlated with disease activity (Section 2.2.; Pers *et al.*, 2005). Thus, increased frequency of self-reactive mature naive B cells in SLE and RA could be associated with high amounts of BAFF, and the efficacy of monoclonal

anti-BAFF antibody therapy is being addressed in clinical trials (Stohl, 2005). Keeping BAFF levels normal may be of special importance in conditions associated with lymphopenia or after therapeutic B-cell depletion until the peripheral B-cell pool is repopulated (Lesley *et al.*, 2004; Thien *et al.*, 2004). Under these circumstances, there is less competition for space and survival factors in the naive B-cell compartment and cells with self-reactive receptors are more likely to survive (Lesley *et al.*, 2004; Thien *et al.*, 2004). Significantly elevated serum BAFF levels were measured in patients after anti-CD20-mediated B-cell depletion and might be predicted to support the survival of reemerging autoreactive B cells after treatment (Cambridge *et al.*, 2006; Tangye *et al.*, 2006).

3. MARGINAL ZONE B CELLS

3.1. Mouse marginal zone B cells

In addition to developing into mature naive B cells, transitional B cells in the spleen of mice can differentiate into marginal zone (MZ) B cells, which are found adjacent to the red pulp and the marginal sinus that borders the white pulp follicle (Lopes-Carvalho and Kearney, 2004). These cells are enriched in self-reactive antibodies, and positive selection by interaction with self-antigen plays an important role in the generation of this compartment (Li *et al.*, 2002b; Martin and Kearney, 2000). MZ B cells are noncirculating B cells; participate in immune responses with T-cell-independent, primarily blood-borne, antigens; become readily activated by low levels of antigen; and are therefore considered to play an important role in innate resistance to blood-borne infection (Martin *et al.*, 2001). However in mice, the MZ may also serve as a reservoir for self-reactive B cells that might contribute to autoimmunity. For example, allelically included self-reactive and polyreactive B cells expressing two Ig light chains are enriched in the MZ in a murine model of graft versus host disease (GVHD) and in transgenic mice with self-reactive Ig heavy chains (Li *et al.*, 2002a,b; Witsch *et al.*, 2006).

3.2. The human MZ and circulating IgM^+ $CD27^+$ B cells

In humans, the anatomy of the spleen is decisively different from mice. Most obvious is the fact that the marginal sinus and metallophilic macrophages are absent, and thus MZ B cells are not separated from the follicular mantle zone harboring naive B cells (Steiniger *et al.*, 2006). In contrast to mice, the human MZ is populated with IgM^+ B cells that express the memory B-cell marker CD27 and harbor somatic mutations (Dunn-Walters *et al.*, 1995; Tangye *et al.*, 1998). Cells with this phenotype and similar Ig mutational spectrum are also found in circulation,

suggesting that human MZ B cells also differ from mouse MZ B cells in that they are not a sessile population (Weller *et al.*, 2004). Nevertheless, there is evidence that these cells are important in T-independent immune responses to blood-borne infection with encapsulated microorganisms (Kruetzmann *et al.*, 2003; Weller *et al.*, 2004; Zandvoort and Timens, 2002).

The origin of human IgM^+ memory B cells and MZ B cells is still debated; among the ideas currently being tested is the possibility that these cells are of GC origin, which does not seem likely since the cells exist in patients harboring mutations in the CD40–CD40L pathway, which is essential for normal GC formation (Kruetzmann *et al.*, 2003; Weller *et al.*, 2005). A second possibility is that these cells represent a distinct B cell lineage, which acquires mutations outside of GCs as part of an innate diversification reaction akin to that found in sheep and birds (Weill *et al.*, 2004). Where and when during their development they acquire somatic mutations is not known, but mutated IgM^+ $CD27^+$ B cells are found in X-linked hyper-IgM patients lacking GCs and class-switched B cells due to a mutation in the CD40L gene (Weller *et al.*, 2001). This finding suggests that somatic mutations in IgM^+ memory B cells are at least in part introduced independently of T-cell help and in the absence of a normal GC environment. It is also possible that the somatic mutations found in IgM^+ $CD27^+$ B cells are independent of antigen and serve to diversify the antibody repertoire (Weill *et al.*, 2004; Weller *et al.*, 2005).

Our own analysis of over 100 recombinant monoclonal antibodies cloned from circulating IgM^+ $CD27^+$ B cells from healthy human donors showed that this population almost completely lacks self-reactive or polyreactive antibodies (Fig. 1; Tsuiji *et al.*, 2006). If human IgM^+ $CD27^+$ B cells were circulating MZ B cells and participated in immune responses to T-cell-independent antigens in a manner similar to that proposed for MZ B cells in mice, one could expect to find antibacterial antibodies with low levels of reactivity and specificity in this population. However, relative to mature naive B-cell antibodies, we could not find antibodies with broad reactivity for T-cell-independent bacterial antigens or whole bacteria (Tsuiji *et al.*, 2006). Instead, we found antibodies with high levels of specificity and reactivity against a *Streptococcus pneumoniae* polysaccharide antigen in one donor that was previously vaccinated with the same antigen. The frequency of these antibodies was high (2/27) and their Ig genes displayed somatic mutations. Both antibodies used identical, rare Ig light-chain V genes but they did not originate from clonally related cells suggesting strong antigenic selection (Tsuiji *et al.*, 2006).

To determine whether transitional B cells expressing self-reactive and polyreactive antibodies are excluded from the IgM^+ $CD27^+$ B-cell compartment before or after the onset of somatic mutations, we reverted the mutated Ig heavy-chain and light-chain genes of a large number of these antibodies into their corresponding germ line sequences (Tsuiji *et al.*, 2006).

When expressed and tested for self-reactivity and polyreactivity, none of these antibodies showed any reactivity including two antibodies that showed low levels of self-reactivity and polyreactivity in their mutated form. We conclude that self-reactive and polyreactive antibodies are efficiently excluded from the IgM^+ $CD27^+$ B-cell pool in humans and that counterselection occurs before the onset of somatic mutation. Taken together with the observation that high numbers of unmutated naive B cells in bone marrow and periphery express self-reactive antibodies, the data suggest that a third checkpoint for self-reactivity regulates the entry of naive B cells into the IgM^+ $CD27^+$ B-cell compartment (Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003).

It is important to note that the term IgM^+ ''memory'' B cells originates from the initial finding that they express the memory B-cell marker CD27 and display somatically mutated Ig genes (Agematsu *et al.*, 1997; Klein *et al.*, 1998; Tangye *et al.*, 1998). Whether these cells represent true memory cells remains to be determined. To date, the evidence suggests that they are likely to be circulating human MZ B cells. Since little more is known about their origin, we should reconsider the appropriateness of the term ''memory'' B cell until we know that they are long-lived and participate in recall responses.

4. B-CELL MEMORY

T-cell-dependent immune responses lead to the development of memory B cells, antibody-secreting short-lived plasmablasts, and long-lived plasma cells. Plasmablasts develop in extrafollicular areas of the spleen, whereas memory cells and plasma cells emanate from the GC reaction (MacLennan, 1994). B-cell differentiation along these two pathways depends on separate groups of transcription factors and stimuli. For example, OCA-B (also known as BOB-1 or OBF-1) is required for GC B-cell differentiation but not for plasmablast development (Kim *et al.*, 1996; Qin *et al.*, 1998), and IRF4 and Blimp-1 are required for plasma cell formation but not for GC or memory B-cell development (Calame *et al.*, 2003; Klein *et al.*, 2006; Schubart *et al.*, 1996). Finally, the quality of the initial BCR signal also influences the program of B-cell differentiation. Cells with high-affinity receptors are more prevalent in extrafollicular sites where they differentiate immediately into short-lived plasma cells, whereas low-affinity B cells participate in GC responses (Paus *et al.*, 2006; Shih *et al.*, 2002). Within the GC environment, somatic mutation of Ig genes creates clonally related variant antibodies that are selected by binding to antigen in the form of immune complexes displayed on follicular dendritic cells (FDCs) in the light zone of the GC (Griffiths *et al.*, 1984; Haberman and Shlomchik, 2003; Jacob *et al.*, 1991; Wang and Carter, 2005). While extrafollicular foci last

only for a few days, GCs persist for weeks and evidence exists that only GC B cells that acquired high affinity for the antigen are allowed to differentiate into plasma cells. Extrafollicular plasma cells in spleen contribute to early serum antibodies, but it is believed that only long-lived plasma cells and quiescent memory B cells of GC origin provide long-term immunological memory and play a major role in recall responses (Ellyard *et al.*, 2004; Manz *et al.*, 2005; Tangye *et al.*, 2003). The formation of immunologic memory is the underlying principle for successful vaccination strategies and protective immunity in secondary infections but, if directed against self-antigen high-affinity memory B cells and plasma cells, represents a major threat for the development of autoimmunity (Avery *et al.*, 2003; Manz *et al.*, 2006).

4.1. Germinal centers

Somatic mutations are initiated by AID in cycling centroblasts that populate the dark zone of the GC (Muramatsu *et al.*, 1999, 2000; Revy *et al.*, 2000). The random nature of the mutation process diversifies the antibody repertoire, leading to several possible outcomes: (1) B cells expressing antibodies with neutral or nonbeneficial replacement mutations in terms of antigen affinity are likely to continue the mutation process or will be lost from the repertoire by apoptosis (Pulendran *et al.*, 1995; Shokat and Goodnow, 1995), (2) clones bearing beneficial replacement mutations leading to increased Ig antigen affinity will be selected for differentiation into plasma cells or memory B cells (McKean *et al.*, 1984), and (3) somatic mutation may also change the antigen specificity and produce self-reactive antibodies (Ray *et al.*, 1996; van Es *et al.*, 1991; Wellmann *et al.*, 2005). The frequency of each of these events remains to be determined, as do the mechanisms that regulate cells that develop aberrant receptors in the GC.

It is believed that changes in antibody specificity and affinity are tested when B cells exit the proliferation–mutation phase in the dark zone, and move to the light zone of the GC (Allen *et al.*, 2004). In the light zone, competition for antigen seems to be the driving force for selection or preferential survival of high-affinity clones, which can then return to the dark zone to be amplified before differentiation into memory B cells and plasma cells and emigration into the periphery (Phan *et al.*, 2006).

In vivo studies of GC dynamics by two-photon laser scanning microscopy have revealed that GC B cells do indeed migrate back and forth between the dark zone and the light zone, suggesting that they can undergo successive rounds of proliferation/mutation and selection (Allen *et al.*, 2007; Schwickert *et al.*, 2007). Interactions of GC B cells with immune complex bearing FDCs in the light zone are mostly transient and only rare stable contacts with follicular T helper cells are observed,

suggesting that T-cell help is a limiting factor for the selection of high-affinity B-cell clones (Allen *et al.*, 2007). However, the amount of traffic between GC zones is equivalent and therefore selection cannot be limited to the light zone (Allen *et al.*, 2007; Schwickert *et al.*, 2007).

Furthermore, data from the Nussenzweig laboratory showed that GCs are open structures, frequently visited by follicular B cells, which if they show high affinity for the cognate antigen, can be recruited into ongoing GC responses (Schwickert *et al.*, 2007). This finding is consistent with the observation that B cells with unmutated and unrelated Ig genes are detected within individual GCs in human tonsils (Kuppers *et al.*, 1993). Given the fact that GCs persist for up to 3 weeks, it is likely that over time B cells pass through a large number of them not only within one lymphoid organ but also within the whole body (Allen *et al.*, 2007; Schwickert *et al.*, 2007). This previously unknown level of GC complexity is likely to significantly enhance competition for antigen in order to ensure the quality of ongoing immune responses. Although it has not been addressed by intravital microscopy, it is possible that circulating memory B cells can also be recruited into ongoing GC reactions. If so, this may explain why memory B cells generated late in the response have a selective advantage over those emigrating early as they can continue to increase their Ig affinity.

4.2. Activation and selection of autoreactive B cells

In the context of autoimmunity, open access to the GC environment might be dangerous as it could induce epitope spreading. Furthermore, the observation that self-reactive and polyreactive antibodies are significantly enriched in mature naive B cells of patients with SLE and RA suggests that these cells, together with all other follicular B cells, can enter the GC environment (Samuels *et al.*, 2005; Schwickert *et al.*, 2007; Yurasov *et al.*, 2005). The target antigens of lupus-associated autoantibodies are diverse, but complex chromatin and RNA-containing antigens as well as antigens exposed on the surface of dying cells, such as phospholipids, are predominant (Sherer *et al.*, 2004). Antigen complexes of nucleic acids and proteins are potent stimulators of immune responses as they contain large and repetitive nucleic acid structures that can facilitate BCR cross-linking. At the same time, these antigens include protein antigens that can be efficiently presented to T cells. Another feature is their ability to activate pattern recognition receptors such as TLRs expressed on innate as well as adaptive immune cells. TLRs, a subset of which are expressed intracellularly and recognize microbial as well as endogenous nucleic acid containing ligands, have been the focus of intensive research to address their potential role in autoimmunity (Marshak-Rothstein, 2006).

A current model for the development of TLR-dependent autoimmunity suggests that complexes of serum autoantibodies with endogenous TLR

ligands are internalized on interaction with FcγRIIa or by cross-linking self-reactive surface Ig molecules on B cells, leading to stimulation of nucleic acid-specific TLRs in endosomes (Leadbetter *et al.*, 2002). Thus, it is feasible that TLRs can support the development of autoimmunity if sufficient amounts of self-antigen-containing immune complexes are available as might occur when clearance of apoptotic cells is defective or when TLR expression is altered. In fact, genetic abnormalities in molecules involved in the removal of self-antigen such as complement, C-reactive protein or DNase, and overexpression of TLR7 in mice predispose to the development of SLE (Graham *et al.*, 2006; Pisitkun *et al.*, 2006; Wakeland *et al.*, 2001). This model postulates the existence of serum (IgG) autoantibodies as prerequisite for the generation of self-antigen-containing circulating immune complexes. It remains to be determined if normal low-level self-reactive serum antibodies participate in the formation of such immune complexes or if defective B-cell tolerance checkpoints and abnormal levels of circulating autoantibodies are initially required.

TLR stimulation with microbial antigens could also lead to (cross-) activation of self-reactive adaptive immune cells during acute infection. In support of this idea, viral and bacterial infections are frequently associated with the development of autoantibodies in nonautoimmune individuals and with disease flares in patients with autoimmunity (Zandman-Goddard and Shoenfeld, 2005).

The hallmark autoantigen in SLE is dsDNA (Hahn, 1998). However, evidence suggests that antinucleosome antibodies are better disease indicators than anti-dsDNA antibodies (Reveille, 2004). Pure dsDNA can be seen as a T-cell-independent antigen, whereas nucleosomes contain proteins and can therefore mediate T-cell activation to generate high-affinity class-switched autoantibodies typical for SLE. The mechanisms by which self-tolerance is broken at this late stage during B-cell activation are not known, but FcγRIIb-mediated negative-feedback mechanisms as well as inflammatory cytokines, such as IFNα/β and IL6, play an important role in SLE (Banchereau and Pascual, 2006; Floto *et al.*, 2005; Mackay *et al.*, 2006; Xiang *et al.*, 2007).

4.3. Autoreactivity and B-cell memory

We have determined the level of self-reactivity in the circulating IgG^+ memory B-cell pool of healthy humans (Fig. 1; Tiller *et al.*, 2007). Heavily mutated antibodies were cloned from single memory B cells isolated based on the expression of IgG and $CD27^+$. We were surprised to find that self-reactive and polyreactive antibodies were significantly increased in these cells when compared to mature naive B-cell antibodies. About 10% of the self-reactive antibodies recognized nuclear antigens reminiscent of ANAs from patients with autoimmune diseases (Tiller *et al.*, 2007).

When we reverted these mutations and tested a number of IgG^+ memory B-cell antibodies for self-reactivity and polyreactivity with diverse antigens, we found that most of the antibodies were no longer autoreactive in the germ line form (Tiller *et al.*, 2007). Thus, these antibodies acquired their reactivity in the GC. We concluded that somatic mutation contributes significantly to the generation of autoantibodies during GC reactions and that memory B cells expressing antibodies with potentially low level of autoreactivity are allowed to exit from the GC into the periphery.

Antibodies can show cross-reactivity with multiple antigens. Thus, it is possible that the self-reactive and polyreactive IgG memory B-cell antibodies represent a by-product of affinity maturation and interact at higher affinity with unknown foreign antigens not tested in our assays. Low-level polyreactive antibodies may also play an important role in the immediate response to infection, as suggested by increased susceptibility to bacterial infection of patients lacking IgG and its reversal by intravenous IgG transfer. Alternatively, self-reactive and polyreactive IgG antibodies may be involved in clearance of self-antigen from apoptotic cells. The latter is supported by the observation that patients with hyper-IgM type 2 syndrome lacking serum IgG antibodies due to AID deficiency are prone to autoimmunity (Quartier *et al.*, 2004).

Long-term immunologic memory is associated with the development and persistence of a relatively small number of long-lived plasma cells in the bone marrow (Radbruch *et al.*, 2006). Despite a limited number of survival niches, a relatively restricted memory repertoire is able to maintain stable titers of specific antibodies over years (Bernasconi *et al.*, 2002). If specific for pathogens, long-term humoral memory can be life saving. However, if directed against autoantigens nondividing long-lived bone marrow plasma cells may be a major source of high-affinity class-switched pathogenic autoantibodies in patients with autoimmune diseases and represent an important target for therapies (Manz *et al.*, 2006).

How the pool of plasma cells is maintained, that is, for how long the cells survive in their respective niches, and how they can be generated from persistent circulating memory B cells is not understood, and the relative contribution of memory B cells and long-lived plasma cells to specific antibody titers remains to be determined (Bernasconi *et al.*, 2002; Manz *et al.*, 2005; Odendahl *et al.*, 2005; Radbruch *et al.*, 2006).

5. CONCLUDING REMARKS

Since the first description of serum SLE autoantibodies against DNA and deoxyribonucleoproteins by Henry Kunkel, we have gained tremendous insight in the molecular details associated with this devastating autoimmune disease. Extensive research in humans and in mouse models has

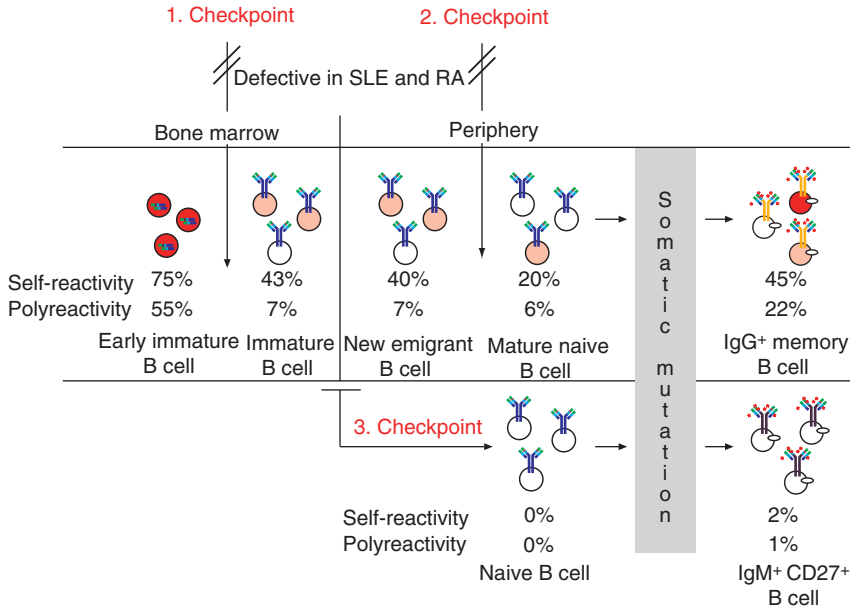

FIGURE 3 B-cell self-tolerance checkpoints under normal circumstances and in autoimmunity. The majority of newly generated early immature B cells in bone marrow of healthy donors express self-reactive antibodies (75%; red) including antibodies polyreactive with diverse antigens (55%). Highly polyreactive and self-reactive antibodies (dark red) are efficiently counterselected at the first checkpoint for self-tolerance in immature B cells in the bone marrow (7% polyreactivity), but a significant fraction of immature B cells retains low levels of self-reactivity (43%; light red). Self-reactive immature B cells leave the bone marrow and are referred to as new-emigrant B cells in the blood stream (40% self-reactivity; 7% polyreactivity). Counterselection of self-reactive new emigrant B cells occurs again in the periphery before maturation into naive B cells (20% self-reactivity; 6% polyreactivity). Self-reactive and polyreactive B cells are efficiently excluded from the circulating IgM^+ $CD27^+$ B-cell compartment at a third checkpoint for self-tolerance before the onset of somatic mutation. In contrast, self-reactive and polyreactive antibodies are frequently expressed by circulating IgG^+ memory B cells (45% and 22%, respectively), the majority of which acquired self-reactivity by somatic mutation (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003). Defective early self-tolerance is associated with high levels of naive B cells in peripheral blood of SLE and RA patients (Meffre *et al.*, 2004; Tiller *et al.*, 2007; Tsuiji *et al.*, 2006; Wardemann *et al.*, 2003).

identified an ever-growing number of molecules, immune cell types, and genetic and environmental factors that are involved in the pathogenesis of SLE. Nevertheless, high autoantibody titers and thus a major breakdown in B-cell self-tolerance remain a hallmark feature of SLE. Understanding where and when autoreactive B cells develop and how they are regulated

under normal circumstances and in SLE is a prerequisite for the development of novel, highly efficient therapies. Our finding that the majority of newly assembled V(D)J genes expressed by immature bone marrow B cells are self-reactive and that somatic mutation contributes significantly to the generation of self-reactive memory B cells in healthy individuals suggests that deregulation at any stage during B-cell development and maturation enhances the risk to develop autoantibodies (Fig. 3). Genetic predisposition and polymorphisms in a large number of genes that directly or indirectly regulate B cells and environmental factors, such as infections, are likely to contribute significantly to the abnormal regulation of autoreactive B cells at early and/or late self-tolerance checkpoints in affected individuals. The finding that defective early self-tolerance checkpoints frequently persist during clinical remission suggests that current treatment protocols fail to reset the B-cell system and predicts that effective strategies should aim at targeting early as well as late B-cell differentiation stages in SLE.

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CHAPTER 4

Manipulation of Regulatory T-Cell Number and Function with CD28-Specific Monoclonal Antibodies

Thomas Hünig*

Contents

*Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany

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Abstract

Suppressor or "regulatory" CD4 T cells play a key role in the control of autoimmunity and overshooting immune responses to foreign antigens, but can also obstruct effective anticancer therapies. The homeostasis and activation of these regulatory T cells (Treg cells) is tightly connected to that of effector CD4 T cells via the costimulatory receptor CD28 and the cytokine IL-2: Both subsets require costimulation to be activated by antigen, and Treg cells additionally depend on IL-2 produced by effector CD4 T cells in a costimulation-dependent fashion. Depending on the therapeutic aim, blockade, or stimulation of CD28 with monoclonal antibodies (mAb) can therefore profoundly affect the size and activity of the Treg compartment. In this chapter, experiments performed in rodents with distinct types of CD28-specific mAb, and the recent failure to translate CD28-driven Treg activation into humans, are discussed.

1. AIMS OF THIS REVIEW

The rediscovery of suppressor T cells, now termed regulatory T cells (henceforth referred to as Treg cells), has led to a surge in basic and applied research aimed at understanding Treg-cell biology and at developing tools for their clinical application in autoimmunity and inflammation (Sakaguchi *et al.*, 2006). Two main avenues are being explored: the induction, propagation, and application of antigen-specific Treg-cell populations, thought to allow the most specific approach to dampening unwanted immune responses (Masteller *et al.*, 2005; von Boehmer, 2005), and polyclonal Treg activation which relies on the preexisting ''natural'' Treg repertoire. Moreover, inhibition or even elimination of Treg cells is considered an important therapeutic option in cancer immunology, with the accepted risk of autoimmune side effects.

The costimulatory receptor CD28 plays a decisive role in Treg-cell biology (Sansom and Walker, 2006). Accordingly, monoclonal antibodies (mAbs) manipulating the CD28 pathway have profound effects on the Treg compartment *in vivo*. In particular, stimulatory or ''superagonistic'' CD28-specific mAbs are potent polyclonal activators of Treg cells, and have proven highly effective in a wide range of rodent models for autoimmune and inflammatory diseases (Hünig and Dennehy, 2005). The high hopes raised by these preclinical data were shattered when the first-in-man application of the human CD28 superagonist TGN1412 resulted in a life-threatening cytokine release syndrome in six healthy volunteers early in 2006 (Suntharalingam *et al.*, 2006).

It is the aim of this chapter to discuss the role of CD28, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and IL-2 in the generation and maintenance of Treg cells, to bring into focus the work done with CD28-specific mAb in rodent models of autoimmunity and inflammation, to suggest a Treg-based mechanism for the therapeutic effects of CD28-specific mAb in these models, and to look at and beyond the tragic outcome of the TGN1412 trial, with regard to the feasibility of using ''Treg expanders'' as a general principle in the treatment of autoimmune pathologies.

2. POSITIVE AND NEGATIVE REGULATION OF T-CELL RESPONSES BY THE CD28/CTLA-4 SYSTEM

Antigen-driven activation of naive T cells is controlled by ''professional'' antigen-presenting cells, usually dendritic cells (DC), which provide an essential second or costimulatory signal (Sharpe and Freeman, 2002) (Fig. 1). There is general agreement that the biological function of this

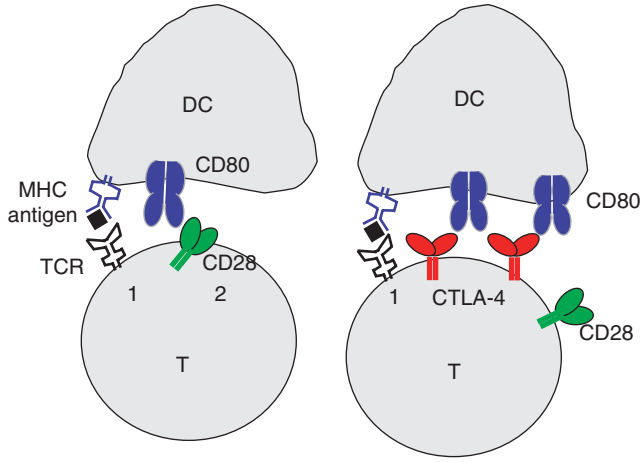

FIGURE 1 Costimulation of the T-cell response by CD28 and its counterregulation by CTLA-4. Note that besides the displacement of CD28 by bivalent CTLA-4 molecules, intracellular signaling events contribute to counterregulation by CTLA-4.

control mechanism is to incorporate the information which DC receive about the nature of an invading pathogen through their innate receptors into the decision on the necessity for and quality of an adaptive immune response (Steinman and Hemmi, 2006). This information is relayed to the naive T cells by the degree of upregulation of costimulatory ligands, and by cytokines that influence the differentiation of costimulated T cells into functionally specialized subsets.

The most important costimulatory receptor on T lymphocytes is the homodimeric glycoprotein CD28 (Hara and Fu, 1985), a member of the Ig superfamily expressed on all rodent T cells, the vast majority of human CD4, and about half of human CD8 T cells (Lum *et al.*, 1982). Its ligands, CD80 and CD86, also belong to the Ig superfamily (Sharpe and Freeman, 2002). They interact with CD28 in the central part of the immunological synapse [the central supra-molecular activation cluster (c-SMAC)] during T-cell activation by DC (Bromley *et al.*, 2001). Like CD28, CD80 is a homodimer, whereas CD86 exists as a monomer. Importantly, however, interaction of CD28 with either CD80 or CD86 is monovalent due to steric constraints (Collins *et al.*, 2002; Evans *et al.*, 2005).

CTLA-4 (CD152) is a negative regulator opposing CD28 stimulation (Chambers *et al.*, 2001; Krummel and Allison, 1995) (Fig. 1). It binds to the same ligands as CD28, albeit with a 20-fold (for CD80) and even 100-fold (for CD86) higher affinity than CD28 (Collins *et al.*, 2002). Moreover, the bivalency of CTLA-4 in ligand interaction boosts this superior affinity by a dramatic avidity effect (Collins *et al.*, 2002), allowing the negative

regulator CTLA-4 to effectively override the positive regulator CD28. Accordingly, CTLA-4 is not constitutively expressed but is induced as a result of T-cell activation, with the notable exception of Treg cells (Alegre *et al.*, 1996). Once formed, CTLA-4 protein is primarily found intracellularly but is transported toward the immunological synapse in response to T-cell receptor (TCR) stimulation, where it dampens and eventually inhibits T-cell activation (Pentcheva-Hoang *et al.*, 2004). Further complexity is added to the CD28/CTLA-4 system by differences in the preference of these two receptors for the ligands CD80 and CD86 (Collins *et al.*, 2002; Pentcheva-Hoang *et al.*, 2004), and by differential regulation of those ligands on DC and other antigen-presenting cells (APC) (Sharpe and Freeman, 2002). The precise mechanism by which CTLA-4 interferes with the activation of conventional T cells (besides ligand competition at the cell surface) is incompletely understood. Since these aspects have no direct bearing on the issue of Treg manipulation with CD28-specific mAbs, they will not be considered further (Chikuma and Bluestone, 2003; Sansom and Walker, 2006).

3. THE IMPORTANCE OF CD28 AND CTLA-4 FOR THE GENERATION AND HOMEOSTASIS OF TREG CELLS

3.1. "Natural" regulatory cells

3.1.1. Phenotype and derivation

''Natural'' Treg cells are essential guardians against autoimmunity and other forms of immunopathology such as allergies (Sakaguchi *et al.*, 2006). They are a subset of CD4 T cells which is selected in the thymus for recognition of self-antigens presented by major histocompatibility complex (MHC) class II molecules (Hsieh *et al.*, 2004). In this selection process, medullary epithelial cells promiscuously expressing tissue-specific antigens are thought to play an important role (Kyewski and Klein, 2006). The autoreactive repertoire of Treg cells functionally translates into immunosuppression when self-antigens are recognized in peripheral lymphoid organs or in inflamed tissues.

A unique feature of Treg cells is their expression of the transcription factor Foxp3 (Fontenot and Rudensky, 2005). The impotance of Foxp3 in directing Treg lineage development is underscored by its ability to confer the suppressor phenotype to conventional CD4 T cells by gene transfer and by the autoimmune phenotype of Foxp3-deficient (scurfy) mice. In contrast, none of the cell surface markers currently used to identify Treg cells are unique to this lineage. Among those, CD25 (IL-2Rα), the marker originally used to characterize murine Treg cells, shows the highest concordance with Foxp3 in a resting immune system. Even under immune stimulation (as is the case in a ''normal'' human donor), high CD25 expression is largely

confined to Treg cells, reflecting the importance of IL-2 in the regulation of Treg-cell numbers and function (Section 3.2).

In addition to their thymic derivation as a fully differentiated T-cell subset, Treg cells can also be induced from conventional CD4 T cells by chronic antigenic stimulation in the periphery (von Boehmer, 2005).

3.1.2. Suppressor function

The functional importance of Treg cells in preventing autoimmune and inflammatory disease has been impressively demonstrated in a broad spectrum of rodent models, and the evidence for a similarly important role in the control of human immunopathies is compelling (Sakaguchi *et al.*, 2006). Accordingly, antigen-specific or polyclonal expansion of the Treg compartment is currently a major focus of preclinical and clinical immunological research.

In vitro, the suppressive function of Treg cells is usually measured as their ability to inhibit proliferation of effector T cells. This effect is strictly dependent on TCR engagement, requires cell–cell contact between Treg cells and effector T cells or APC (if involved), and cannot be replaced by the Treg-derived suppressor cytokine IL-10 (Thornton and Shevach, 2000). *In vivo*, however, IL-10 plays an essential role in some models of immunopathology (Maloy *et al.*, 2003; Singh *et al.*, 2001). Transforming growth factor beta (TGF-β), also thought to be involved in Treg-mediated control of adverse immune responses, seems to act as a survival and differentiation factor for Treg cells rather than as an effector suppressor cytokine (Marie *et al.*, 2005).

3.2. IL-2 dependence of Treg cells

IL-2 was among the first cytokine genes to be inactivated by gene targeting. The observed phenotype of an autoimmune-lymphoproliferative disease was totally unexpected at that time (Sadlack *et al.*, 1993), but was soon confirmed by similar results obtained in mice lacking the α- or β-chains of the IL-2R, CD25, and CD122, respectively, which succumbed to a similar ''IL-2 deficiency syndrome'' (reviewed in Hünig and Schimpl, 1997). In the meantime, it has become firmly established that paracrine IL-2 has a nonredundant role in Treg-cell survival and activation, explaining the puzzling overreaction of the immune system in the absence of what was considered the major T-cell growth factor (Furtado *et al.*, 2002; Krämer *et al.*, 1995; Maloy and Powrie, 2005; Papiernik *et al.*, 1998; Schimpl *et al.*, 2002; Wolf *et al.*, 2001).

This conclusion was formally tied to Foxp3-expressing Treg cells through their marked reduction in the periphery of the respective IL-2 and IL-2R knockout strains (D'Cruz and Klein, 2005; Fontenot *et al.*, 2005; Maloy and Powrie, 2005), and from a gradual loss of Treg cells in normal

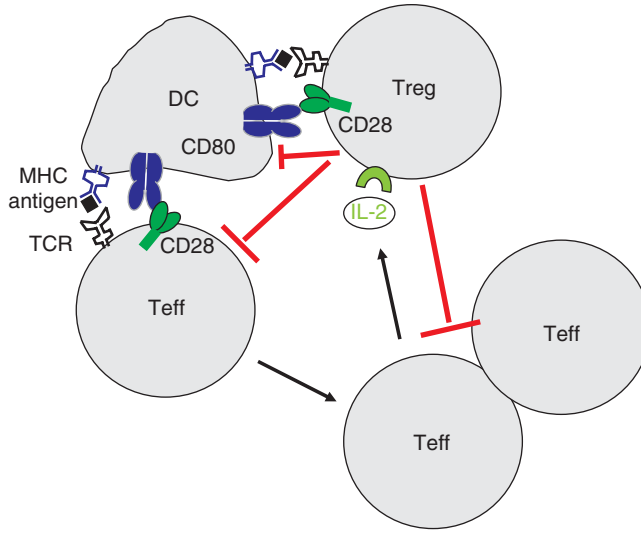

FIGURE 2 Roles of CD28 and IL-2 in Treg homeostasis. Treg cells require CD28 in *cis* for both thymic generation and peripheral homeostasis. For survival, expansion and activation in the periphery, they additionally need CD28 in *trans* on effector CD4 T cells to enable production of paracrine IL-2.

mice treated with neutralizing anti-IL-2 mAb (Setoguchi *et al.*, 2005). Moreover, mixed bone marrow chimeras constructed from IL-2- and IL-2R-deficient donors have established that the Treg cells themselves depend on functional IL-2R genes but, in keeping with their own inability to produce IL-2, are not affected by IL-2 gene inactivation. In contrast, the effector cells which they control do not require IL-2R to be properly regulated, but instead have to express an intact IL-2 gene, indicating that Treg cells thrive on the IL-2 provided by effector T cells (Furtado *et al.*, 2002; Krämer *et al.*, 1995).

Importantly, this IL-2-mediated regulatory circuit (Fig. 2) appears to operate both in Treg-cell homeostasis (where IL-2 would be provided by inapparent T-cell responses to environmental and self-antigens) and in the control of overt (auto)immune responses (Sections 3.4 and 3.5).

3.3. Significance of CTLA-4 for Treg function

In the present context, a possible function of CTLA-4 in Treg-mediated suppression is of interest because of the shared ligands with CD28 and the consequences of their blockade. Treg cells are the only T-cell subset constitutively expressing CTLA-4 (Takahashi *et al.*, 2000). It is a matter of ongoing debate whether this ''just'' reflects their continuous

confrontation with self-antigens and thus is indicative of counterregulation in *cis*, or whether CTLA-4 has a specific role in Treg-mediated immunosuppression (reviewed in Sansom and Walker, 2006). The former view is supported by the presence of Foxp3-expressing Treg cells in CTLA-4-deficient mice which, at least *in vitro*, are functional (Tang *et al.*, 2004a). Since, however, these CTLA-$4^{-/-}$ Treg cells seem to adapt by upregulating TGF-β and IL-10 production (and possibly other mechanisms of suppression) (Read *et al.*, 2006; Tang *et al.*, 2004a), these results do not exclude a role for CTLA-4 in suppression mediated by wild-type Treg cells. This possibility is indeed supported by mixed bone marrow chimeras in which the lymphoproliferative phenotype conferred by CTLA-4 deficiency is corrected by wild-type bone marrow—an effect of suppression ''in *trans*'' most easily explained through Treg cells (Bachmann *et al.*, 1999). Moreover, a nonredundant function for CTLA-4 expressed by Treg cells has been demonstrated in a mouse model of inflammatory bowel disease using a combination of CTLA-4 blockade by Fab fragments of anti-CTLA-4 antibodies and gene-targeting approaches (Read *et al.*, 2000, 2006). In conclusion, there is convincing evidence that in at least some experimental setups, CTLA-4 expressed by Treg cells does exert an important function in suppressing immune pathology. Several mechanisms by which CTLA-4 would mediate this suppressor function have been proposed. As an example, evidence has been presented that Treg cells may upregulate the immunosuppressive enzyme indoleamine 2,3-dioxygenase in DC by CTLA-4–CD80/CD86-mediated ''back-signaling'' (Fallarino *et al.*, 2003).

3.4. Role of CD28 in generation and homeostasis of Treg cells

Like all rodent and most human CD4 T cells, Treg cells constitutively express CD28. There is overwhelming evidence indicating an important and nonredundant role for CD28 in the generation, homeostasis, and functional activation of Treg cells. Most obviously, this is illustrated by the reduced numbers of Treg cells in CD28-deficient mice (Salomon *et al.*, 2000), a finding corroborated by a similar deficiency in mice lacking the CD28 ligands CD80/CD86 (Bour-Jordan and Bluestone, 2002; Lohr *et al.*, 2003) or undergoing chronic ligand blockade by mAb (Salomon *et al.*, 2000; Tang *et al.*, 2003). The interpretation of the latter finding is, however, confounded by the double role of CD80 and CD86 as ligands for both CD28 and CTLA-4.

As mentioned previously, it has been known for some time that mice deficient in IL-2 or IL-2-specific components of the IL-2R lack Treg cells. Since IL-2 production is impaired in the absence of CD28-mediated costimulation, the question arises whether Treg-cell reduction in CD28-deficient mice is due to the absence of CD28 on the Treg cells themselves, on the

IL-2-producing effector CD4 T cells, or on both. This question has recently been answered: For thymic Treg differentiation, CD28 expression on the Treg cells themselves is necessary and sufficient; whereas for peripheral T-cell homeostasis, CD28 is required on both conventional and Treg cells.

Thus, the deficiency of *thymic* Treg cells in $CD28^{-/-}$ (Tai *et al.*, 2005; Tang *et al.*, 2003), but not $IL\text{-}2^{-/-}$ mice (D'Cruz and Klein, 2005; Fontenot *et al.*, 2005) (which are Treg deficient in the periphery) already suggested that during thymic generation, a CD28 signal in *cis* (most likely given by medullary expression of CD80), but not IL-2 provision in *trans*, is required. Furthermore, mixed bone marrow radiation chimeras constructed from CD28 and IL-2-deficient donors have established that IL-2 derived from wild-type cells supports development of a peripheral Treg compartment derived from IL-2-deficient but not from CD28-deficient precursors, formally proving the dual requirement for CD28 on effector and Treg cells (Tai *et al.*, 2005).

3.5. Role of CD28 and IL-2 in antigen-driven expansion and activation of Treg cells

Most likely, the numerical expansion of Treg cells in response to antigen *in vivo* is controlled by the same set of interactions involving CD28 and IL-2 which are required for Treg-cell homeostasis. Thus, IL-2-producing effector T cells are thought to facilitate the expansion and activation of Treg cells which, in turn, switch off IL-2 production in the effectors and subsequently undergo clonal contraction and functional quiescence as a result of IL-2 withdrawal (Figs. 2 and 3A). Direct evidence for such sequential waves of effector and Treg-cell activation has been presented in a transgenic mouse model of systemic autoimmune disease (Knoechel *et al.*, 2005).

As discussed previously with respect to T-cell homeostasis, the initial expansion and activation of Treg cells in this negative feedback loop depends on CD28 at two checkpoints (Fig. 2): the induction of IL-2 production in the conventional or effector CD4 T cells, and the costimulation of the Treg cells themselves after encounter of the relevant self- or environmental antigen. While CD28 dependence of IL-2 production is a classic means to study costimulation, evidence for a role of CD28-mediated costimulation in driving antigen-specific Treg proliferation comes from experiments with DC, either lacking or overexpressing CD28 ligands (Tang *et al.*, 2003; Yamazaki *et al.*, 2003).

An important feature of this model is that besides counteracting expansion and effector function of conventional T cells in the secondary lymphoid organs themselves, a subset of the expanded Treg cells will scan the tissues and remain at sites of inflammation, presumably retained on the basis of their antigen specificities (Fig. 3A). These ''tissue-seeking'' Treg cells are marked by the integrin $\alpha_E\beta_7$ and are equipped with a special set of

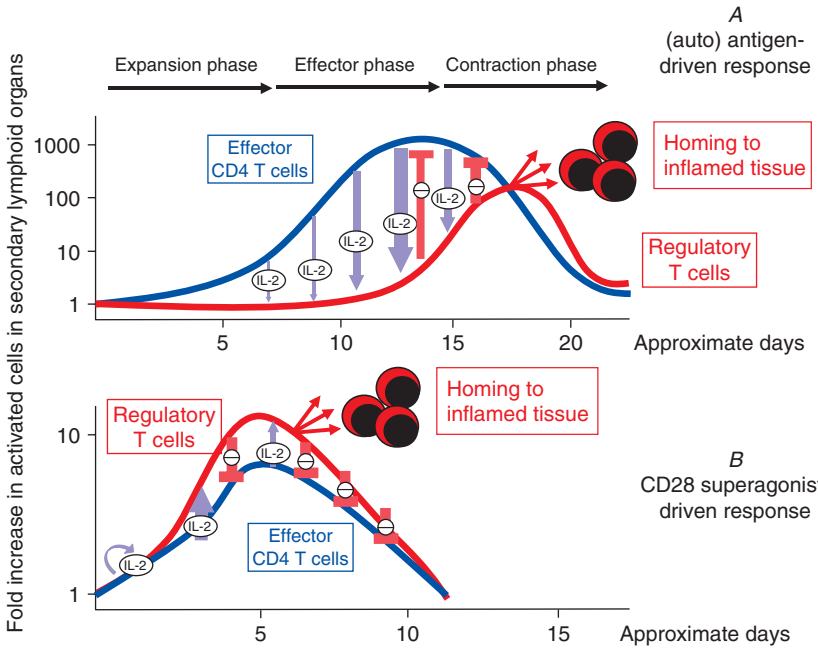

FIGURE 3 Interrelationship of clone sizes of effector and Treg cells. (A) In antigen-driven responses, expansion of IL-2-producing effector T cells is followed by that of Treg cells, which then turn off IL-2 production and clonal expansion in the effector T cells and contract themselves for lack of paracrine IL-2. (B) In CD28 superagonist-driven responses, activation of effector and Treg cells occurs simultaneously and favors that of Treg cells. Note that in both settings, Treg cells are dispatched to inflamed sites where they are retained and combat immunopathology.

cell adhesion molecules and receptors for proinflammatory chemokines (Huehn and Hamann, 2005; Huehn *et al.*, 2004). As long as they receive the appropriate antigenic stimuli and survival signals, they will remain on-site to control inflammation and effector T-cell responses.

4. MANIPULATING THE CD28 PATHWAY: GENERAL CONSIDERATIONS

The key role of CD28 in controlling the activation of both effector and Treg cells makes it an attractive target for manipulating desired or pathologic immune responses. Both recombinant soluble natural receptors or ligands and mAbs have been employed and are under experimental and/or clinical investigation. These can be viewed either as blocking or as activating agents, although that distinction may require modification as new data on the biological function of the cell surface receptors addressed are

emerging. For example, soluble recombinant proteins constructed by fusing the extracellular domain of CTLA-4 to the Fc portion of IgG are effective blockers of CD28-mediated costimulation (Linsley *et al.*, 1991), due to the manyfold higher avidity of CTLA-4 as compared to CD28 for their shared ligands CD80 and CD86 (Collins *et al.*, 2002). Indeed, such recombinant fusion proteins have been successfully introduced into the treatment of rheumatoid arthritis (Nogid and Pham, 2006). However, a report suggests that the immunosuppressive function of CTLA-4-Ig constructs observed in animal models (Turka *et al.*, 1992) may, in part, be due to back signaling of the ligated costimulatory ligands, CD80/CD86, into the DC, where the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) is upregulated (Grohmann *et al.*, 2002). In addition, binding of CTLA-4-Ig fusion proteins to CD80/CD86 is likely to interfere with both ligation of the costimulatory receptor CD28 and that of its counterregulator CTLA-4 itself, which is constitutively expressed by Treg cells and induced after activation on conventional T cells. As a result, the fusion protein may interfere with both positive and negative signals controlling T-cell activation.

This example may serve to illustrate the complexity of the results obtained with artificial ligands to the four components of the CD28/CTLA-4–CD80/CD86 system, which cannot be exhaustively discussed within this chapter. Rather, I will specifically focus on the results obtained in rodent models using CD28-specific mAbs of two types: ''conventional'' and ''superagonistic.''

5. CONVENTIONAL AND SUPERAGONISTIC mAb TO THE COSTIMULATORY RECEPTOR CD28

5.1. Epitope–function relationship

CD28 was first identified by mAb in humans as a homodimeric cell surface glycoprotein (Tp44). The original mAb 9.3 as well as numerous additional mAbs to human CD28 have costimulatory activity as originally assessed by their ability to complement phorbol myristate acetate (PMA) in polyclonal T-cell activation (Hara *et al.*, 1985). Research on CD28 function in rodent models became possible when the first mouse-specific (Gross *et al.*, 1990) and rat-specific (Tacke *et al.*, 1995) mAb became available 5 and 10 years after identification of the human molecule, respectively. As described for the human system, these CD28-specific mAb displayed costimulatory function *in vitro*, that is they synergized with mAb to the TCR complex in the activation of resting T cells but were *per se* not stimulatory *in vitro*. This type of CD28-specific mAb will be referred to as ''conventional.''

The use of the mouse as a highly efficient host for the production of rat CD28-specific mAb has permitted the isolation of a large number of

hybridomas, which were systematically investigated for functional activity, using purified resting T cells and TCRβ-specific mAbs as signal 1. Surprisingly, these novel mAb fell into two functionally distinct categories: conventional ones that would costimulate together with signal 1, hence providing signal 2; and other, ''superagonistic'' antibodies which fully activated resting T cells even without TCR ligation (Tacke *et al.*, 1997). This functional difference was not explained by antibody class or differential binding avidity. Rather, systematic epitope mapping using rat/mouse CD28 chimeras revealed a consistent pattern of epitope–function relationship (Lühder *et al.*, 2003): Conventional CD28-specific mAb bound to an epitope that was sensitive to a mutation close to the ligand-binding site at the ''top'' of the CD28 molecule, whereas binding of CD28 superagonists required an intact loop protruding laterally from the Ig-like domain of CD28, the C″D loop. Importantly, this function–epitope correlation held true for conventional and superagonistic human CD28-specific mAb that were subsequently isolated (Lühder *et al.*, 2003). When recently, mouse anti-mouse CD28-specific mAb were generated in CD28-deficient mice, again conventional and superagonistic mAb (called E18 and D665, respectively) were identified which fitted in the previous scheme (Dennehy *et al.*, 2006) (Fig. 4).

Because of their specificity for the ligand-binding region of CD28, conventional (but not superagonistic) mAb to CD28 may, on one hand, mimic the natural receptor–ligand interaction and, on the other hand, interfere with it. We have directly tested the effects of the conventional mouse CD28-specific mAb E18 and the superagonist D665 on ligand binding (Beyersdorf, T. Kerkau, and T. Hünig, unpublished data). As expected, binding of recombinant CD80-Ig fusion protein to mouse T cells was unimpaired by the CD28 superagonist D665, but was efficiently blocked by the conventional mAb E18. With regard to the commonly used conventional hamster anti-mouse CD28 mAb 37.51, a previous report (Yu *et al.*, 2004) failed to observe binding (when mixing the mAb and a CD28-Ig fusion protein in solution, before incubation with CD80-expressing B-cell blasts), and in our hands this antibody is much less efficient than E18 in blocking the binding of soluble CD80 to cell-bound CD28, a finding of some relevance for the interpretation of results obtained by *in vivo* treatment with this reagent (see Section 7).

5.2. Mode of mAb binding: A clue to superagonism?

In the case of a human CD28-specific superagonist, cocrystallization of Fab fragments with recombinant CD28 revealed a lateral binding mode (Evans *et al.*, 2005), which predicts extensive cross-linking between the homodimeric CD28 molecules through bivalent interaction with the mAb. Since in their interactions with the natural ligands CD80 and CD86, CD28

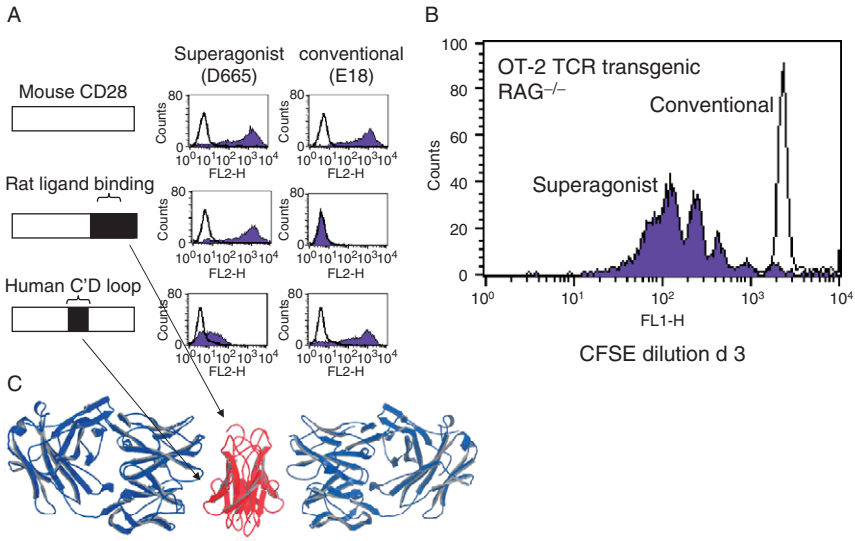

FIGURE 4 Epitope–function relationship of CD28-specific mAb. (A) Genetic epitope mapping of conventional and superagonistic mouse anti-mouse mAb (Dennehy *et al.*, 2006). Identical results were found for rat and human CD28 (Lühder *et al.*, 2003). (B) Induction of proliferation in TCR transgenic, MHC class II-restricted T cells by superagonistic but not conventional mouse anti-mouse CD28 mAb (Dennehy *et al.*, 2006). (C) Lateral binding of Fab fragments from a mouse anti-human CD28 superagonist to CD28. The extracellular domain of CD28 is viewed from the side (Evans *et al.*, 2005).

is monovalent whereas CTLA-4 is bivalent (Collins *et al.*, 2002), we hypothesized that a bivalent, CTLA-4-like interaction of CD28 with the superagonistic mAb may contribute to the superagonistic signaling properties of these antibodies; or conversely, that monovalency of CD28 in its interaction with natural ligands is important to keep the activating signal under the control of the TCR.

We have presented evidence in support of this view (Dennehy *et al.*, 2006): First, the stoichiometry of binding of conventional (E18) and superagonistic (D665) mouse CD28-specific mAb to CD28, as assessed by Biacore analysis, follows this prediction. More intriguingly, it was also possible to ''directly'' activate T cell lines with recombinant CD80 if the T cells expressed chimeric receptors allowing bivalent interactions (such as binding of CD80-Ig to a chimeric receptor consisting of extracellular CTLA-4 and intracellular CD28).

As a competing hypothesis for the stimulatory effects of superagonistic antibodies, the proximity of the epitope recognized by CD28 superagonists to the cell membrane has been proposed as the essential factor.

Thus, within the framework of the ''kinetic segregation model'' of T-cell activation at the immunological synapse (Davis and van der Merwe, 2006), it was suggested that mAb binding at a membrane-proximal, but not at a membrane-distal site of CD28, would lead to the exclusion of large phosphatases (mainly CD45), during mAb-mediated apposition of T cells to either a plastic surface or to an APC presenting the mAb via Fc receptors (FcR) (Evans *et al.*, 2005).

5.3. Signaling pathways

The molecular basis of costimulation in the sense of intracellular signal transduction is only partially known (Rudd and Raab, 2003). From the comparison of TCR-only versus costimulated responses, it is clear that costimulation-independent signals (such as an initial calcium flux or NFAT translocation) can emanate from the TCR; conversely, our results using CD28 superagonists suggested that signaling cascades resulting in full T-cell activation can be transduced by CD28 alone, if appropriately addressed (Bischof *et al.*, 2000; Hünig and Dennehy, 2005). This view of truly CD28 autonomous signals leading to full T-cell activation was supported by our findings that the proximal components of the TCR signaling machinery TCRζ and Zap-70 are not [and the linker for activation of T-cells (LAT) is only marginally] phosphorylated above background levels as a result of CD28 superagonist stimulation (Dennehy *et al.*, 2003, 2007; Lühder *et al.*, 2003).

However, we made the surprising observation that constitutive proximal TCR signaling is an absolute requirement for the mitogenic effects of CD28 superagonists (Dennehy *et al.*, 2007). Genetic and biochemical evidence suggests that these ''tonic'' TCR signals are amplified at the level of the SLP-76 signalosome and that the participation of Vav1 in this signalosome is essential (Dennehy *et al.*, 2007). Furthermore, studies using small-molecule inhibitors (Bischof *et al.*, 2000; Dennehy *et al.*, 2007) as well as mutant mice with altered cytoplasmic CD28 signaling motifs (unpublished data) indicate that initiation of signaling by CD28 superagonists is dependent on lck but not (and unexpectedly so) on PI3 kinase.

As compared to costimulation, proliferative responses and IL-2 production induced by CD28 superagonists *in vitro* can be of similar magnitude, indicating that the signaling pathways required for cell cycling and IL-2 production are fully active in both modes of stimulation. There are, however, differences in the kinetics of calcium fluxing (Dennehy *et al.*, 2003), translocation of distinct members of the nuclear factor of activated T-cells (NFAT) family of transcription factors (Bischof *et al.*, 2000) and of GATA-3 (Rodriguez-Palmero *et al.*, 1999).

Qualitative differences in T-cell responses to costimulation versus CD28 superagonist stimulation observed *in vitro* further underscore the incomplete identity of the signaling pathways that are activated in

the two modes of T-cell stimulation. Thus, rat CD28 superagonist stimulation of resting T cells is much more efficient than costimulation in priming T cells for IL-4-driven Th2 differentiation (Rodriguez-Palmero *et al.*, 1999). Furthermore, the antiapoptotic effect of CD28-mediated costimulation on TCR-activated T cells is more pronounced if the TCR is not ligated by inducing proliferation with CD28 superagonists, a phenomenon most likely explained by the marked upregulation of Bcl-xL and lack of CD95L expression under these conditions (Kerstan and Hünig, 2004).

In summary, T-cell activation by CD28 superagonists is dependent on, but does not address, the proximal TCR signaling machinery. It is as effective as costimulation with regard to the induction of proliferation and IL-2 production while leading to some distinct functional effects including a predisposition toward Th2 differentiation and increased resistance to apoptosis.

Finally, it should be noted that at least some ''conventional'' mAb also have signal-transducing capacity in the absence of TCR ligation, resulting in transcription of ''CD28-only'' genes via the Vav-SLP76 signalosome (Diehn *et al.*, 2002; Nunes *et al.*, 1994; Raab *et al.*, 2001; Riley *et al.*, 2002). This will also be a recurrent theme in discussing the *in vivo* effects of CD28-specific mAb.

6. *IN VITRO* EXPANSION OF TREG CELLS WITH THE HELP OF CD28-SPECIFIC mAb

6.1. Costimulation

For efficient *in vitro* expansion, Treg cells require costimulation in *cis* together with a source of IL-2. For the former, synthetic beads coated with mAb to the TCR complex and (conventional) mAb to CD28 have become the most widely employed tool in experimentation and clinical use (Hoffmann *et al.*, 2004; Tang *et al.*, 2004b). IL-2 may either be derived from conventional CD4 T cells contained in the same culture, or be externally provided. Since overgrowth by conventional CD4 cells presents a major problem in large-scale expansion of Treg cells, and a separation is difficult once all T cells express CD25 as a result of *in vitro* stimulation, it is desirable to start cultivation with highly purified Treg cells. As a potentially useful marker for the depletion of conventional CD4 cells, IL-7Rα (CD127), which is absent from human Treg cells, was identified (Liu *et al.*, 2006; Seddiki *et al.*, 2006).

6.2. CD28 superagonists

Treg cells respond very well to CD28 superagonists, although as in costimulation, IL-2 is additionally required for long-term expansion. In a comparison of rat Treg-cell expansion by costimulation and superagonist

activation in the presence of IL-2, the latter was found superior (Beyersdorf *et al.*, 2006). Possibly, this has to do with the marked antiapoptotic effects of CD28 superagonists and the avoidance of pro-apoptotic effects of TCR-stimulation (Kerstan and Hünig, 2004). Importantly, CD28 superagonist plus IL-2 expanded Treg cells proved highly active in standard *in vitro* suppression assays. Of note, exertion of suppressive function by these long-term expanded Treg cells still relied on triggering of the TCR and on cell–cell contact (Beyersdorf *et al.*, 2006). The conservation of these salient features of Treg effector function is important with regard to the use of expanded Treg populations as a therapeutic agent.

7. *IN VIVO* EFFECTS OF CONVENTIONAL CD28-SPECIFIC mAb

7.1. General considerations

Immunomodulation with conventional CD28-specific mAb may, theoretically, have three distinct outcomes: (1) antibody-mediated costimulation, as is observed *in vitro*, may activate T cells, including autoreactive (pathogenic and regulatory) T cells encountering self-antigens on tissues devoid of costimulatory ligands; (2) CD28 modulation and prevention of physiologic CD28–ligand interactions may result in a loss of costimulation and hence in immunosuppression; (3) long-term provision of signal 2 (CD28) without signal 1 (TCR) might exhaust the T-cell response to a subsequent antigen encounter on professional APCs. In view of the critical requirement of CD28 for the homeostasis and function of Treg cells outlined previously, one might expect that inhibitory effects mediated by conventional CD28-specific mAb (outcomes 2 and 3) may not only result in transient immunosuppression, but could also lead to a loss of Treg cells, followed by autoimmunity once treatment is terminated.

7.2. Experimental findings

7.2.1. Results obtained in mice

In the mouse system, the widely used hamster anti-mouse mAb 37.51 was initially shown to costimulate T-cell responses *in vitro* (Gross *et al.*, 1992), but to interfere with superantigen-driven T-cell expansion *in vivo* (Krummel and Allison, 1995). Since this mAb does not block ligand binding, the inhibitory effect could result from an ''exhaustion'' of costimulatory signals during the pre-exposure time of the animals to the antibody before injection of the superantigen, to a hindrance of CD28 participation in the immunological synapse, or to the activation of a regulatory phenomenon, which would inhibit superantigen-driven clonal expansion.

An activating rather than a blocking mechanism underlying the effects of mAb 37.51 *in vivo* is indeed suggested by studies in which protection

from spontaneous development of diabetes was achieved by chronic treatment of young non-obese diabetic (NOD) mice via an IL-4-dependent pathway (Arreaza *et al.*, 1997). Similarly, lipopolysaccharide (LPS)-induced toxic shock was prevented in mice through an IL-10-dependent mechanism by pretreatment with this mAb (Wang *et al.*, 1997). Both of these results are reminiscent of the effects observed with CD28 super-agonists with single and lower antibody doses (see below).

T-cell activation rather than blockade was also proposed as an essential step in graft versus host (GvH) inhibition by mAb 37.51. Here, it was concluded that the mAb provides costimulation to the few alloreactive T cells inoculated, which then leads to their depletion via an IFN-γ-dependent mechanism (Yu *et al.*, 2004).

Finally, the observation that treatment with mAb 37.51 stimulates the migration of memory cells into tissues illustrates another outcome of activating effects of a conventional CD28-specific mAb *in vivo* (Mirenda *et al.*, 2006).

Using a different hamster anti-mouse CD28-specific mAb (PV.1; Abe *et al.*, 1995) in a prophylactic treatment protocol, prevention of autoimmune uveitis was observed albeit without the induction of long-term tolerance (Silver *et al.*, 2000). In mouse experimental autoimmune encephalomyelitis (EAE), both prevention and intervention with ongoing disease were achieved (Perrin *et al.*, 1999). In the latter set of experiments, the efficacy of Fab fragments unable to signal but shown to block CD28–ligand interaction indicated that a therapeutic effect can also be obtained by blockade, rather than by stimulation leading to immune deviation or activation of Treg cells. Thus, selective blockade of CD28 (as opposed to that of both CD28 and CTLA-4 via blockade of their common ligands) appears to be a valid option for transient, nondepleting immunosuppression.

In our own unpublished studies, we have used the conventional mouse anti-mouse CD28-specific mAb E18 (Dennehy *et al.*, 2006), which efficiently blocks CD80 binding (unpublished data). Besides this functional characteristic, this mAb (being of mouse origin) has the advantage of not eliciting an anti-Ig response even under chronic application. *In vivo*, mAb E18 blocks superantigen-driven T-cell proliferation without CD28 modulation or detectable T-cell activation, compatible with signal exhaustion, blockade, or sequestration of CD28 molecules from productive interactions in the synapse (Ding, Beyersdorf, Kerkau, and Hünig, unpublished data). Furthermore, chronic application (6 months) of this mAb reduces the representation of Treg cells within the CD4 T-cell compartment by about two-thirds, along with an about fourfold reduction of the CD4 compartment at large (Blank and Hünig, unpublished data). Importantly, however, no signs of autoimmunity were detected during or after release from anti-CD28 treatment, during which Treg numbers swiftly recover (Blank and Hünig, unpublished data). Since mAb E18 effectively prevents

and treats EAE (Lühder, Gold, and Hünig, unpublished data), these results confirm the therapeutic effects obtained with hamster anti-mouse Fab fragments and extend them by demonstrating the feasibility of prolonged treatment with blocking CD28-specific mAb, without lasting damage to the Treg compartment.

7.2.2. Results obtained in rats

In the rat system, the conventional CD28-specific mAb JJ319 (Tacke *et al.*, 1995), which was previously mapped to the ligand-binding region of CD28 but has not been formally shown to block CD80/CD86 interactions, has been effectively used to prolong the survival of vascular allografts (Dengler *et al.*, 1999; Otto *et al.*, 2002) and to prevent type 1 diabetes in biobreeding (BB) rats (Beaudette-Zlatanova *et al.*, 2006). In the vascularized heterotopic heart transplantation model, it was shown that the duration of graft acceptance correlated with modulation of CD28 from the T-cell surface without T-cell depletion, and that alloreactivity and graft rejection returned after cessation of antibody treatment and reexpression of CD28 (Dengler *et al.*, 1999).

7.2.3. Conclusions on treatment with conventional Anti-CD28 mAb

In summary, conventional CD28-specific mAbs require further subdivision with regard to their *in vivo* effects. Among these, transient inhibition of T-cell activation through either modulation or functional blockade of CD28, and activating effects leading to either depletion of transplanted alloreactive T cells in a lymphopenic setting or to immune deviation, may provide distinct opportunities for therapeutic intervention.

8. *IN VIVO* EFFECTS OF CD28 SUPERAGONISTS: PREDOMINANCE OF TREG-CELL ACTIVATION

8.1. Studies in rats

When rats were initially injected with superagonistic anti-CD28 mAb, a dose-dependent and transient lymphocytosis, which was well tolerated, was observed (Tacke *et al.*, 1997). In keeping with an apparent lack of toxicity, the cytokines induced most prominently at the peak of the response (as measured by RNase protection analysis on day 3) were IL-10 and, to a lesser degree, IL-4 both of which have anti-inflammatory properties (Rodriguez-Palmero *et al.*, 1999).

In further studies in the rat model, the source of IL-10 was traced to a subset of CD4 T cells which coexpressed CD25, high levels of CTLA-4, and the transcription factor Foxp3 and hence were identical with the ''natural'' Treg cells discussed previously (Beyersdorf *et al.*, 2005; Lin and Hünig, 2003). The frequency of these cells within the CD4 compartment

transiently increased from 5% before injection to about 20% on day 3 and gradually decreased to normal levels by day 10. Importantly, the Treg cells harvested at the peak of stimulation had an activated phenotype ($CD25^{high}$, $CTLA\text{-}4^{high}$) and showed about tenfold increased suppressor function on a per cell basis as compared to Treg cells isolated from unstimulated rats (Beyersdorf *et al.*, 2005; Lin and Hünig, 2003).

8.2. Studies in mice

Similar experiments were performed with the novel mouse CD28-specific superagonist D665 (Dennehy *et al.*, 2006). As had been observed in rats, treatment with superagonistic (but not with conventional) CD28-specific mAb led to a marked enhancement of relative and absolute numbers of regulatory CD4 T cells expressing Foxp3 (as assessed by staining with mAb FJK-16s) (Fig. 5) (Na and Hünig, unpublished data). Thus, in comparison to CD4 T cells at large, the natural Treg-cell subset is dramatically ''over-expanded'' by the application of CD28 superagonists, resulting in a 10- to 20-fold increase in absolute cell number. Furthermore, the functional activation of these disproportionately expanded Treg cells observed in rats was confirmed in the mouse system as well (unpublished data).

Significantly, close monitoring of circulating cytokine levels between 30 min and 5 days revealed no proinflammatory cytokines (IFN-γ, TNF, IL-12) above background, whereas low levels of IL-4 and IL-10 were present on day 3 after mAb injection (Elflein and Hünig, unpublished data). These results suggest that the dramatic activation of Treg cells by the superagonistic mAb effectively suppressed the production of proinflammatory cytokines by effector T cells. This is in marked contrast to the cytokine release syndrome induced in mice by polyclonal activation of T cells with TCR-specific reagents, such as superantigens (Miethke *et al.*, 1992), or in humans by a CD3-specific antibody (Abramowicz *et al.*, 1989).

8.3. A model for preferential expansion of Treg cells in CD28-superagonist-stimulated rodents

8.3.1. Experimental basis

In both rats (Lin and Hünig, 2003) and mice (unpublished data), we have excluded by transfer experiments using carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled $CD4^+$ $CD25^-$ cells that the dramatic increase in Treg cells is due to conversion of conventional CD4 cells to Treg cells. This implies that the preexisting ''natural'' Treg cells are in fact the source of the enlarged suppressor cell population. Why, then, do they expand faster than conventional T cells? In brief, we believe that CD28 superagonists shortcut the CD28- and IL-2-dependent regulatory circuit for antigen-driven T-cell responses illustrated in Figs. 2 and 3A.

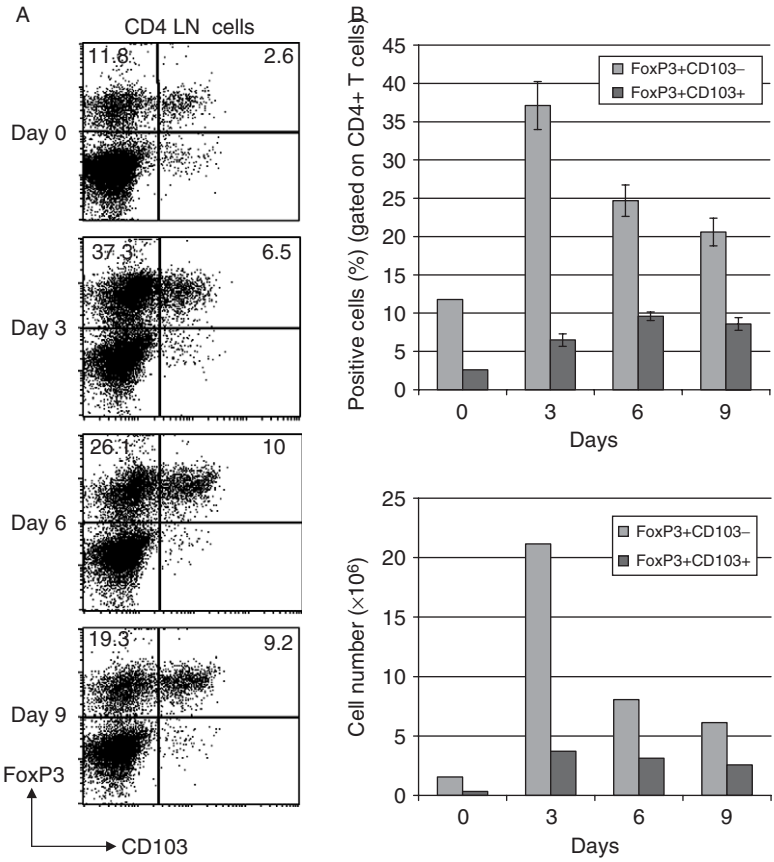

FIGURE 5 Transient expansion of mouse Treg cells after CD28 superagonist stimulation. Mice were i.p. injected with a single dose of mAb D665 at 5 mg/kg. (A) Two color stainings of CD4 lymph node cells. (B) Calculation of frequencies and absolute numbers of Treg cells in total lymph nodes. Note that both CD103-negative (lymph node seeking) and CD103-positive (tissue or inflammation seeking) Treg cells are transiently expanded.

The kinetics of conventional CD4 and Treg-cell expansion and contraction, shown in Fig. 3B, are based on our experimental data obtained in rats (Lin and Hünig, 2003; Tacke *et al.*, 1995) and mice (Fig. 4).

8.3.2. The role of CD28 and IL-2 signals in CD28-driven Treg expansion

Our data suggest that the rapid expansion of the Treg-cell compartment in CD28 superagonist-stimulated rats and mice is explained by the fact that these cells are preactivated, memory-like T cells which are poised for immediate responsiveness if the appropriate signals (here, CD28

superagonist stimulation and IL-2) are provided. Furthermore, the pronounced antiapoptotic effect of CD28 superagonist stimulation (Kerstan and Hünig, 2004) may counteract apoptosis associated with the rapid physiological turnover of Treg cells and thus further contribute to clonal expansion of this subset.

More precisely, we suggest that in contrast to antigen-driven responses, where Treg activation lags behind that of conventional T cells (Knoechel *et al.*, 2005) (Fig. 3A), CD28 superagonist stimulation simultaneously addresses both conventional and regulatory CD4 T cells, resulting in accelerated kinetics of Treg activation (Fig. 3B). As part of this concept, it is proposed that CD28 stimulation of conventional CD4 cells rapidly provides sufficient amounts of IL-2 for the expansion and activation of Treg cells, most likely before clonal expansion of conventional CD4 T cells even begins. Our failure to detect circulating IL-2 in the plasma of CD28-stimulated mice (Elflein and Hünig, unpublished data) does not contradict this hypothesis. Rather, it may reflect the immediate consumption of this cytokine by Treg cells, a notion born out by our own *in vitro* experiments (Lin and Hünig, 2003) and supported by data from other groups demonstrating that Treg cells efficiently act as an ''IL-2 sink'' (Barthlott *et al.*, 2005; Scheffold *et al.*, 2005), although this does not suffice to explain their suppressive effect (D'Cruz and Klein, 2005; Fontenot *et al.*, 2005; Maloy and Powrie, 2005).

The proposed involvement of CD28 signals in *cis* and *trans*, and of paracrine IL-2, in the rapid expansion of the Treg compartment is currently under investigation, using the appropriate knockout strains and neutralizing mAb. Initial results are in full accord with the previous scheme.

8.3.3. Control of effector T-cell function by activated Treg cells and clonal contraction

As a result of rapid Treg-cell activation and a fourfold greater numerical expansion than seen in the conventional (potential effector) CD4 T cells (Lin and Hünig, 2003; Tacke *et al.*, 1997), it is proposed that the latter are pushed into clonal contraction and functional inactivation before reaching their effector phase in the sense of effector cytokine (e.g., the proinflammatory cytokines IFN-γ and TNF) production. Since IL-2 production from the conventional CD4 cells also ceases (Lin and Hünig, 2003), Treg cells are deactivated and clonally contract in parallel. As noted previously for the antigen-driven regulatory circuit, however (Fig. 3A), the activated Treg cells will scan the body for sites of inflammation and remain at those sites to combat immunopathology (Chen *et al.*, 2005; Huehn and Hamann, 2005; Tischner *et al.*, 2006). It is important to emphasize in this context that *in vivo* stimulation with CD28 superagonists leads to an expansion of both CD103-negative (lymph node seeking) and CD103-positive (tissue seeking) Treg

subsets. In fact, the analysis shown in Fig. 5 is likely to underestimate the increase in tissue-seeking Treg cells since it was performed on lymph node cells. The therapeutic effects of CD28 superagonist therapy in rodent models of autoimmunity and inflammation described in Section 8.4 may therefore entail Treg-mediated suppression in both the secondary lymphoid organs and the inflamed tissues themselves.

8.4. A rationale for transient polyclonal Treg-cell activation as a general therapeutic principle in autoimmunity and inflammation

As mentioned previously, our animal studies showed that the expanded pool of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells found after CD28 superagonist stimulation *in vivo* is not derived from previously CD25-negative, that is, ''conventional'' CD4 T cells, but rather from the preexisting pool of CD25-positive ''natural'' Treg cells (Lin and Hünig, 2003). Since these cells are generated in the thymus by positive selection for autoreactivity, the activated Treg-cell compartment is self-reactive and, via its functional properties, autoprotective when self-antigens are recognized in the various tissues of the body (Sakaguchi *et al.*, 2006). This, in principle, makes polyclonal Treg-cell stimulation by CD28 superagonists an extremely powerful tool to counteract inflammatory and autoimmune disease, without the need to know the target antigens of autoimmune responses. This has, indeed, been born out in a number of rodent disease models.

9. TREATMENT OF AUTOIMMUNE AND INFLAMMATORY MODEL DISEASES WITH CD28 SUPERAGONISTS

9.1. Overview

So far, CD28 superagonists have been successfully used to prevent or treat the following experimentally induced or genetically predetermined diseases in rats and mice (Table 1): (1) experimental autoimmune neuritis of the LEW rat (Schmidt *et al.*, 2003); (2) EAE induced either as a monophasic disease by immunization of LEW rats with myelin basic protein (MBP), or as a biphasic disease by immunization of DA rats with myelin oligodendrocyte glycoprotein (MOG) (Beyersdorf *et al.*, 2005); (3) MOG peptide-induced EAE in C57BL/6 and S/JL mice, respectively (Lühder, Gold, and Hünig, unpublished data); (4) spontaneously developing type 1 diabetes in the BB rat (Beaudette-Zlatanova *et al.*, 2006); and (5) spontaneously developing CD8 T-cell-mediated EAE in a new transgenic model (Na and Hünig, unpublished data). Moreover, CD28 superagonist treatment-promoted tolerance of transplanted allogeneic vascularized heart (Otto *et al.*, 2002) and liver (Urakami *et al.*, 2006) grafts in rats, and suppressed GvH disease

TABLE 1 Rodent models of autoimmunity and transplantation in which CD28 superagonist treatment has shown preventive and/or therapeutic efficacy

Strain/species	Model	References
LEW rat	Experimental autoimmune neuritis	Schmidt *et al.*, 2003
LEW, DA rat	Experimental autoimmune encephalomyelitis	Beyersdorf *et al.*, 2005
Wistar rat	Adjuvant arthritis	Rodriguez-Palmero *et al.*, 2006
LEW, DA rat	Allogeneic liver transplantation	Urakami *et al.*, 2006
LEW, DA rat	GvH disease	Li, X.-K., unpublished data
LEW, DA rat	Allogeneic heterotopic heart graft	Li, X.-K., unpublished data; Otto *et al.*, 2002
BB rat	Spontaneous autoimmune diabetes	Beaudette-Zlatanova *et al.*, 2006
C57Bl/6 mouse	Experimental autoimmune encephalomyelitis	Lühder, Gold, and Hünig, unpublished data
Balb/C, C57Bl/6 mouse	GvH disease	Beyersdorf, Hünig, and Kerkau, unpublished data
ODC-OVA mouse	CD8-mediated autoimmune encephalomyelitis to OVA-expressing oligodendrocytes	Na and Hünig, unpublished data

in mice (Beyersdorf, Kerkau, and Hünig, unpublished data) and rats (Kitazawa *et al.*, unpublished data).

In two of these disease models, we obtained direct proof for the identity of Treg cells as the underlying therapeutic principle. In ''classical'' EAE of the LEW rat (Beyersdorf *et al.*, 2005) as well as in the CD8 T-cell-mediated EAE observed in transgenic mice (Na and Hünig, unpublished data), protection was achieved by transferring $CD4^+$ $CD25^+$ $Foxp3^+$ T cells from syngeneic animals which had received a single injection of CD28 superagonist 3 days earlier. In contrast, the CD25-negative fraction of CD4 T cells from the same animals did not confer protection. As an example, our results obtained in LEW rat EAE will be summarized in the following section.

9.2. Prevention and treatment of EAE in the LEW rat

In the well-studied system of monophasic EAE of the LEW rat, disease is induced by immunization with MBP from guinea pigs emulsified in complete Freünd's adjuvant (CFA). After ~9 days, the resulting inflammatory autoimmune response leads to ascending limb paralysis and weight loss, which peak around day 13 and are overcome by the end of the third week. There is ample evidence that the effector cells in this model are mainly proinflammatory (Th1, Th17) CD4 cells. In the adoptive transfer version of EAE (AT-EAE), MBP-specific Th1 cell lines or clones are transferred i.v., leading to an accelerated disease onset with similar monophasic kinetics as observed in ''active'' EAE (reviewed in Gold *et al.*, 2006).

CD28 superagonist treatment at the time of immunization or cell transfer completely prevents all aspects of disease development in both active and AT-EAE (Beyersdorf *et al.*, 2005). Moreover, therapeutic intervention, even after near-maximum clinical scores are reached, results in rapid remission and cessation of weight loss (Beyersdorf *et al.*, 2005). Importantly, the spinal cords of rats treated with CD28 superagonist while undergoing EAE, but not those either undergoing EAE or superagonist treatment alone, contain high frequencies of Treg cells (Fig. 6) (Tischner *et al.*, 2006). This is in line with the ''dispatchment'' model outlined previously, where transient polyclonal activation of Treg cells stimulates their tissue-seeking subset to home to sites of inflammation. Besides this local accumulation of Treg cells under CD28 superagonist therapy, we have, however, also documented their ability to prevent homing of EAE-effector T cells to the CNS (Tischner *et al.*, 2006). Thus, as has been described for antigen-driven Treg activation, Treg cells polyclonally activated by CD28 superagonists can act both at the levels of the local draining lymh nodes, inhibiting effector cell multiplication and export to the affected organ, and in the inflamed tissue itself.

Interestingly, even low doses of the CD28 superagonist (0.1 mg/rat) were fully effective in reaching the maximum therapeutic effect in active LEW rat EAE (Beyersdorf *et al.*, 2005). At this dose, an increase in Treg-cell numbers is observed without generalized lymphocytosis (Beyersdorf *et al.*, 2005). However, we assume that under these circumstances, conventional T cells are sufficiently activated to provide paracrine IL-2 required to upregulate the suppressive activity of Treg cells (Maloy and Powrie, 2005).

Finally, the importance of Treg-cell activation, rather than mere numerical expansion, became clear in adoptive transfer experiments designed to identify the cell type mediating suppression of EAE. Here, as little as 6 million Treg cells from CD28 superagonist-stimulated donors

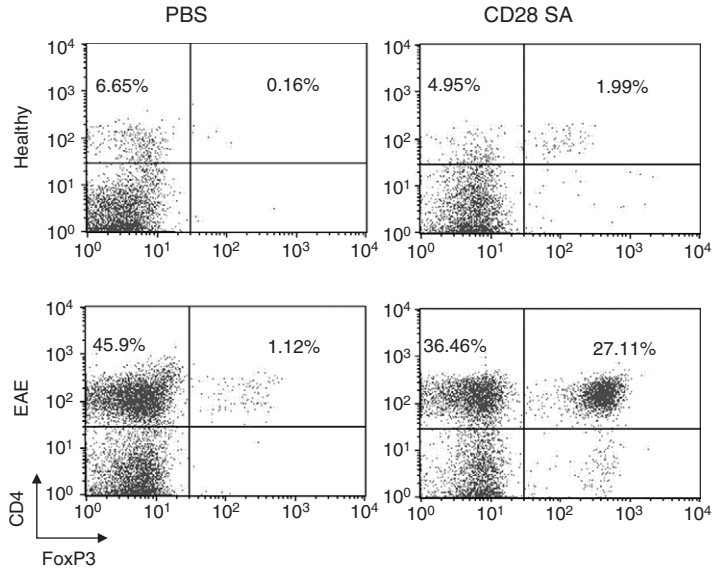

FIGURE 6 Polyclonally activated Treg cells home to inflamed tissue. Lymphocytes were isolated from the spinal cords of healthy LEW rats (top) or LEW rats undergoing AT-EAE (day 6). Note that CD28 superagonist treatment leads only to mild Treg accumulation in healthy, and to a massive accumulation in inflamed nervous tissue (Tischner *et al.*, 2006).

sufficed to protect recipients undergoing active EAE—a small fraction (<10%) of the Treg-cell number already present in these hosts (Beyersdorf *et al.*, 2005).

In summary, treatment of LEW rat EAE with CD28 superagonists is highly efficacious even at doses not resulting in the expansion of conventional T cells, and acts via Treg cells which counteract the autoimmune response at both induction and effector sites. Most importantly, however, it does not require knowledge of the autoantigen in question—a tremendous advantage for therapy of human autoimmune disease.

10. TGN1412—A SUPERAGONISTIC mAb TO HUMAN CD28

10.1. Introductory remarks

On March 13, 2006, the human CD28-specific superagonist TGN1412 was administered to six healthy volunteers at a clinical trial unit located at Northwick Park Hospital, London. This first-in-man study failed tragically

due to a life-threatening cytokine release syndrome encountered by the trial participants; the reasons that led to this failure are yet unclear.

For a thorough assessment of the preclinical data submitted by the sponsor to the regulatory authorities and of the trial itself, the reader is referred to the final report prepared by the British Expert Scientific Group (ESG) on phase I clinical trials (Duff, 2006) and a publication from the medical team at the Northwick Park Hospital (Suntharalingam *et al.*, 2006). With regard to the ethical aspects of the study, refer to the expert analysis by Emanuel and Miller (2007). The following discussion is based on the preclinical data submitted by TeGenero which were released by the British regulatory authority MHRA into the public domain, and on pertinent scientific publications in peer-reviewed journals.

10.2. Development of TGN1412

The therapeutic effects observed in the rodent models outlined previously suggested that superagonistic anti-CD28 antibodies would also be effective in treating, *inter alia*, human autoimmune and inflammatory diseases. Accordingly, TeGenero initiated a program to isolate a human CD28 superagonist. A set of mouse anti-human CD28 mAb was generated and screened for the ability to induce T-cell proliferation in the presence or absence of TCR ligation. Both conventional and superagonistic mAbs were isolated. Importantly, these novel mAbs conformed to the scheme of epitope–function relationship described in Section 5 for mAb to rat and mouse CD28 (Lühder *et al.*, 2003).

From this collection, a superagonistic mouse anti-human CD28 antibody was developed into a fully humanized IgG4 mAb by genetic engineering. Since IgG4 mAbs are less likely to recruit cytotoxic effector mechanisms via their Fc portion, the company proceeded to develop the human superagonist of this isotype, termed TGN1412, with the aim of opening novel treatment options for multiple sclerosis, rheumatoid arthritis, and B-cell chronic lymphocytic leukemia (Duff, 2006).

10.3. Biophysical properties of TGN1412

As mentioned previosly, TGN1412 is a human CD28-specific mAb of the IgG4 subclass. Cocrystallization of Fab fragments derived from a related mouse anti-human CD28 mAb 5.11.A1 with recombinant human CD28 had confirmed the lateral binding mode predicted by genetic epitope mapping (Lühder *et al.*, 2003) (Fig. 4) and, more importantly, had for the first time allowed the formation of CD28 crystals and hence the solution of the CD28 extracellular domain structure (Evans *et al.*, 2005).

The affinity of TGN1412 for human CD28 as determined by both Biacore and flow cytometry-based measurements lies in the nanomolar range and, thus, in the ''normal'' range of antibody affinities

(Table 2) (Hanke, 2006). Importantly, affinity of TGN1412 for CD28 on T cells of cynomolgus monkeys, the species used for preclinical safety/toxicity testing, was also determined and found to be very similar to the affinity for human CD28. This was not unexpected because sequence analysis of the extracellular domain of cynomolgus CD28 had revealed 100% identity at the amino acid level to its human counterpart (Hanke, 2006).

Furthermore, FcR are highly conserved between humans and cynomolgus monkeys, including motifs important for signal transduction and Fc binding (Presta and Namenuk, 2003). Of particular relevance for preclinical development of TGN1412, the binding of IgG4 antibodies to the various human and cynomolgus FcR was found to be virtually identical. Thus, differences in affinity of TGN1412 neither for the target antigen nor for the FcR provide a satisfactory explanation for the absence of toxic effects in cynomolgus monkeys during TGN1412 preclinical development reported by TeGenero and reproduced by the National Institute for Biological Standards and Control (NIBSC) study, commissioned by the Expert Commission on Phase One Trials (Duff, 2006), and the devastating cytokine release syndrome observed in humans receiving 500-fold less mAb than the highest dose tested in the monkeys.

For comparison, the two rodent CD28 superagonists discussed previously were also analyzed with regard to their affinity for CD28 (T. Hanke, personal communication; Table 2). In the flow cytometry-based assays using primary T cells, the rat CD28-specific superagonist JJ316 (Tacke *et al.*, 1997) showed a similar affinity as TGN1412, while the mouse superagonist D665 (Dennehy *et al.*, 2006) displayed a tenfold higher affinity.

10.4. Cytokine release syndrome in humans but not in animal models

Despite its superior affinity, extensive titrations of the mouse CD28 superagonist D665 have failed to reveal toxic effects in mice but instead showed a remarkable expansion of Treg cells (Fig. 5). Furthermore, an optimally Treg-activating dose of 5 mg/kg body weight did not result in the release of detectable circulating proinflammatory cytokines (Elflein and Hünig, unpublished data; Section 8).

This is in marked contrast to the life-threatening cytokine storm observed in the TGN1412 study, but not in TGN1412-treated cynomolgus monkeys in which only weak induction of IL-2, IL-4, and IL-5, but no increase of IFN-γ or TNF-α, had been observed. As documented in detail in the case report on the TGN1412 trial (Duff, 2006), high levels of proinflammatory cytokines, including TNF-α and IFN-γ, were observed in the plasma of the trial volunteers within an hour after antibody infusion, followed by subsequent multiorgan failure necessitating intensive care measures.

TABLE 2 Affinity of CD28 superagonists for human, rat, and mouse CD28 (Source: Hanke, 2006, and T. Hanke, personal communication)

Species	Antibody	Isotype	Reactivity with CD28 C″D loop	Affinity for CD28 (K_d in M)
Human	TGN1412 (anti-human CD28)	Human IgG4κ	Yes	$\sim 2 \times 10^{-9}$ (Bia.[a]) $\sim 4 \times 10^{-9}$ (Flow.[b])
Cyno monkey	TGN1412 (anti-human CD28)	Human IgG4κ	Yes	$\sim 2 \times 10^{-9}$ (Bia.[a]) $\sim 4 \times 10^{-9}$ (Flow.[b])
Rat	JJ316 (anti-rat CD28)	Mouse IgG1κ	Lühder *et al.*, 2003, JEM	$\sim 3 \times 10^{-9}$ (Flow.[b])
Mouse	D665 (anti-mouse CD28)	Mouse IgG1κ	Dennehy *et al.*, 2006	$\sim 0.3 \times 10^{-9}$ (Flow.[b])

[a] Biacore measurement using recombinant CD28.
[b] Flow cytometry-based measurement using primary T cells.

In summary, the severe mode-of-action-related toxicity of TGN1412 in the human volunteers remained unpredictable by careful evaluation of both analogous rodent models and by a primate model expressing a target structure identical to the human one and in which TGN1412 itself was tested over a wide range of concentrations, including the clinical trial dose and a dose 500-fold higher than the one used in TGN1412-HV trial itself (Duff, 2006).

10.5. Follow-up *in vitro* studies

While at present, no conclusive explanation for the discrepancy between the response of humans and cynomolgus monkeys to TGN1412 has been provided, the outcome of the NIBSC studies published by the ESG (Duff, 2006) has suggested the possibility of endothelial cell involvement in the hyperactivation of the human immune system by TGN1412.

First, this study confirmed the failure to elicit cytokine production in cultures of human peripheral blood mononuclear cells or of whole blood by soluble TGN1412, an approach taken to ensure representation of all circulating immune cells in the assay system. In the same tests, mAb UCHT1 to CD3, a component of the TCR complex, and lectin stimulation efficiently induced cytokine release.

In an attempt to further address this puzzling discrepancy between the *in vitro* and *in vivo* activity of TGN1412, peripheral blood mononuclear cells (PBMC) were cultured on a monolayer of human endothelial cells, mimicking interactions available within the blood vasculature. Importantly, this protocol did result in the release of the proinflammatory cytokines: IL-8, TNF, and IL-6, although no precise data have been published yet (Duff, 2006). This result raises the possibility that the unexpected cytokine storm in response to soluble TGN1412 antibody observed *in vivo* might have involved cell surface receptors expressed on human endothelial cells such as ligands for additional costimulatory receptors that may have synergized with CD28 stimulation. Of note, a publication describes such synergistic interactions for costimulated naive CD4 T cells and, as an additional stimulus, the ligand for the inducible costimulator (ICOS) (Mesturini *et al.*, 2006). It is thus tempting to speculate that the ICOS-ligand, LICOS, or another functionally effective costimulatory ligand is expressed on human but not monkey endothelial cells.

An alternative suggestion to explain the differential responses of cynomolgus monkeys and humans to TGN1412 has been brought forward in another post-TGN1412 trial publication from an independent research group (Nguyen *et al.*, 2006). These authors observed a correlation between hyperresponsiveness of human as compared to nonhuman primate T cells in polyclonal T-cell activation, with the absence of CD33-related inhibitory

siglecs in humans, and showed that transduction of human T cells with Siglec-5 dampened their responsiveness.

In summary, first data are emerging from retrospective studies aimed at explaining the failure of preclinical *in vitro* data as well as those from animal models to predict the cytokine release syndrome experienced by the healthy human volunteers who were administered TGN1412. No definitive statement on the actual nature of the relevant differences between the preclinical settings and the human trial can, however, be made yet, and the mechanisms discussed previously may not be causally related to the acute inflammatory effects observed in the TGN1412 trial.

10.6. Lessons from the TGN1412 trial

10.6.1. First-in-man studies

The current set of rules governing clinical trials has been developed over time, often in response to unexpected adverse reactions and with the aim to avoid those in future trials. Based on the unexpected outcome of the TGN1412 study, the ESG has made a number of recommendations for future first-in-man studies with biologicals (Duff, 2006). These recommendations should and will modify current regulations to further increase the safety of future phase I trials without blocking the development of innovative drugs for unmet medical needs.

10.6.2. Human CD28 superagonists: Future perspectives?

Initial toxicity observed with various biologicals has not always precluded their reentry into the clinic, though this generally occurred in a modified form. The example most closely related to the adverse effects observed with TGN1412 is that of the anti-CD3ε mAb OKT3, used as an immunosuppressant to prevent graft rejection in various settings, and originally found to induce a massive cytokine release syndrome (Abramowicz *et al.*, 1989; Chatenoud *et al.*, 1991). After two decades of further clinical research and experience, genetically modified forms of OKT3 are again being tested for tolerance induction in humans (reviewed by Chatenoud, 2005). Of note, accumulating evidence from both animal models and the clinic implicates Treg cells in the long-lasting effects of transient anti-CD3 therapy (Bisikirska *et al.*, 2005; Chatenoud, 2005; Herold *et al.*, 2005). While it is too early to discuss such future perspectives for CD28 superagonists, a balanced view should keep this parallel in mind.

11. CONCLUSIONS

Its key role in the generation and control of Treg cells makes the costimulatory receptor CD28 an attractive target for mAb-mediated manipulation of self/non-self-discrimination by the immune system. The sharing of

natural ligands by CD28 and its counterregulator, CTLA-4, indicates that selective interruption of costimulation is only achieved by addressing CD28 itself. Indeed, some mAbs to rodent CD28 or Fab fragments thereof block costimulation *in vivo* including that of Treg cells.

On the other hand, activating CD28-specific mAb protects against autoimmunity and inflammation in rodents by promoting Treg-cell activation and expansion. While to some degree, this effect is observed *in vivo* with certain ''conventional,'' that is, costimulatory mouse CD28-specific mAb, it is dramatic when ''superagonistic'' mAbs that do not require TCR ligation are used for their activating properties. Such CD28 superagonists are also useful for the *in vitro* expansion of Treg cells in therapeutic strategies based on cell transfer.

The life-threatening cytokine release syndrome encountered during the first-in-man study of the human superagonist TGN1412 has shattered the translation of this very promising approach into human therapy. It remains to be seen, however, if different or modified approaches to transient Treg activation—with or without the involvement of CD28—will eventually contribute to the therapy of human immunopathologies. For example, treatment with vitamin D3 receptor agonists alone or together with corticosteroids seems to favor expansion and activation of Treg cells in mouse models (Adorini *et al.*, 2004; Barrat *et al.*, 2002). Futhermore, application of IL-2, the major growth and survival factor of Treg cells *in vivo*, can be effectively used in mice for Treg activation *in vivo*. Here, targeting of IL-2 to CD25-expressing (Treg) cells as opposed to CD8 cells expressing CD122, the β-chain of the IL-2R, was achieved with the help of certain mAb to IL-2 (Boyman *et al.*, 2006), and concomitant application of IL-2 and dexamethasone was shown to promote Treg expansion and functional activity in mouse EAE (Chen *et al.*, 2006). Finally, a report of human Treg expansion by costimulation with CD52-specific mAb (Watanabe *et al.*, 2006) suggests that beyond activating CD28- and CD3-specific mAbs, other cell surface receptors on T cells may be targeted by mAbs for the transient expansion of the Treg compartment.

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CHAPTER 5

Osteoimmunology: A View from the Bone

Jean-Pierre David*

Contents

*Group Bone Cell Differentiation, Deutsches Rheuma-Forschungszentrum (DRFZ), Cahritéplatz 1, Berlin, Germany

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Abstract

Osteoimmunology can be defined in a very broad sense as the field of research focusing on interrelations between bone and the immune system. This is a rather opened field that covers at least three different issues. The first one is developmental, that is, organogenesis of the bones and immune systems. The second is post-developmental, that is, the role of the bone in the regulation of the immune response and role of the immune cells on the regulation of bone homeostasis. The third one is related to pathologies: Can immune cells be involved in the development of bone-related pathology? Can deregulation of the bone be causing immune-related diseases? I will not review in detail the bibliography covering osteoimmunology. This has been extensively done in Immunological Reviews (Vol. 208, December 2005) and Current Opinion in Rheumatology (Vol. 18, 2006). I will rather critically comment on hypotheses and concepts in osteoimmunology from a bone biologist's point of view.

1. OPENING REMARKS: THE OLD AND THE NEW

Osteology is often presented as a new field of research. In fact, the role of immune cells on bone biology has been for long time investigated by bone biologists. Their interest was driven by few observations. First, bone is a hematopoietic organ from which lymphoid and myeloid cell progenitors are originating. Second, one of the cells necessary for bone remodeling is itself of hematopoietic origin: These are the bone-specialized macrophages or osteoclasts. Like other macrophages, osteoclasts are regulated by inflammatory cytokines that are often abundantly produced by immune cells. Indeed, bone remodeling (i.e., the balance between the bone-forming and the bone-resorbing activities) that is necessary for proper maintenance of bone homeostasis is often considered as a controlled inflammatory reaction. Therefore, the effects of numerous inflammatory cytokines on the osteoclast or osteoblast differentiation or activities have been more or less systematically investigated *in vitro* or *in vivo*. This is the case for IL-1, IL-4, IL-6, IL-10, the TNF family or more recently the interferons (IFNs) (Goldring, 2003; Lee and Lorenzo, 2006).

Newer is the interest that immunologists have for a few years for bone. Immunologists have in the past intensively analyzed the structural organization and the relation of immune cells with the other cellular components of soft immune organs such as spleen, lymph nodes, or thymus. In contrary, they have, for the most, neglected the bone. This was mainly a consequence of the mineralization of bone that rendered histological analyses of the native structures difficult. In addition, demineralization

which allows classical immunohistology destroys the structure of the bone tissue and therefore tends to generate potential artifacts.

2. OSTEOIMMUNOLOGY: A DEVELOPMENTAL ENCOUNTER

2.1. Bone: Five cell types and two mechanisms of formation

Bone is a highly specialized form of connective tissue that, together with cartilage, constitutes the skeleton. At the cellular level, bone is formed and remodeled by the activity of five different cell types: chondrocytes, osteoblasts, and osteocytes, all derived from mesenchymal stem cells; and chondroclasts and osteoclasts, derived from the monocyte–macrophage lineage (Karsenty and Wagner, 2002). Osteoblasts are the bone-forming cells secreting and mineralizing the bone matrix. Osteocytes are the most abundant cells of the bone and are the ultimate stage of osteoblast differentiation. Embedded in the mineralized bone and forming an extensive network of connections between each other, they are believed to serve as biomechanical sensors. Chondroclasts are responsible for resorbing mineralized matrix generated by the chondrocytes, whereas osteoclasts resorb the matrix mineralized by osteoblasts.

During development, two types of ossification can be distinguished. In intramembraneous ossification that occurs in the developing flat bones of the skull, condensations of mesenchymal cells of the embryonic connective tissue directly differentiate into osteoblasts, which produce the extracellular matrix that subsequently becomes mineralized. In endochondral bone formation, bone anlagen are first formed by cartilaginous condensation of chondrocytes shaping the future bone. Following resorption of the mineralized cartilaginous matrix by the chondroclasts, these structures will progressively be colonized by both the bone-forming cells (osteoblasts) and the bone-resorbing cells (osteoclasts) brought by blood vessels. A cartilaginous structure called growth plate that permits elongation of the bone will subsist at the extremities of the long bones, during fetal development and throughout childhood, and regress with age. In contrary to human, this growth plate will stay active in adult animals that are often used as models to study bone biology, that is, mouse and rat.

2.2. The developmental encounter

A primitive hematopoiesis characterized by the presence of limited cell types, that is, embryonic erythroblasts and few myeloid cells, is first taking place during early embryonic development in the yolk sac. The primitive hematopoiesis will progressively be replaced by the definitive hematopoiesis, first in the fetal liver, then in the spleen, and finally accompanying the

formation of the bone marrow cavity into the bone. The bone will then become the main hematopoietic organ of the adult. The resorbing activity of the osteoclasts is required to dig the bone marrow cavity, therefore establishing the first relationship between bone and immune cells.

2.3. Developmental consequences

The implications of these developmental events are multiple.

- First, the bone will provide the local environment for the hematopoietic stem cells (HSCs) from which all hematopoietic cells including T cells, B cells, dendritic cells, and macrophages are differentiating. Therefore, it can be expected that alteration of bone homeostasis could affect hematopoiesis and, thus, the immune response. Indeed, both osteopetrosis caused by defect in the bone-resorbing activity of osteoclasts and osteosclerosis due to increased bone formation by osteoblasts lead to a drastic reduction of the bone marrow space and development of secondary extramedullary hematopoiesis in the spleen or liver.
- Second, the bone is not required for hematopoiesis, and therefore, perturbation of bone homeostasis would not always affect the immune response.
- Third, the close vicinity between immune cells and bone cells strongly suggests that immune cells could also regulate bone homeostasis.

3. THE TNF SUPERFAMILY: A DEVELOPMENTAL LINK BETWEEN BONE AND IMMUNE SYSTEM

3.1. Osteoclasts differentiation

Osteoclasts are multinuclear cells generated by differentiation of hematopoietic precursors along the monocyte–macrophage lineage (Karsenty and Wagner, 2002). Analyses of bone phenotypes in mutated mice have allowed the identification of the two essential osteoclastogenic molecules as the macrophage-colony stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL) (Teitelbaum and Ross, 2003). The respective role played by each cytokine in osteoclast differentiation is so far understood as follows: While M-CSF promotes proliferation and the survival of the osteoclast progenitors at the expense of the other myelomonocytic lineages (dendritic cells or granulocytes) (Miyamoto *et al.*, 2001), RANKL prompts the cells to differentiate along the osteoclast lineage. RANKL also acts as an activating and survival factor for the mature osteoclasts (Wong *et al.*, 1999). Thus, RANKL is the osteoclast differentiating factor. It was first characterized as a ligand for the osteoclast inhibitory factor osteoprotegerin (OPG), a circulating decoy receptor

belonging to the TNF-α receptor family (Simonet *et al.*, 1997). RANKL also belongs to the TNF superfamily of cytokines. Following its binding to a receptor called RANK and via intracellular coupling that involves TNF receptor activating factors (TRAFs), among which TRAF6 is playing a determinant function (Lomaga *et al.*, 1999; Naito *et al.*, 1999), RANKL-induced NF-κB, AP-1, and NFATC1 activation, all transcription factors required for osteoclast differentiation (David *et al.*, 2002; Franzoso *et al.*, 1997; Grigoriadis *et al.*, 1994; Iotsova *et al.*, 1997; Takayanagi *et al.*, 2002a).

3.2. Immune and bone developmental phenotypes in RANK axe mutated mice

3.2.1. Bone phenotypes

OPG was first identified by sequence homology as a potential secreted member of the TNF-receptor (TNFR) superfamily that, when overexpressed, induced strong osteopetrosis caused by decreased osteoclast number *in vivo*. *In vitro*, the recombinant protein was shown to directly inhibit osteoclast differentiation (Simonet *et al.*, 1997). In opposite, OPG-deficient mice developed osteoporosis due to increased number of resorbing osteoclasts (Bucay *et al.*, 1998). The natural ligand for OPG (OPG-L) was characterized as RANKL/TRANCE (Kong *et al.*, 1999; Yasuda *et al.*, 1998). The essential functions of RANKL, RANK, and the adaptator protein TRAF6 in osteoclastogenesis were confirmed by gene inactivation. All three deficient mice developed severe osteopetrosis due to impaired osteoclast differentiation (Dougall *et al.*, 1999; Kong *et al.*, 1999).

3.2.2. Immune system phenotypes

Histological analysis of RANK- or RANKL-deficient mice also revealed their essential function in the development of secondary hematopoietic organs. Both mice were characterized by an absence of lymph node (Dougall *et al.*, 1999; Kong *et al.*, 1999). A similar phenotype was also observed in TRAF6-deficient mice (Naito *et al.*, 1999). Therefore, it can be concluded that RANK–RANKL–TRAF6 pathway is a key regulator of both bone and lymph node organogenesis. However, if the upstream signaling events are shared between bone and immune system, a perfect relationship between the two organs cannot be drawn. Indeed, lymph nodes are present in c-Fos or in NF-κB1/NF-κB2-deficient mice that all developed severe osteopetrosis due to the complete absence of osteoclasts (Caamano *et al.*, 1998; Johnson *et al.*, 1992; Sha *et al.*, 1995; Wang *et al.*, 1992). In addition, the absence of lymph node does not imply the development of a bone phenotype as illustrated by the absence of reported bone defect in lymphotoxin pathway-deficient mice (Locksley *et al.*, 2001).

3.3. TNF-α: A nonessential regulator of bone Involved in autoimmune-induced bone pathology

While not being essential for bone development, the potential role for TNF-α on bone homeostasis was noticed long time ago based on both *in vitro* works and on studies of inflammation-mediated bone diseases. TNF-α is a strong proinflammatory cytokine produced by activated T cells that is involved in autoimmune-induced bone destruction (Walsh and Gravallese, 2004). *In vivo*, no clear bone phenotype has been reported in TNF-α- or TNFR-deficient mice. However, transgenic mice overexpressing a stabilized form of the human TNF-α (*hTNF-α* Tg) developed severe rheumatoid arthritis with all the hallmarks of the disease including bone and cartilage destruction (Keffer *et al.*, 1991) and general osteopenia (Schett *et al.*, 2003). Administration of OPG can protect the mice against bone destruction (Redlich *et al.*, 2002a). In addition, when crossed with mice lacking osteoclasts which do not show any clear immune defect (*c-Fos* knockout), the *hTNF-α* Tg mice were protected against bone destruction but still developed strong inflammation and cartilage destruction (Redlich *et al.*, 2002b). These data clearly demonstrated that osteoclasts are required for TNF-α-induced bone loss and argued in favor of an indirect stimulatory effect of TNF-α mediated via RANKL on osteoclast differentiation. Whether TNF-α can also directly induce osteoclast differentiation is controversial. While it has been shown that, when combined with IL-1α, TNF-α can drive osteoclast differentiation and activation in the absence of RANKL (Kobayashi *et al.*, 2000), other studies concluded that the presence of RANKL was required (Lam *et al.*, 2000; Zhang *et al.*, 2001).

3.4. RANKL, TNF-α: In search for differences

While both RANKL and TNF-α can induce osteoclast differentiation *in vitro*, it seems that only RANKL can efficiently do it *in vivo*. The reasons for the discrepancy are not clear. At the molecular level, TNF-α can bind to two different receptors, TNFR1 or p55 and TNFR2 or p75. TNFR1 but not TNFR2 contains a death domain that can directly couple TNF-α to apoptosis. The two receptors were found to have opposite function in osteoclasts. While TNFR1 promote osteoclastogenesis, TNFR2 is inhibitory (Abu-Amer *et al.*, 2000). Thus, it can be hypnotized that the balance between TNFR1 and TNFR2 in favor of TNFR2 may be one of the reasons for the lack of efficiency of TNF-α *in vivo*. RANKL binds to RANK, a member of the TNFR that lacks the death domain and therefore should not be able to directly mediate apoptosis. Indeed, RANK acts as a survival factor for osteoclasts, a function that was found to be mediated in part via RANKL-induced Jun N-terminal kinase 1 (JNK1) activity (David *et al.*, 2002).

The TRAFs are key components in the transduction of the various members of TNFR superfamily. Not all receptors can bind to all TRAFs (Aggarwal, 2003; Chung *et al*., 2002). The recruitment of a specific TRAF may explain the differences between TNF-α and RANKL. Indeed, while the pro-osteoclastogenic activity of RANKL and TNF-α required TRAF6 and the participation of TRAF5 (Kaji *et al*., 2001; Kanazawa *et al*., 2003), TRAF2 that is not mediating RANK signaling in osteoclasts is required for TNFR1 differentiating activity (Kanazawa and Kudo, 2005). TNF-α not only acts on the bone-resorbing cells, but also blocks bone formation by osteoblasts (Bertolini *et al*., 1986), most likely via up-regulation of the Wingless and INT-1 (Wnt) signaling inhibitor Dickkopf-1 (DKK-1) (Diarra *et al*., 2007). These functions, not described for RANKL, are also mediated via TNFR1.

Thus, it appears that the two TNF-like pathways are regulating bone homeostasis. RANKL/RANK is the required mediator of osteoclastogenesis during development and in normal physiological conditions. TNF-α/TNFR1, TNFR2 that may act as modulators of bone homeostasis when an increased bone resorption is needed are involved in the development of pathology leading to bone destruction such as rheumatoid arthritis or psoriasis (Ritchlin *et al*., 2003; Walsh and Gravallese, 2004; Zenz *et al*., 2005; Zwerina *et al*., 2004).

4. IFNs: Linking Bone Homeostasis to Immunity and T Cells

The role of another family of immune-related cytokines, the IFNs, was in the last few years extensively analyzed *in vitro* and *in vivo*. This work was mainly performed by Takahashi and colleagues who analyzed the bone properties of mice strains carrying inactivating mutations of various components of the IFN responses.

4.1. Type I IFNs establish an autoinhibitory loop in osteoclasts

Type I IFNs are produced by most of the cells of the organism in response to viral aggression and are important mediators of the antiviral response (Dunn *et al*., 2006). A genomic screen in which the response to M-CSF and RANKL stimulation of c-Fos-deficient monocytes was compared to wild-type cells identified IFN-β as potential c-Fos target genes in osteoclasts. RANKL-induced up-regulation of IFN-β was shown not to utilize the antiviral response system. Surprisingly, IFN-β could block M-CSF and RANKL-induced osteoclast differentiation *in vitro*. The inhibitory mechanism was analyzed in detail and revealed that c-Fos transcriptionally induced IFN-β synthesis that in turn post-transcriptionally down-regulated c-Fos expression, therefore, establishing a c-Fos-dependent negative autoregulatory loop.

A low bone mass phenotype due to increased osteoclast activity was found when the bone of mice carrying genetic inactivation of one of the components of the type I IFNR, IFNAR1, or lacking IFN-β were analyzed. The anti-osteoclastogenic effect of IFN-β was found to be dependent on the transcription factor interferon-stimulated gene factor 3 (ISGF3) as shown by the lack of effect of IFN-β on the osteoclast differentiation of monocytes lacking Stat1 or IRF9, two components of ISGF3. It involved the activation of PKR, a kinase regulating translation (Takayanagi *et al.*, 2002b) and seemed to be independent of c-Fos phosphorylation by RSK2, a kinase modulating c-Fos function in osteoblasts (David *et al.*, 2005). The inhibitory effect of IFN-β was shown to depend on the level of expression of the suppressor of cytokines signaling (SOCS) that block IFN signaling. Interestingly, both SOCS1 and SOCS3 were found to be up-regulated by RANKL treatment (Hayashi *et al.*, 2002), therefore, establishing a second negative loop counterbalancing the first one.

4.2. Type II IFN: A potent inhibitor of osteoclastogenesis secreted by T cells

By supporting osteoclastogenesis, activated T cells expressing RANKL are involved in autoimmune-induced bone destruction and in metastatic invasion of the bone (Horwood *et al.*, 1999; Jones *et al.*, 2006; Kong *et al.*, 1999; Zenz *et al.*, 2005). The same Th1 cells that produce RANKL are also secreting high amounts of type II IFNs (IFN-γ) that strongly inhibits osteoclast differentiation *in vitro* (Fox and Chambers, 2000; Takayanagi *et al.*, 2000). *In vivo*, mice lacking IFNGR1, one of the components of the IFN-γ receptor were shown to be more sensitive to lipopolysaccharide (LPS)-induced bone destruction. Activated T cells but not the resting ones could suppress M-CSF and RANKL-induced osteoclastogenesis. Mechanistically, IFN-γ was shown to induce ubiquitin-mediated TRAF6 degradation therefore shunting RANKL signaling (Takayanagi *et al.*, 2000). Thus, the role of activated T cells on osteoclast differentiation appears to be paradoxical. They can both stimulate and inhibit osteoclastogenesis *in vitro*. *In vivo*, IFN-γ was proposed to stimulate osteoclast-mediated bone loss via a mechanism involving T-cell activation (Gao *et al.*, 2007). The paradox was reinforced by the fact that IFN-γ-producing Th1 cells were proposed to be the T cells supporting osteoclastogenesis *in vitro* (Kotake *et al.*, 2005). This view was challenged in a publication that identified the Th17 cells as osteoclastogenic T helper cells (Sato *et al.*, 2006). In this work, Th1-producing IFN-γ and Th2-producing IL-4 were both shown to inhibit RANKL-induced osteoclast differentiation *in vitro*. These data were in agreement with the known anti-osteoclastogenic activity of IL-10 and IL-4, both cytokines produced by the Th2 cells (Abu-Amer, 2001; Owens *et al.*, 1996; Wei *et al.*, 2002). Th17 cells do

not directly induce osteoclast differentiation. They produce IL-17 that was found to be required *in vitro* and *in vivo* to enhance the supportive activity of the osteoblasts (Sato *et al.*, 2006).

From the works published so far, it clearly appears that both bone and T cells have established a very complex relationship that is involved in positive and negative regulation of bone remodeling and may be of immune response. While the connection between T cells and bone destruction is quite well understood, the potential contribution of the regulatory T cells that can be regulated by RANKL (Loser *et al.*, 2006) has not yet been properly addressed. In addition, the role of T cells in modulating bone formation is still poorly documented.

5. B CELLS AND BONE

In contrary to T cells, we know very little about the interrelationship of B cells with the bone. All we know is based on correlative analysis of phenotype developing in mutated mice and on experimental work that tested the possibility to transdifferentiate lymphoid cells into myeloid lineages (Horowitz *et al.*, 2005; Laiosa *et al.*, 2006). More documented is the interplay between bone and the tumor cells mediating bone destruction in multiple myeloma.

5.1. The hypothesis of myeloid lineage switch

The transcription factor Pax5 is a key regulator of B-cell commitment. This function was revealed by the analysis of Pax5-deficient mice where B-cell development is blocked at the pro-B-cell stage (Urbanek *et al.*, 1994). Surprisingly, Pax5-deficient pro-B cells can differentiate in all hematopoietic lineages including osteoclasts (Nutt *et al.*, 1999). Pax5 was shown to decide the B-cell fate and to suppress the expression of genes involved in the commitment of the other hematopoietic lineages. *In vivo*, Pax5-deficient mice were shown to develop osteopenia due to increased number of osteoclast progenitors (Horowitz *et al.*, 2004). Interestingly, myeloid cell differentiation could be obtained when committed B cells were forced to overexpress members of the C/EBP transcription factor family. This effect was correlated with a down-regulation of Pax5 (Xie *et al.*, 2004). However, similar experiments performed by another group did not lead to the same conclusion. Indeed, Heavey *et al.* (2003) could only reprogram Pax5-deficient progenitors but not the wild-type cells therefore challenging the transdifferentiation hypothesis. Klotho-deficient mice developed all syndromes of premature aging including low bone turnover osteopenia where decreased bone formation exceeded decreased bone resorption (Kawaguchi *et al.*, 1999; Kuro-o *et al.*, 1997). The analysis of osteoclast

differentiation in Klotho-deficient mice indicated that B cells could regulate osteoclastogenesis by two different mechanisms. In the first one, B cells were shown to directly support osteoclastogenesis by producing RANKL. In the second one, it was shown that osteoclasts could be generated from B220 positive cells that argued in favor of a potential B-cell reprogramming (Manabe *et al.*, 2001). More work will be needed to clearly establish the role of B cells in bone.

5.2. Multiple myeloma: An osteolythic tumor

Multiple myeloma is a clonal malignancy of the terminally differentiated plasma cells. This is the second most common adult hematopoietic tumor, and the most common cancer directly affecting the bone (Giuliani *et al.*, 2006; Heider *et al.*, 2005). Multiple myeloma is leading to osteolysis, fractures, and hypercalcemia caused by impaired bone remodeling. The bone characteristic is an increased bone resorption by osteoclasts and a decreased bone formation by osteoblasts. *In vitro*, osteoclasts are supporting survival and proliferation of myeloma cells that can directly induce osteoclast differentiation. The plasma cells isolated from multiple myeloma expressed osteoclastogenic factor such as RANKL and the chemokine MIP1-α which enhances RANKL activity. In addition of being pro-osteoclastogenic, the tumor cells are blocking osteoblast differentiation, thereby inhibiting bone formation (Giuliani *et al.*, 2006). This effect is most likely due to the expression by the plasma cells, of DKK-1. Therefore, a vicious circle is established in which the plasma cells induced bone destruction by osteoclast that mobilized factors promoting proliferation and survival of the tumor cells that also inhibit osteoblast differentiation and stimulate the osteoclastogenic activity of the pre-osteoblasts. This circle can be disrupted by anti-osteoclastogenic drugs such as biphosphonates.

6. A BONE QUALITY CONTROL OF THE IMMUNE RESPONSE

The most exciting new concept emerging from osteoimmunology is that the bone would exert a quality control on the immune response by controlling the availability of the HSCs. This function would require a tight control of bone remodeling by both the bone-forming cells or osteoblasts and the bone-resorbing cells or osteoclasts. There are several indications that suggested it. The active osteoblasts are bone lining cells secreting and mineralizing the bone matrix. They express the stem cells chemoattractant and survival factor stromal-cell derived factor-1 (SDF1) (Ponomaryov *et al.*, 2000). A subset of spindle-shaped cells that colocalized with the lining osteoblasts on the trabecular bone, expressing N-cadherin and $CD45^{-}$ [spindle-shaped N-cadherin+CD45-osteoblastic (SNO) cells],

were identified as the niche for the HSCs (Calvi *et al*., 2003; Zhang *et al*., 2003). SNO cells maintain HSCs in quiescence stage. Osteoblasts can negatively control the pool of HSCs available by producing osteopontin that inhibits HSCs proliferation (Nilsson *et al*., 2005; Stier *et al*., 2005). In addition, osteopontin is required for the proper localization of the HSCs (Nilsson *et al*., 2005). Stimulation of bone resorption increases the number of immature hematopoietic cells in the circulation, suggesting that osteoclasts, by destroying the architecture of the niche, play a key function in mobilizing HSCs (Kollet *et al*., 2006). This function involves the production by osteoclasts of peptidases (MMP-9, cathepsin K) (Engsig *et al*., 2000; Gelb *et al*., 1996) that can cleave the adhesion molecule and factor essential for the maintenance of the HSC niche such as SDF1. When mobilized, HSCs need to migrate through the bone marrow to meet the epithelial cells of sinusoidal vessel. These epithelial cells define a new niche for the HSCs, called vascular niche, that support their proliferation, differentiation, as well as their passage into the circulation (Yin and Li, 2006). Therefore, compartmentalization and polarization of the bone are established in order to properly control hematopoiesis.

How can bone be modulating immunity? The HSC niche in the bone can be expanded by treatment increasing the number of osteoblasts, while it decreased when the trabecular bone volume or the number of osteoblasts is reduced (Calvi *et al*., 2003; Zhang *et al*., 2003). Changes in bone quality can reversibly affect hematopoiesis (Visnjic *et al*., 2004). Mobilization of the HSC can be performed either by activating osteoclast activity or by inducing the degradation of essential homing factors (Adams *et al*., 2007; Petit *et al*., 2002). It is therefore tempting to think that treatment modifying the balance between bone formation and bone resorption could be used to improve immune response. However, there are several observations arguing against this option. First, the bone is homing not only the HSCs but also the long-lived plasma cells important for the memory of the immune response that may also be perturbed (Moser *et al*., 2006). Second, the strategy may be difficult to apply due to the difference of kinetics between bone formation and destruction. Indeed, while bone destruction by osteoclasts is a rather quick event; bone formation is a slow process that is not easily stimulated. The option of stimulating bone resorption to mobilize HSCs should be taken with care. Inducing bone resorption can have definitive deleterious consequences for the bone. Indeed, if bone trabecular structures can be reversibly destroyed in mice which are continuously growing due to the persistence of an active growth plate (Corral *et al*., 1998; Visnjic *et al*., 2004), this is probably not possible in human where the growth plate is progressively becoming inactive with age. Finally, a change of localization more than a change in the quality of hematopoiesis has so far been reported in most of the animal models with impaired bone remodeling. Osteopetrosis or osteosclerosis as

well as osteoblast deprivation that impaired the hematopoietic function in the bone marrow all resulted in compensatory extramedullary hematopoiesis (Jochum *et al*., 2000; Okada *et al*., 1994; Visnjic *et al*., 2004; Wang *et al*., 1992). Therefore, challenging the immune response in mice developing various bone diseases will be needed before speculating about any therapeutic application.

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CHAPTER 6

Mast Cell Proteases

Gunnar Pejler,* Magnus Åbrink,* Maria Ringvall,* and **Sara Wernersson***

Contents

* Department of Anatomy, Physiology and Biochemistry, The Biomedical Centre, Swedish University of Agricultural Sciences, Uppsala, Sweden

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Abstract

Mast cells (MCs) are traditionally thought of as a nuisance for its host, for example, by causing many of the symptoms associated with allergic reactions. In addition, recent research has put focus on MCs for displaying harmful effects during various autoimmune disorders. On the other hand, MCs can also be beneficial for its host, for example, by contributing to the defense against insults such as bacteria, parasites, and snake venom toxins. When the MC is challenged by an external stimulus, it may respond by degranulation. In this process, a number of powerful preformed inflammatory "mediators" are released, including cytokines, histamine, serglycin proteoglycans, and several MC-specific proteases: chymases, tryptases, and carboxypeptidase A. Although the exact effector mechanism(s) by which MCs carry out their either beneficial or harmful effects *in vivo* are in large parts unknown, it is reasonable to assume that these mediators may contribute in profound ways. Among the various MC mediators, the exact biological function of the MC proteases has for a long time been relatively obscure. However, recent progress involving successful genetic targeting of several MC protease genes has generated powerful tools, which will enable us to unravel the role of the MC proteases both in normal physiology as well as in pathological settings. This chapter summarizes the current knowledge of the biology of the MC proteases.

ABBREVIATIONS

Ang	angiotensin
α_1-AC	α_1-antichymotrypsin
BMMC	bone marrow-derived mast cell
CGRP	calcitonin gene-related peptide
CPA	carboxypeptidase
CTAP-III	connective tissue-activating peptide III
CTMC	connective tissue type mast cell
GAG	glycosaminoglycan
HDL	high-density lipoprotein
KO	knockout
LDL	low-density lipoprotein
MC	mast cell
MMC	mucosal mast cell
mMCP	mouse mast cell protease
MMP	matrix metalloprotease
NAP-2	neutrophil-activating peptide 2
NDST	*N*-deacetylase/*N*-sulfotransferase
PAR	protease activated receptor
PG	proteoglycan
PHM	peptide histidine-methionine
α_1-PI	α_1-proteinase inhibitor
rMCP	rat mast cell protease
SCF	stem cell factor
SG	serglycin
SMC	smooth muscle cell
TGF	transforming growth factor
TMT	transmembrane tryptase
VIP	vasoactive intestinal peptide

1. INTRODUCTION

Mast cells (MCs) originate from the bone marrow, circulate in the blood as immature precursors, and then migrate into various tissues in which they undergo terminal differentiation under influence of local growth factors, in particular stem cell factor (SCF) (Gurish and Boyce, 2002). MCs are widely distributed throughout the body with a particular preponderance at sites close to the exterior, for example, mucosal surfaces and skin. This anatomic distribution is clearly in line with the notion of the MC as an important player in first-line defense toward external insults. For various general aspects of MC biology, the reader is referred to excellent review articles (Galli *et al.*, 2005a,b; Metcalfe *et al.*, 1997).

When MCs mature, they acquire numerous electron dense cytoplasmic granules, which can be released following an appropriate stimulus. Out of such stimuli, cross-linking of IgE molecules bound to the high-affinity IgE receptor, FcεRI, by polyvalent antigen is the most well characterized (Blank and Rivera, 2004). It has also been known for many years that MCs can be activated in response to several other types of stimuli, including anaphylatoxins C5a and C3a, neuropeptides (e.g., substance P), endothelin 1 (ET-1), and engagement of Toll-like receptors or FcγRIII (Galli *et al.*, 2005b).

MCs are undoubtedly most well known for their harmful effects in connection with immediate hypersensitivity reactions (Yu *et al.*, 2006), but MCs are also implicated in various additional disorders such as multiple sclerosis (Secor *et al.*, 2000), arthritis (Lee *et al.*, 2002a), bullous pemphigoid (Chen *et al.*, 2001), congestive heart failure (Hara *et al.*, 2002), and squamous carcinoma (Coussens *et al.*, 1999). Although reports describing harmful effects of MCs dominate the literature, it is important to emphasize that MCs can also be beneficial for their host by contributing to the innate immune defense toward certain pathogens, including bacteria (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996), parasites (Ha *et al.*, 1983), and even toward snake venom poisoning (Metz *et al.*, 2006). Much of this knowledge is based on experiments using a mouse strain that lacks MCs (the W/W^v strain), as a result of a mutation in the SCF receptor, c-kit. By comparing the response to a certain stimulus, for example an experimentally induced disease, between wild-type and MC-deficient mice, it has been possible to specifically pinpoint an involvement of the MC in a variety of pathological settings. Importantly however, the exact mechanism by which MCs contribute to the various pathological conditions listed above has in many cases not been clarified.

When MCs degranulate, they release a number of preformed components to the exterior. MC activation may also cause *de novo* synthesis and release of additional compounds, including PGD_2, PGE_2, LTB_4, and LTC_4 as well as cytokines (Galli *et al.*, 2005a,b; Metcalfe *et al.*, 1997). Out of the preformed MC mediators, histamine is by far the most thoroughly characterized in terms of biological function. In addition, it has been known for a long time that MC granules contain proteoglycans (PGs), that is, protein ''cores'' to which glycosaminoglycan (GAG) side chains are attached, and it is now well established that MCs also contain preformed cytokines, for example, TNF-α (Gordon *et al.*, 1990). Finally, MC granules contain a number of MC-specific proteases: tryptases, chymases, and MC carboxypeptidase A (MC-CPA). Tryptases and chymases belong to the serine protease class, while MC-CPA is a zinc-dependent metalloprotease. The designation of these proteases relates to their substrate specificities, with tryptases having trypsin-like cleavage specificities, while chymases have chymotrypsin-like specificities. MC-CPA has acquired its designation

through its specificity for cleaving proteins/peptides from their C-terminal end. The term ''MC proteases'' usually refers to the chymases, tryptases, and CPA that are specifically expressed by MCs and stored within MC granules. However, in addition to these MC-specific proteases, MCs may contain and secrete other proteases, for example, matrix metalloproteases (MMPs) (Baram *et al.*, 2001), cathepsin D, C, and E (Dragonetti *et al.*, 2000; Henningsson *et al.*, 2005; Wolters *et al.*, 2000). In this chapter, the term ''MC proteases'' will refer to the tryptases, chymases, and CPA that are present as releasable compounds within MC secretory granules.

Mature MCs contain conspicuously large amounts of stored proteases. For example, it has been calculated that human skin MCs contain altogether ~16 μg of tryptase and chymase per 10^6 cells (Schwartz *et al.*, 1987). Thus, MC degranulation will result in release of very large quantities of proteases and it is therefore not far fetched to assume that they may profoundly affect any process in which MCs are involved and in which MC degranulation is a component. Indeed, a number of potential functions for various MC proteases have been outlined during the years. Noteworthy however, only in a few cases has any suggested function of a MC protease been confirmed *in vivo*, for example, in experiments taking advantage of genetically modified animals. In this chapter, the aim has been to summarize current knowledge of the MC proteases, including novel insights into their biological function.

2. EXPRESSION OF MC PROTEASES

The specific expression patterns for the MC proteases vary considerably between species and also among different MC subclasses found within a certain species (Table 1). Moreover, the repertoire of expressed MC proteases is also a function of the degree of cellular differentiation and can be modified following, for example, the course of a disease. The phylogenetic relationships between the various MC tryptases and chymases are indicated in Figs. 1 and 2, respectively. As indicated in Fig. 1, MC chymases are classified as belonging to either the α- or β-class, based on phylogenetic relationships (Chandrasekharan *et al.*, 1996).

2.1. Human MC proteases

In humans, MCs are actually classified according to their content of MC proteases, with MCs of the ''MC_T'' class expressing tryptase only and the ''MC_{TC}'' subclass expressing both tryptase and chymase. The MC_{TC} subclass also expresses MC-CPA (Table 1). The MC_T subclass is mainly present in mucosal surfaces, for example, in lung and gut, whereas the MC_{TC} subclass dominates, for example, in skin and intestinal submucosa

TABLE 1 Expression of MC proteases by different MC subclasses in human, mouse, and rat

	Tryptase	Chymase	MC-CPA
Human			
MC_T	β, α	–	–
MC_{TC}	β, α	Chymase	MC-CPA
Cord blood-derived MCs	β, α	Chymase	?
Rat			
CTMCs	rMCP-6, -7	rMCP-1, -5	MC-CPA
MMCs	–	rMCP-2, -3, -4, -8, -9, -10	–
BMMCs	?	rMCP-2	–
Mouse			
CTMCs	mMCP-6, -7	mMCP-4, -5	MC-CPA
MMCs	–	mMCP-1, -2	–
BMMCs-IL-3 (WEHI-3B)	mMCP-6, -7	mMCP-5	MC-CPA
BMMCs-IL-3/SCF	mMCP-6, -7	mMCP-4, -5	MC-CPA
BMMCs-IL-3/IL-9/TGF-β1/SCF	mMCP-6, -7	mMCP-1, -2, -4, -5	MC-CPA

(Metcalfe *et al.*, 1997). Importantly, only one human chymase gene, belonging to the α-family, has been identified (Caughey *et al.*, 1991; Johnson *et al.*, 1986; Urata *et al.*, 1991), whereas two classes of secreted tryptases, α and β, are expressed by human MCs (Miller *et al.*, 1989, 1990; Pallaoro *et al.*, 1999; Schwartz *et al.*, 1981a; Smith *et al.*, 1984; Vanderslice *et al.*, 1990). Three similar β-tryptases have been identified, βI, βII, and βIII, of which βII and βIII may be allelic variants at one locus, and βI and α-tryptase allelic variants at a neighboring locus (Pallaoro *et al.*, 1999). Accordingly, it has been found that as much as 29% of the human population are genetically deficient in α-tryptase (Soto *et al.*, 2002). Human MCs also express a transmembrane tryptase (denoted γ-tryptase or hTMT) (Caughey *et al.*, 2000a; Wong *et al.*, 2002), which appears to get exposed at the cell surface after MC degranulation (Wong *et al.*, 2002). In addition, human MCs have been shown to express an enzyme denoted δ-tryptase, a protease of trypsin-like cleavage specificity but so far of unknown biological significance (Wang *et al.*, 2002). Like in all other species investigated, only one MC-CPA gene has been identified in humans (Goldstein *et al.*, 1989; Reynolds *et al.*, 1989a, 1992). It has also been reported that

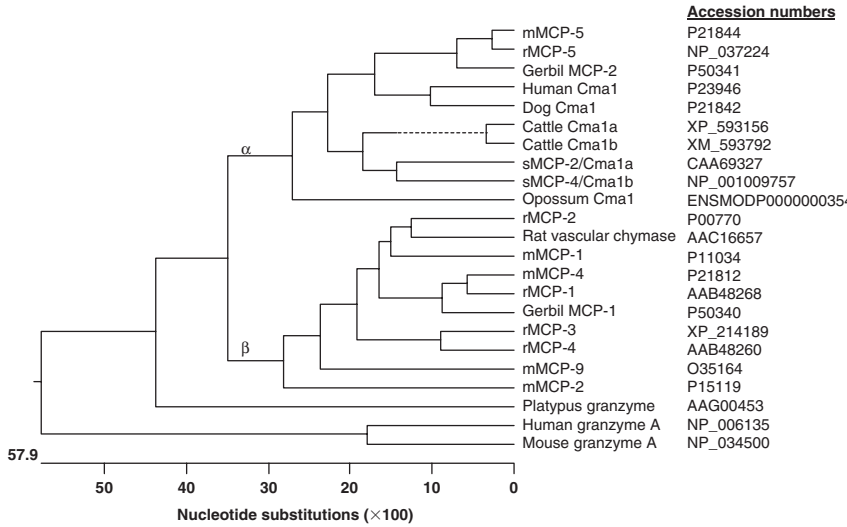


FIGURE 1 Dendrogram of mammalian α- and β-chymases. Phylogenetic analyses of the amino acid sequence of mature proteins were performed, using the CLUSTAL W algorithm implemented in the DNAStar™ software. The length of each branch is proportional to the number of mismatched residues between groups or pairs of sequences. Granzyme A serves as an out-group. Accession numbers from the protein database of NCBI (or Ensembl for Opossum) are listed to the right. m, murine; r, rat; s, sheep; MCP, mast cell protease; and CMA, α-chymase.

human MCs contain and express neutrophil cathepsin G, a serine protease with chymotrypsin-like cleavage specificity. This finding was originally based on immunohistochemical evidence (Schechter *et al.*, 1990). However, immunoblot analysis revealed that the MC cathepsin G antigen had a higher molecular weight than that of reference cathepsin G. In a subsequent study, cathepsin G mRNA was identified by RT-PCR analysis of skin tissue from patients with urticaria pigmentosa (Schechter *et al.*, 1994). However, the MCs were not purified, and it is therefore difficult to completely rule out that the cathepsin G mRNA could be derived from other cell types found within the skin. To fully establish cathepsin G as a MC protease, it would thus be important to identify cathepsin G mRNA expression in purified MCs.

2.2. Murine MC proteases

In rodents, MCs are usually divided into two major subclasses: mucosal MCs (MMCs) and connective tissue type MCs (CTMCs). As this designation indicates, MMCs are preferentially located in mucosal surfaces, for example, in gut, whereas CTMCs are located in, for example, skin, peritoneum, and intestinal submucosa (Metcalfe *et al.*, 1997).

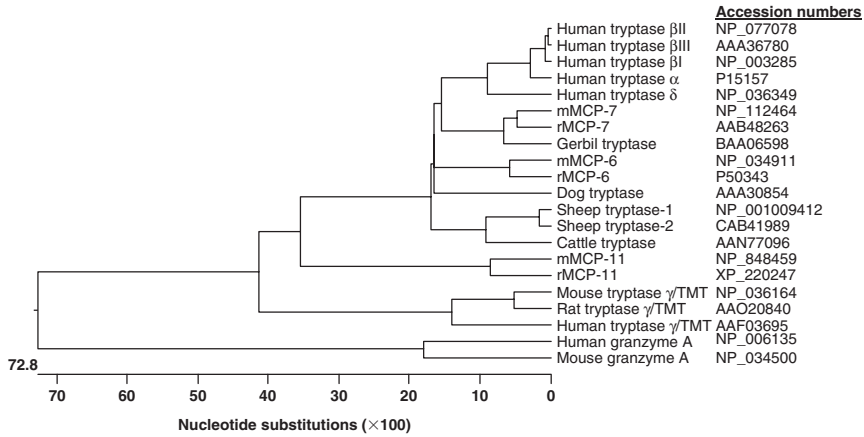


FIGURE 2 Dendrogram of mammalian tryptases. Phylogenetic analyses of the amino acid sequence of mature proteins were performed, using the CLUSTAL W algorithm implemented in the DNAStar™ software. The length of each branch is proportional to the number of mismatched residues between groups or pairs of sequences. Granzyme A serves as an out-group. Accession numbers from the protein database of NCBI are listed to the right. m, murine; r, rat; s, sheep; MCP, mast cell protease; and TMT, transmembrane tryptase.

In mouse, MMCs mainly express two β-chymases, mouse MC protease 1 (mMCP-1) and mMCP-2 (Chu *et al.*, 1992; Reynolds *et al.*, 1990; Serafin *et al.*, 1990; Trong *et al.*, 1989), but do not express tryptase or MC-CPA. CTMCs also express chymases, but instead of mMCP-1 and-2 they predominantly express mMCP-4 (β) and one α-chymase, mMCP-5 (Huang and Hellman, 1991; Reynolds *et al.*, 1990). Yet another chymase, mMCP-9, has been identified and shown to be specifically expressed by MCs in the uterus (Hunt *et al.*, 1997). In addition to chymases, mouse CTMCs express two tryptases: mMCP-6 and -7 (Huang and Hellman, 1991; McNeil *et al.*, 1992; Reynolds *et al.*, 1990, 1991), and also MC-CPA (Reynolds *et al.*, 1989b). Similar to human MCs, mouse MCs express a transmembrane form of tryptase, designated mTMT (Wong *et al.*, 1999). More recently, it was found that murine MCs express an additional trypsin-like enzyme, mMCP-11. However, similar to mMCP-7, mMCP-11 was mostly expressed at very early stages of MC differentiation (Wong *et al.*, 2004).

2.3. Rat MC proteases

Rat MCs display a protease expression pattern highly similar to that of murine MCs. However, for historical reasons, the designation of rat MC proteases is not in perfect analogy with the nomenclature used in mice. Rat

MMCs express predominantly the β-chymase rMCP-2, which is structurally most similar to mMCP-1 in mice, as well as two additional β-chymases: rMCP-3 and -4 (Lützelschwab *et al.*, 1997, 1998). In addition, rat MMCs express several members of the rMCP-8 family (rMCP-8, -9, and -10), a protease family of unknown substrate specificity and biological function (Lützelschwab *et al.*, 1997, 1998). One member of this protease family, mMCP-8, is expressed in mouse, but it appears that mMCP-8 is expressed by basophils rather than MCs (Poorafshar *et al.*, 2000b). Similar to the situation in mouse, rat MMCs do not express tryptases or MC-CPA. Rat CTMCs express two chymases, rMCP-1 (β) and rMCP-5 (α), which are homologues to mMCP-4 and -5, respectively (Le Trong *et al.*, 1987b; Lützelschwab *et al.*, 1997). Thus, despite their discrepancy in nomenclature, rMCP-1 and mMCP-4 are the corresponding mouse and rat β-chymases. In addition, rat CTMCs express MC-CPA (Cole *et al.*, 1991; Lützelschwab *et al.*, 1997) and two tryptases, rMCP-6 and -7 (Lützelschwab *et al.*, 1997), with the latter being the corresponding homologues to mMCP-6 and -7. No detectable mRNA expression of cathepsin G was found in a cDNA library derived from purified rat peritoneal MCs (Lützelschwab *et al.*, 1997).

2.4. MC proteases from other species

In sheep, two tryptases have been identified, tryptase-1 and -2 (Pemberton *et al.*, 2000), and two other MC proteases have also been characterized, sMCP-1 and -3. Interestingly, sMCP-1 has dual trypsin- and chymotrypsin-like cleavage specificity (McAleese *et al.*, 1998). An MC protease of such dual cleavage specificity has also been isolated from parasite-infected goat jejunum (Macaldowie *et al.*, 1998). Canine MCs express one chymase, belonging to the α-family (Caughey *et al.*, 1990). In addition, a dog MC tryptase has been characterized (Vanderslice *et al.*, 1989). Two similar tryptases have been isolated and characterized from bovine sources (Gambacurta *et al.*, 2003). Porcine MC tryptase with properties similar to those of human β-tryptase has also been isolated (Chen *et al.*, 2000). In gerbil MCs, tryptase activity has been identified and two different chymases have been characterized: a β-chymase homologous to mMCP-4 and an α-chymase homologous to mMCP-5 (Itoh *et al.*, 1996).

2.5. MC protease expression profiles as MC markers

The specific expression pattern for MC proteases is a useful way to determine the subclass of MCs. In addition, cells can actually be identified as MCs based on their expression of MC proteases. The reason for this is that MC proteases are highly specific for MCs. However, in some cases, proteases classified as ''MC proteases'' can be expressed in other cell

types. One example is the basophil lineage, which can express α-tryptase, β-tryptase, and chymase, although at much lower levels than in MCs (Jogie-Brahim *et al.*, 2004; Li *et al.*, 1998). Another example is the reported expression of both α- and β-tryptases by human cell lines of monoblast-like characteristics (Huang *et al.*, 1993). Moreover, a chymase highly similar to rMCP-2 has been reported to be expressed by rat smooth muscle cells (SMCs), and has consequently been named SMC chymase (Guo *et al.*, 2001; Ju *et al.*, 2001).

3. GENETIC ORGANIZATION AND REGULATION OF TRANSCRIPTION

3.1. The chymase locus

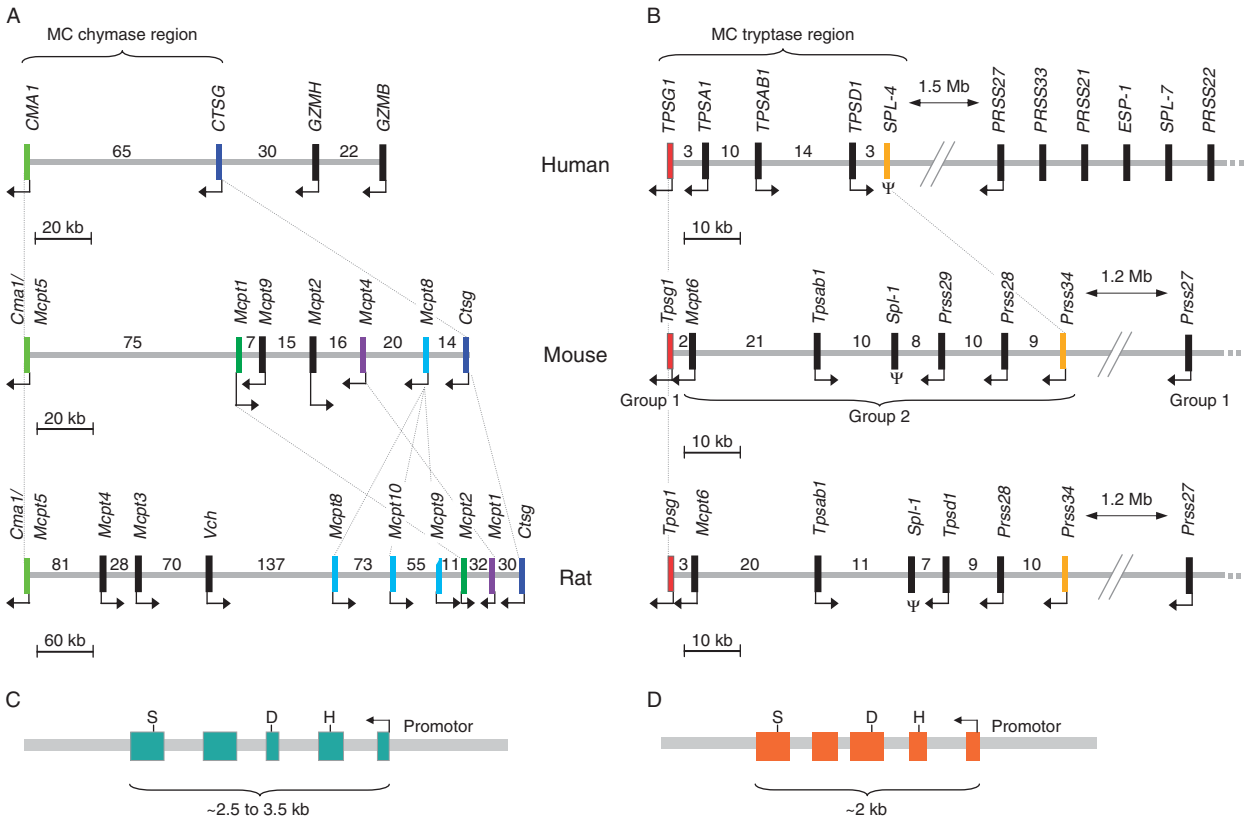
The human MC α-chymase gene (*CMA1*) is located on chromosome 14q11.2 at one end of a small cluster of four genes, covering ∼130 kb (Caughey *et al.*, 1993b). This cluster contains the neutrophil-specific cathepsin G gene (*CTSG*) 65 kb upstream of *CMA1* and the T-cell-specific granzyme H and B genes (*GZMH* and *GZMB*) located 30 and 55 kb upstream of *CTSG* (Table 2; Fig. 3A). The transcriptional orientations of these genes are the same, and their overall genetic organization is conserved in rodents, dog, and cattle (Gallwitz and Hellman, 2006; Gallwitz *et al.*, 2006). However, species-specific expansion of the locus has occurred. The locus can be divided into a chymase and a granzyme region, and particularly in rodents, a massive expansion of the chymase region is evident. The exon/intron organization of a MC chymase gene is depicted in Fig. 3C.

In rat, one α-chymase and eight β-chymase genes are clustered on chromosome 15p12. The complete locus has expanded to 1.1 Mb of which the chymase region covers ∼550 kb. The genetic organization and transcriptional orientations of *Ctsg*, the rMCP-5 (*Cma1/Mcpt5*), and rMCP-1 (*Mcpt1*) genes are the same as the orientation of the genes in the human chymase locus but, interestingly, all other chymase genes, that is, *Mcpt2*, *-3*, *-4*, *-8*, *-9*, and *-10*, transcribe in the opposite orientation (Fig. 3A). *Mcpt8*, *-9*, and *-10* are found in rat but not in human and only one member (*Mcpt8*) is found in mouse.

In mouse, the MC chymase genes are closely clustered on chromosome 14C3, covering ∼160 kb of the complete 325-kb locus (Fig. 3A). Again, the transcriptional orientations of the mMCP-5 gene (*Cma1/Mcpt5*) and *Ctsg* are the same as in humans and also the mMCP-4, -8, and -9 genes (*Mcpt4*, *-8*, and *-9*) are transcribed in the same direction. In contrast, the mMCP-1 and -2 genes (*Mcpt1* and *-2*) transcribe in opposite orientation. Possibly, the opposite transcriptional direction of mouse *Mcpt1/2* versus *Mcpt4/5* may be related to their preferential expression in MMCs and CTMCs, respectively.

TABLE 2 Gene designation, chromosomal location, and homologues of human, mouse, and rat MC proteases

MC proteases	Gene designation	Chromosomal location	Homologues
MC chymases			
Chymase 1	*CMA1*	14q11.2	mMCP-5, rMCP-5
rMCP-1	*Mcpt1*	15p12/13	mMCP-4
rMCP-2	*Mcpt2*		mMCP-2
rMCP-3	*Mcpt3*		mMCP-9
rMCP-4	*Mcpt4*		mMCP-1
rMCP-5	*Cma1/ Mcpt5*		mMCP-5
rMCP-8	*Mcpt8*		mMCP-8
rMCP-9	*Mcpt9*		
rMCP-10	*Mcpt10*		
mMCP-1	*Mcpt1*	14C1/2	
mMCP-2	*Mcpt2*		
mMCP-4	*Mcpt4*		
mMCP-5	*Cma1/ Mcpt5*		
mMCP-9	*Mcpt9*		
MC tryptases			
hTMT/Tryptase γ	TPSG1	16p13.3	mTMT, rTMT
hTryptase αI/βI	*TPSA1*		mMCP-6, rMCP-6
hTryptase βII/βIII	*TPSAB1*		mMCP-6, rMCP-6
hTryptase δ	*TPSD1*		mMCP-7, rMCP-7
	hSPL-4		mMCP-11, rMCP-11
rTMT	*Tpsg1*	10q12	mTMT
rMCP-6	*Mcpt6*		mMCP-6
rMCP-7	*Tpsab1*		mMCP-7
rMCP-11	*Prss34*		mMCP-11
mTMT	*Tpsg1*	17A3.3	
mMCP-6	*Mcpt6*		
mMCP-7	*Tpsab1*		
mMCP-11	*Prss34*		
MC-CPA			
hMC-CPA	*CPA3*	3q24	rMC-CPA, mMC-CPA
rMC-CPA	*Cpa3*	2q24	
mMC-CPA	*Cpa3*	3A3	


A
MC chymase region
CMA1
CTSG
GZMH
GZMB
65
30
22
20 kb
Cma1/
Mcpt5
Mcpt1
Mcpt9
Mcpt2
Mcpt4
Mcpt8
Ctsg
75
7
15
16
20
14
20 kb
Cma1/
Mcpt5
Mcpt4
Mcpt3
Vch
Mcpt8
Mcpt10
Mcpt9
Mcpt2
Mcpt1
Ctsg
81
28
70
137
73
55
11
32
30
60 kb
B
MC tryptase region
Human
TPSG1
TPSA1
TPSAB1
TPSD1
SPL-4
1.5 Mb
PRSS27
PRSS33
PRSS21
ESP-1
SPL-7
PRSS22
3
10
14
3
Ψ
10 kb
Mouse
Tpsg1
Mcpt6
Tpsab1
Spl-1
Prss29
Prss28
Prss34
1.2 Mb
Prss27
2
21
10
8
10
9
Ψ
Group 1
Group 2
Group 1
10 kb
Rat
Tpsg1
Mcpt6
Tpsab1
Spl-1
Tpsd1
Prss28
Prss34
1.2 Mb
Prss27
3
20
11
7
9
10
Ψ
10 kb
C
S
D
H
Promotor
~2.5 to 3.5 kb
D
S
D
H
Promotor
~2 kb

In support for such a notion, the rat MMC and CTMC chymase genes are also transcribed in opposite directions (Fig. 3A).

3.2. The tryptase locus

The human tryptase locus resides on chromosome 16p13.3 and spans ~1.6 Mb. This locus harbors at least 11 genes (Fig. 3B). In mouse, the tryptase locus covers 1.5 Mb, harbors 14 genes, and is situated on chromosome 17A3.3 (Fig. 3B; note that the genes beyond *mPancreasin* are not depicted in the figure). It contains 13 functional tryptic genes and 1 nonfunctional gene, *mSpl*. Both the human and rodent tryptase loci are organized into two separate clusters spaced by ~1.2–1.5 Mb (Fig. 3B). The genes have been divided into group 1 and group 2 tryptases due to conserved amino acid motifs in their propeptides (Wong *et al.*, 2004). Group 1 tryptases share, in their propeptide, a conserved upstream Cys-Gly sequence and a conserved Arg/Lys at position –1. Group 2 genes share a conserved Gly residue at position –1. In human and mouse, the γ-tryptase/TMT gene (*TPSG1*/*Tpsg1*), a group 1 gene, defines one border of the locus, whereas human ϵ-tryptase (*TPSE1*) or mouse *mT6* (not shown in the figure) defines the other end. The group 2 genes are clustered close to *TPSG1*/*Tpsg1* and have average sizes of only 2 kb. In contrast to the chymase locus, the tryptase locus shows a fairly high degree of conservation as regards MC-specific transcripts, without any evident species-specific expansion of homologous genes [Ensembl, March 2007 (Wong *et al.*, 2004)]. Both the mouse and rat tryptase loci contain four MC-specific genes (Table 2): a gene for γ-tryptase/TMT (a group 1 tryptase; *Tpsg1*) and genes for three group 2 tryptases: mMCP-6/rMCP-6 (*Mcpt6*), mMCP-7/rMCP-7 (*Tpsab1*), and mMCP-11/rMCP-11 (*Prss34*). The exon/intron organization of an MC tryptase gene is depicted in Fig. 3D.

3.3. The MC-CPA locus

The MC-CPA gene organization is well conserved in human (*CPA3*), mouse, and rat (*Cpa3*). In human and mouse, *CPA3*/*Cpa3* is situated on chromosome 3q24 and 3A3 and contains 11 exons that span over 32 and

FIGURE 3 Genetic organization of MC proteases. An overall organization comparison of the chymase (A) and tryptase (B) locus in human, mouse, and rat. Note the expansion of chymase genes in rodents. Numbers indicate the distances in kilobases (kb) between genes. Arrows at standing bars show the transcriptional orientation of respective gene. Ψ indicates a nonfunctional gene. The scale is indicated by a bar and a number (kb); note that the rat chymase locus is displayed at a size 1:3 to the human and mouse locus. The human, mouse, and rat tryptase locus scale is 10 kb. The overall exon/intron structures of chymase and tryptase genes are depicted in (C) and (D), respectively. The positions of the serine protease catalytic triad residues, His, Asp, Ser, are indicated.

27 kb, respectively. In rat, *Cpa3* is situated on chromosome 2q24 and is built from 12 exons that span over 34 kb (Ensembl, March 2007).

3.4. Transcriptional regulation

During differentiation, MCs depend on different cytokines for proliferation, maturation, and survival (Gurish and Boyce, 2002). To study various aspects of MC differentiation, including regulation of MC protease expression, many studies have used protocols in which bone marrow cells are differentiated *in vitro* into MC phenotype, so-called bone marrow-derived MCs (BMMCs). In a hallmark study, Gurish *et al.* (1992) developed murine BMMCs in the presence of conditioned medium from WEHI-3B cells (a murine macrophage-like myelomonocytic cell line) and showed that *Mcpt5*, *-6*, and *Cpa3* were upregulated within the first week of culture. WEHI-3B-conditioned medium thus contains factors that drive expression of MC proteases. Two of these factors were identified as IL-3 and SCF, of which IL-3 seems to promote expression of *Mcpt5* and *Cpa3*. *Cpa3* is actually one of the first MC protease genes to be expressed and detected in MCs differentiating from bone marrow progenitors (Rodewald *et al.*, 1996). SCF induced similar protease expression as IL-3 but also promoted expression of *Mcpt4*, which is considered to be a marker of terminally differentiated CTMCs. Interestingly, when IL-3 and SCF were combined, *Mcpt4* was not expressed, indicating that IL-3 arrests the MCs in a more immature state that SCF cannot override (Gurish *et al.*, 1992). Other cytokines that influence the transcriptional activity of MC proteases include TGF-β1, activin A, IL-4, IL-9, and IL-10. TGF-β1 and IL-9 seem to promote the *in vitro* differentiation of bone marrow cells into more mucosal-like MCs, enhancing the expression of the characteristic MMC chymase genes, *Mcpt1* and *-2* (Miller *et al.*, 1999). Whereas *Mcpt4* expression appears to be a late differentiation marker for CTMCs, *Mcpt2* expression has been suggested to be a late differentiation marker for MMCs (Serafin *et al.*, 1990). A combination of IL-9 or IL-10 and SCF induces expression of *Mcpt1*, *-2*, and *-4*, an induction that was counteracted when IL-4 was added to the cultures (Eklund *et al.*, 1993). So, how is the transcription and the protein levels of the MC proteases controlled? Is it dictated by specific transcription factors or by posttranscriptional events? In one study, it was reported that IL-10 induces expression of *Mcpt1* and *-2* in BMMCs (Xia *et al.*, 1996). However, when using a nuclear run-on analysis, transcripts for *Mcpt1* and *-2* were found even in the absence of IL-10, indicating that *Mcpt1* and *-2* are constitutively transcribed by the MCs. Based on these findings, it was suggested that IL-10 stimulation results in higher levels of *Mcpt1* and *-2* transcripts by increasing the mRNA stability (Xia *et al.*, 1996). In contrast, glucocorticoids seem to inhibit IL-10-induced stabilization of *Mcpt1* and *-2* transcripts by

promoting mRNA degradation. The mRNA levels for *Mcpt6* and *-5* were not affected to a major extent by glucocorticoids but, strikingly, transcriptional levels for the serglycin (SG) gene and *Cpa3* were upregulated (Eklund *et al*., 1997).

In mouse, the 5 exon/4 intron organization of the chymase genes is well conserved, and also the promotor/enhancer regions display strong conservation (Huang and Hellman, 1994). In fact, the upstream regions (up to –1200) of *Mcpt1*, *-2*, *-4*, and *-9* all show very strong sequence conservation, pointing to a common theme in their transcriptional regulation. In contrast, the upstream region of *Mcpt5* displays only partial homology to the rest of the chymase locus. The upstream region of *Mcpt8* shows no homology to the rest of the chymase locus (Lunderius and Hellman, 2001; Tchougounova *et al*., 2003).

In a series of elegant studies, Kitamura and coworkers identified that the mi transcription factor (MITF), a basic helix-loop-helix-leucine zipper transcription factor, controls and enhances the transcriptional activity of most of the MC protease genes. MITF regulates transcription through specific E-boxes (CANNTG motifs) located –100 to –1200 upstream of the respective gene. These studies took advantage of a mouse strain with a mutant form of MITF which is a poor transactivator of the MC protease genes (Ge *et al*., 2001; Jippo *et al*., 1999; Morii *et al*., 1994, 1996, 1997). It was reported that the leucine zipper (ZIP) domain of MITF is important for normal MC development and that overexpression of normal MITF rescued expression of the MC protease genes (Morii *et al*., 2001a). Of the chymase genes, *Mcpt4* was shown to be regulated directly by MITF as a targeted deletion of the E-box, at position –383 to –378, abrogated the transcriptional activity (Jippo *et al*., 1999). In contrast, *Mcpt5* was indirectly regulated by MITF as MITF did not bind to the promotor region of *Mcpt5*. Here, addition of SCF rescued the transcriptional activity of *Mcpt5* in mi mutant MCs (Morii *et al*., 1997). Also *Mcpt2* and *-9* are regulated by MITF (Ge *et al*., 2001), whereas, in contrast, *Cpa3* transcription appears to be MITF independent (Morii *et al*., 1996). Finally, a striking observation in the mi mutant mice was that the mRNA levels for *Ctsg* and *Gzmb* were also diminished. Therefore, it has been suggested that one or several locus control regions, guided by MITF, controls the transcriptional activity of the chymase locus in mice (Jippo *et al*., 1999).

Also MC tryptase expression is regulated by MITF. Kitamura and coworkers demonstrated that the expression of *Mcpt6* was directly regulated by MITF (Morii *et al*., 1994, 1996). On interaction of MITF with polyomavirus enhancer-binding protein 2/core-binding factor (PEBP2/CBF), a synergistic effect was seen on the level of *Mcpt6* transcription. The interaction between PEBP2/CBF and MITF was achieved through the N-terminal region of MITF (Ogihara *et al*., 1999). In another study, the myc-associated zinc finger protein-related factor (MAZR) was found to interact with the ZIP

domain of MITF and synergistically activate *Mcpt6*. The zinc finger domain of MAZR was crucial for the interaction to MITF (Morii *et al.*, 2002). In addition, the transcription factor c-jun has been implicated in regulation of *Mcpt6* through interaction with MITF (Kim and Lee, 2004). In contrast, *Tpsab1* (the gene for mMCP-7) does not seem to be positively regulated by MITF (Ogihara *et al.*, 2001). The levels of *Tpsab1* transcript increase when MCs are activated with TGF-β1 and activin A and decrease in the presence of MITF. The role of MITF in this system may be to inhibit Smad3-mediated transcription (Funaba *et al.*, 2003). Finally, MITF regulates TMT gene expression by binding to two E-boxes located at position –1708 and –1805, respectively (Morii *et al.*, 2001b). In summary, MITF may control MC protease gene regulation either by making direct contact with promotor elements or by interacting with other transcription factors.

Considering that MC proteases may be important components of inflammatory pathways (Section 10), it would be likely that their expression is induced by inflammatory stimuli, for example, MC-activating agents. However, there is to date no firm evidence supporting such a notion. Instead, it appears that the expression of the MC proteases is constitutive. As an example, MCs from various sources (e.g., BMMCs, peritoneal MCs) contain high steady state levels of transcript coding for the various MC proteases. Further, there is no striking evidence that MC activation, for example, by FcεRI cross-linking, results in increased transcription of the MC protease genes (Xia *et al.*, 1997). In many cases, however, it has been shown that the mRNA levels for MC proteases are markedly increased in a tissue during the course of a disease. For example, the infection of rats with *Nippostrongylus brasiliensis* induces upregulation of MMC protease expression (Table 1; Lützelschwab *et al.*, 1997). However, the parasite infection causes a dramatic influx of MMCs into the infected tissue. Hence, it appears likely that the increased mRNA levels arise from the increased numbers of MCs present, rather than upregulated mRNA levels within individual MCs.

4. EVOLUTION OF MC PROTEASES

4.1. MCs in evolution

MCs or MC-like cells have been observed in essentially all vertebrates, as well as in some invertebrates, for example, clams (Ulrich and Boon, 2001) and tunicates (Cavalcante *et al.*, 2002). The widespread presence of MCs indicates that they have indeed played an important beneficial role, also in early evolution. However, most research on MC-derived proteases has been done in mammals. Therefore, it is still an open question whether MC proteases have an ancient origin or if they are restricted to mammalian species.

4.2. MC proteases in early evolution

It has not been clearly established whether MC proteases are present in MCs of primitive vertebrates or invertebrates. A recent study searching specifically for chymase-related genes in bony fish (teleosts) revealed that the closest related group of proteases in teleosts differs significantly in primary structure from their mammalian counterparts (Wernersson *et al.*, 2006). Thus, there are no direct structural homologues in teleosts to MC chymase or to any of the other proteases encoded by the chymase locus, that is, to granzyme B-H, rodent mMCP-8/rMCP-8 family, cattle duodenases, or cathepsin G. In contrast, the T-cell granzyme A (and K) can clearly be traced back to early vertebrate evolution, as demonstrated by the identification of single genes with close sequence homologies in several species of teleosts (Praveen *et al.*, 2004; Wernersson *et al.*, 2006), together with data supporting functional homology (Praveen *et al.*, 2006). The lack of primary structure homologues to MC chymase in teleosts does not exclude the possibility that functional homologues may exist. Indeed, a previous report suggests that chymotrypsin-like activity is conferred by granzyme-like proteases in teleosts (Praveen *et al.*, 2006). Certainly, in order to determine the origin and evolutionary importance of the MC proteases, it will be of great interest to identify proteases with chymotrypsin-like, trypsin-like, or CPA activity in MC-like cells of primitive life-forms.

4.3. MC chymase in mammalian evolution

The phylogenetic relationships of MC proteases in mammals have been more thoroughly investigated (Caughey, 2001; Huang and Hellman, 1994; Lützelschwab *et al.*, 1997). One or two α-chymase genes seem to be present in every mammalian species studied so far, whereas β-chymases are restricted to rodents. Although the rodent α-chymases, mMCP-5 and rMCP-5, cluster together with the single human α-chymase in phylogenetic analyses (Fig. 1), they are most likely not the closest functional homologues to human chymase since they have no chymase activity. Instead they have acquired elastase activity (Karlson *et al.*, 2003; Kunori *et al.*, 2002), presumably as a result of a reduced selective pressure on α-chymases in rodents due to the presence of β-chymases. It is possible that β-chymases have emerged exclusively in rodents. On the other hand, the identification of a dog chymase-like gene, *Cma2*, related to both α- and β-chymases favors the alternative hypothesis that an ancestral β-chymase gene was present, at least before the divergence of carnivores and rodents, which subsequently was lost in primates and ruminants (Gallwitz and Hellman, 2006).

Interestingly, the chymase locus can be dated back to at least 200 Myr ago, that is to early mammalian evolution. This was demonstrated by the

presence of a single chymase-like gene in platypus and two related genes in opossum (Gallwitz *et al.*, 2006; Poorafshar *et al.*, 2000a). In fact, of all the genes within the chymase locus, the α-chymase gene seems to have emerged first since one of the chymase-related genes in opossum clustered firmly within the α-chymase group in phylogenetic analyses, whereas the other gene related nearly equally well to any of the other genes of the chymase locus (Gallwitz *et al.*, 2006). The chymase locus has undergone diverse changes during mammalian evolution, characterized by multiple species-specific gene duplications, deletions, neofunctionalization, or loss of function (Gallwitz *et al.*, 2006). In this context, rodents stand out as their chymase loci have expanded remarkably in relation to that in other mammals. For instance, mice and rats hold at least four and eight expressed chymase genes, respectively, as compared to the single chymase gene in humans. The reason for this difference is not known, but one may speculate that a larger pathogenic exposure in rodent lifestyle has fueled the expansion and diversification of the chymase gene family in mice and rats.

4.4. MC tryptase in mammalian evolution

The MC tryptase locus has undergone extensive gene duplications, similar to that seen in the rodent chymase locus. However, these duplications have most likely occurred already before the separation between humans and rodents, as demonstrated by the presence of similar numbers of corresponding genes in humans, mice, and rats (Wong *et al.*, 2004). It is difficult to trace the origin of the tryptase locus since most investigations have studied the genes only of placental mammals. Yet, the high degree of diversification and homology between different species implies an ancient origin. For example, the closely related γ-tryptase genes in mice, rats, and humans suggests that an ancestor gene must have separated from the other tryptase genes long before the separation of rodents and primates (Caughey, 2001; Fig. 2). The recently completed genome databases from many distantly related species will be rich sources of information in tracking the evolutionary events forming the tryptase locus.

5. PROTEIN ORGANIZATION AND PROCESSING

The MC proteases are all synthesized as preproenzymes, that is, with an N-terminal signal peptide directing them into the ER lumen. Cleavage of the signal peptide then results in generation of proforms of the respective proteases, and subsequent cleavage of the propetide results in formation of mature enzyme. In no case has a proform of any MC protease been isolated from natural sources to allow N-terminal sequencing and

determination of the exact site for cleavage of the signal peptide *in vivo*. However, cleavage sites in the signal peptides have been predicted based on the rules of von Heijne (1984). Hence, mMCP-6 and -7 have been predicted to have 10-amino acid-long propeptides (McNeil *et al.*, 1992; Reynolds *et al.*, 1991), whereas human β-tryptase has been proposed to have a slightly longer signal peptide, 12 amino acids (Sakai *et al.*, 1996a). In chymases, it has been postulated that the preproenzyme is cleaved at the –2 position, leaving a 2-amino acid activation pro/activation peptide (Caughey *et al.*, 1991; Huang *et al.*, 1991; Serafin *et al.*, 1991; Urata *et al.*, 1991). This is in analogy with a number of other serine proteases of hematopoietic origin, for example, granzymes, elastase, and cathepsin G, which all are known to possess 2-amino acid activation peptides. The preproenzymes of both mouse and human MC-CPA have been predicted to have 15-amino acid-long signal peptides, followed by 94 amino acid pro/activation peptides (Reynolds *et al.*, 1989a, 1991).

An unusual feature of the MC proteases is that they are all stored as fully processed enzymes, that is, with the propeptide removed. This is in sharp contrast with, for example, the pancreatic digestive proteases, which are stored as proenzymes and are activated after release into the gut lumen. The storage of such massive amounts of fully active proteases within MC granules probably imposes a major challenge for the host cell, considering that escape of the granular proteases into the cytosol may cause serious damage. Most likely, the MC needs protective systems in form of protease inhibitors that neutralize proteases that escape into the cytosol. The existence of such protective mechanisms has not been investigated to any major extent. A likely possibility is that protection against MC proteases leaking into the cytoplasm is conferred by one or several of the relatively recently identified intracellular protease inhibitors of the serpin class (Silverman *et al.*, 2004). In line with such a notion, Strik *et al.* (2004) showed that an intracellular serpin, PI6, is present in human MCs and forms complexes with monomeric β-tryptase.

The fact that MC proteases are stored in fully active form may have an important impact on the understanding of their biological function. Indeed, the storage of the proteases in active form and in such large amounts strongly suggests that the ability to rapidly release preactivated proteases is functionally important for the MCs. The most obvious implication of this is that the MC proteases are aimed to function in the initial phase during a response toward pathogens.

The exact mechanism and enzymes responsible for propeptide processing, leading to mature MC proteases, are only partly understood and are best characterized for the chymase family. Dikov *et al.* (1994) showed that inhibitors of dipeptidyl peptidase I (DPPI; synonym for cathepsin C), a cysteine protease with specificity for cleaving dipeptides from the N-terminus, prevented prochymase activation in the KiSV-M9 cell line,

suggesting that DPPI could be involved in processing of the N-terminal dipeptide of prochymases. Support for this notion came when it was demonstrated in a purified system that DPPI can process the N-terminal dipeptide of recombinant human prochymase and that this cleavage resulted in active enzyme (Murakami *et al.*, 1995). An important implication of this finding is that the N-terminal processing of the propeptide is itself sufficient for gaining enzymatic activity, which appears to be in contrast to the tryptases (see below). The ability of DPPI to cleave off the propeptide in human prochymase has also been confirmed by other laboratories (McEuen *et al.*, 1998). Further strong support for a role of DPPI in prochymase processing came through the knockout (KO) of the DPPI gene in mice, and the demonstration that mice lacking DPPI fail to generate active CTMC chymases *in vivo*. This was shown both by *in situ* enzymatic determinations and by that mMCP-5 was exclusively present in its proform in DPPI$^{-/-}$ BMMCs (Wolters *et al.*, 2001). Hence, DPPI is essential for activation of the CTMC chymases and is presumably also involved in the propeptide processing of the MMC chymases, mMCP-1 and -2. Moreover, it is likely that DPPI has the corresponding function in processing of human prochymase, although this notion remains to be proven *in vivo*. An interesting finding in this context is a loss-of-function mutation in the DPPI gene in the Papillon–Lefevre syndrome, a rare disorder characterized by increased susceptibility to early onset periodontitis (Toomes *et al.*, 1999). By using material from patients suffering form this disorder, it would be possible to investigate if DPPI indeed activates also the human prochymase.

The N-terminal processing mechanisms involved in tryptase activation are much less characterized than those of the chymases. In one report, it was suggested that, similar to the chymases, also protryptases were N-terminally processed by DPPI (Sakai *et al.*, 1996b). It was proposed that protryptase was capable of autocatalytic processing at the Arg(-3)–Val(-2) bond and that DPPI proceeded by cleaving off the remaining dipeptide. Further support for a role of DPPI in tryptase processing came from studies on the DPPI KO strain in which it was demonstrated that the levels of mature tryptase were somewhat lower in DPPI$^{-/-}$ MCs than in wild-type counterparts (Wolters *et al.*, 2001). However, in contrast to the effect on chymases, there was no corresponding accumulation of protryptase in DPPI$^{-/-}$ cells and it is thus not possible to firmly establish a role for DPPI in protryptase processing.

The processing steps leading to formation of mature MC-CPA are not fully understood. In an early report, it was shown that addition of a cysteine proteases inhibitor (E-64d) to KiSV-MC14 cells, a cell line in which both pro- and mature MC-CPA is detected, resulted in the accumulation of pro-MC-CPA (Springman *et al.*, 1995). However, pro-MC-CPA processing was not affected by a DPPI inhibitor, indicating that the processing is catalyzed

by a cysteine protease other than DPPI. An interesting observation was that the pro-MC-CPA processing occurred within the secretory granule rather than in ER or Golgi (Springman *et al.*, 1995), a finding that is supported by an independent study (Rath-Wolfson, 2001). More recently, we sought to identify the proteases involved in processing of pro-MC-CPA by making use of mouse strains lacking individual cathepsins. In line with the previous observations, we did not see any accumulation of pro-MC-CPA in BMMCs lacking DPPI/cathepsin C (Henningsson *et al.*, 2003). Instead, the KO of DPPI caused an accumulation of mature MC-CPA, suggesting that DPPI may be involved in regulating the amount of stored mature MC-CPA within the MC secretory granule (Henningsson *et al.*, 2003). Further, no accumulation of pro-MC-CPA was detected in MCs from animals deficient in either cathepsin B, -L, or -S (Henningsson *et al.*, 2003, 2005). In contrast, when analyzing MCs from mice lacking cathepsin E, an aspartic protease, we noticed a clear accumulation of pro-MC-CPA (Henningsson *et al.*, 2005). However, mature MC-CPA was present to some extent even in the absence of cathepsin E, indicating that cathepsin E is not essential for pro-MC-CPA processing. Absence of a related aspartic acid protease, cathepsin D, did not alter the levels of pro-MC-CPA. Thus, the ability to process pro-MC-CPA is not a general property of aspartic acid proteases (Henningsson *et al.*, 2005).

6. THREE-DIMENSIONAL STRUCTURE

6.1. Chymase

The crystal structures have been solved both for rMCP-2 (Remington *et al.*, 1988) and human chymase (McGrath *et al.*, 1997; Pereira *et al.*, 1999; Fig. 4A). In addition, the structure of human prochymase was solved (Reiling *et al.*, 2003). The three-dimensional structure of human MC chymase revealed a high degree of similarity with pancreatic chymotrypsin, rMCP-2, and cathepsin G. A striking feature of human chymase, but not of rMCP-2, is the presence of a large number of basic amino acid residues (Lys/Arg) in two distinct clusters on the molecular surface (Fig. 4B). Most likely, these clustered basic residues are involved in the interaction of chymase with anionic GAGs (Section 8). By solving the crystal structure of human prochymase, important clues were given as to the molecular events taking place during the chymase activation process. It was shown that two loops, the autolysis loop (Gly142-Thr154) and the 180 s loop (Pro185A-Asp194), were positioned in a way that may prevent enzymatic activity in the proform, and that rearrangement of these structural elements after propeptide processing unleashes the chymase proteolytic activity (Reiling *et al.*, 2003).

Additional insights into the three-dimensional structures of the chymases have been derived from molecular modeling studies. In one study,

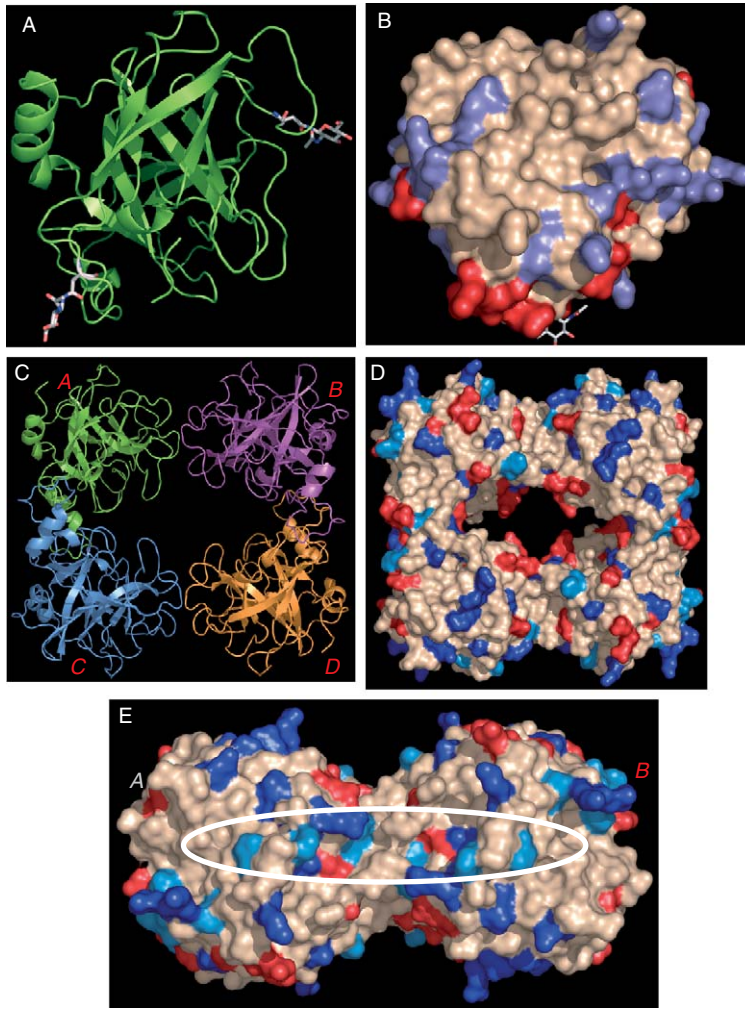


FIGURE 4 (A) Structure of human chymase in ribbon representation. Two N-linked *N*-acetyl-glucosamine units are shown as stick models. (B) Surface representation of human chymase. Positively charged residues (Arg, Lys, His) are indicated in blue and negative residues (Glu, Asp) in red. Note the defined patches of positively charges residues. One N-linked *N*-acetyl-glucosamine unit (Asn 72) is visible (sticks). (C) Structure of tetrameric human β-tryptase in ribbon representation. The four monomer units (*A–D*; red coloring) are represented in different coloring. (D) Surface representation of human β-tryptase viewed down the central cavity. Positively charged residues are indicated in blue, with His residues in light blue and Arg/Lys in dark blue; negative residues in red. (E) Surface representation of tryptase tetramer rotated 90° around the horizontal axis, showing the *A–B* interface. Note the cluster of His residues (indicated by a white oval) spanning over the *A–B* interface.

the four major murine MC chymases, mMCP-1, -2, -4, and -5, were modeled (Sali *et al.*, 1993). An interesting finding in that study was that the murine chymases differed markedly with regard to their electrical charge distribution on the molecular surface. Both mMCP-4 and -5 were, similar to the human chymase (Fig. 4B), predicted to contain two distinct patches of positive charge (Lys/Arg residues), and it was proposed that these regions mediate tight interactions with the highly negatively charged heparin PGs that are present in mMCP-4/5-expressing MCs (i.e., CTMCs). In contrast, mMCP-1 and -2 lacked one of these positive patches, and it was discussed that the lower exposure of positive charge on the mMCP-1/2 surface is related to the lower charge of the PGs resident in mMCP-1/2-expressing MCs (MMCs) than of the CTMC PGs (Enerback *et al.*, 1985).

6.2. Tryptase

In contrast to the chymases, which are active in monomeric form, it was early discovered that the active form of MC tryptase is a tetramer (Schwartz, 1981a). It is now known that MC tryptases from other mammalian species share the tetrameric organization. Further, it was shown that disruption of the tryptase tetramer into monomers caused inactivation of the tryptase (Schwartz and Bradford, 1986). Thus, it was inferred that the tetramerization procedure is necessary for gaining enzymatic activity. Another remarkable feature of MC tryptase is its complete resistance to endogenous macromolecular protease inhibitors (Smith *et al.*, 1984), although the enzyme is readily inhibited by synthetic low molecular weight inhibitors. The latter findings indicated that the active sites of tryptase have limited access to the exterior, but the explanation for these findings remained obscure for a long time. These issues were all resolved when human β-tryptase was crystallized and its three-dimensinal structure solved (Pereira *et al.*, 1998). It was found that the tryptase tetramer was organized as a flat rectangular frame with one tryptase monomer in each of its corners (Fig. 4C). A central pore is formed at the interior of the tetramer and, remarkably, all of the active sites were facing this central pore (Fig. 4C and D). Since the entry into the central pore is quite narrow ($\sim 40 \times 15$ Å), most macromolecular compounds would not be able to enter, thus providing a perfect explanation for the resistance of tetrameric tryptase to macromolecular protease inhibitors. Further, this structural anatomy implies that many macromolecular potential substrates are excluded from access to the active sites. Indeed, many of the identified tryptase substrates are low molecular compounds (Table 6). In addition to β-tryptase, also the structure of human α-tryptase has been solved. α-Tryptase displayed a similar tetrameric organization and general structural features as β-tryptase (Marquardt *et al.*, 2002).

Although it is clear that active tryptase predominantly occurs in a tetrameric form, it has been suggested that tryptase also can be active in a monomeric state (Addington and Johnson, 1996). Further evidence for this came when Hallgren *et al*. (2001b) showed that recombinant mMCP-6, when activated by low molecular weight heparin, gained enzymatic activity without concomitant tetramerization (Fig. 5A). Importantly, it was demonstrated that active monomeric mMCP-6, in contrast to its tetrameric counterpart, was readily inhibited by a macromolecular protease inhibitor. Further, monomeric mMCP-6, in contrast to the tetrameric form, degraded fibronectin (Hallgren *et al*., 2005), a protein that is predicted to be too large to enter the central pore of a tryptase tetramer (Sommerhoff *et al*., 2000). In a subsequent study, it was demonstrated that tetrameric human β-tryptase, under conditions mimicking those that tryptase is exposed to after its exocytosis *in vivo*, can dissociate to functionally distinct active monomers (Fajardo and Pejler, 2003a). The notion that active monomers can be generated from tetrameric human β-tryptase has also been confirmed independently (Fukuoka and Schwartz, 2004). Moreover, similar to mMCP-6, it has been reported that activation of human recombinant β-tryptase with low molecular weight heparin resulted in generation of active tryptase monomers with properties distinct from those of tetrameric tryptase (Hallgren *et al*., 2005).

6.3. MC-CPA

To date, no MC-CPA has been crystallized. Thus, insights into the three-dimensional structure of MC-CPA are so far derived from predictions made by modeling of MC-CPA based on the known structure of other carboxypeptidases. Using such strategy, it was predicted that murine pro-MC-CPA displays high structural similarity to porcine pancreatic pro-CPA and -B (Springman *et al*., 1995). Moreover, it was found that large areas of positive charges were present on the molecular surface, and it was suggested that these areas most likely are involved in interactions with anionic PGs (Springman *et al*., 1995).

7. CLEAVAGE SPECIFICITY

The key to understanding the function of any protease is to identify its *in vivo* substrate(s). Important clues to this issue may come from determining the fine cleavage specificity of a protease, and then search for matching peptide sequences in potential target proteins/peptides. Information regarding the exact cleavage specificity of a protease can also be useful when constructing inhibitors of the protease. In the following, cleavage sites are designated by using the nomenclature of Schechter and Berger (1967),

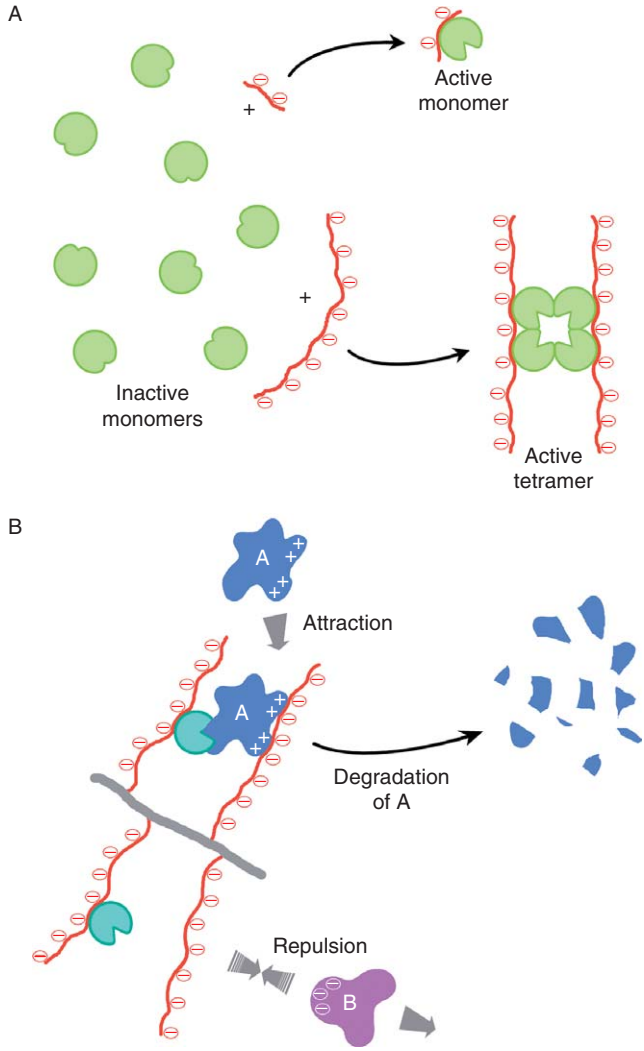


FIGURE 5 Different effects of SG PG on MC tryptase and chymase activity. (A) Model showing cross-linking of inactive tryptase monomers by GAGs, leading to tetramerization and enzymatic activation. GAGs that are of insufficient chain length to accommodate two monomers simultaneously can induce activation but not tetramerization, that is, active monomers. (B) Model for the effect of SG PG on chymase activity. *In vivo*, many MC chymases occur in complexes with SG PG, containing anionic GAG side chains. Proteins/peptides with affinity for GAGs will interact with SG PG, and are thereby presented to the chymase. This results in efficient degradation of GAG-binding compounds, whereas proteins that do not interact with GAGs may be electrostatically repelled and protected from degradation.

that is, where the P1 position corresponds to the residue at the N-terminal side of the cleavage site and the P1′ residue is on the C-terminal side. Hence, the P1–P1′ bond constitutes the cleavage site (scissile bond), P2, P3, and so on, residues are located further to the N-terminal end, and P2′, P3′, and so on, are to the C-terminal end of the cleavage site (Table 3).

Mainly two types of approaches have been employed for determining the substrate specificities of the MC proteases. The first type of approach utilizes chromogenic substrates where a chromofore (usually 4-nitroanilide; pNA) is replacing the P1′ residue and where the P1, P2, and so on, residues are varied. A disadvantage with this methodology is that no information is obtained with regard to the contribution of the P1′, P2′, and so on, residues to the cleavage specificity. Another inherent limitation of this methodology is that a full covering of all possible variants at, for example, the P1–P4 positions would require chemical synthesis of an enormous amount of different chromogenic substrates. Another approach to determine cleavage specificity is to use peptide phage display technique. An advantage of this method is that information is also gained regarding the influence of the P1′, P2′ residues to the cleavage specificity. Further, a peptide phage library will contain far more combinations of sequences than what is practically feasible using a pNA substrate-based approach.

7.1. Chymase

In an early study, Powers *et al.* (1985) investigated the substrate specificities of rMCP-1, -2, human chymase, and dog chymase by using a chromogenic substrate-based approach. They found that all of these chymases showed a preference for Phe at the P1 position, but Tyr, Trp, and Leu were also well tolerated. By varying the P2–P4 positions, a slight preference for hydrophobic residues at the P2 and P3 positions was noted (Powers *et al.*, 1985). Importantly, the substrate specificities of all tested chymases were relatively similar. Karlson *et al.* (2002) employed a peptide phage display-based approach to study the cleavage specificity of the rat chymase, rMCP-4. In accordance with its predicted chymotrypsin-like specificity, rMCP-4 preferred Tyr and Phe at the P1 position. Further, there was a striking preference for bulky/aromatic residues at the P2 position, for Val at P3, for Leu at the P4 position, for residues with small side chains at the P2′ position, and a slight preference for Arg at the P1′ position. Based on the findings, a consensus sequence of Leu-Val-Trp-Phe-Arg-Gly was generated, and the corresponding synthetic peptide was cleaved by rMCP-4 exclusively at the Phe–Arg bond. However, it remains to be shown whether this consensus sequence is the *in vivo* target for rMCP-4. In a follow-up study, Karlson *et al.* (2003) studied the cleavage specificity of rMCP-5 by peptide phage display technique. Prior to the study, it had been predicted that rMCP-5 (and its mouse homologue, mMCP-5)

TABLE 3 Identified cleavage sites for MC chymases

Chymase	↓ P4-P3-P2-P1-P1′-P2′-P3′-P4′	References
Human chymase		
Ang I	I H P F H L	Reilly *et al.*, 1982
Fibronectin	S G P F T D V R	Okumura *et al.*, 2004
-''-	Q M I F E E H G	Okumura *et al.*, 2004
Serum albumin	R E T Y G E M A	Raymond *et al.*, 2003
C1-inhibitor	K M L F V E P I	Schoenberger *et al.*, 1989
Procollagenase	Q F V L T E G N	Saarinen *et al.*, 1994
α_2-macroglobulin	R V G F Y E S D	Walter *et al.*, 1999
IL-1β	N E A Y V H D A	Mizutani *et al.*, 1991
Procollagen 1α	L K S L S Q Q I	Kofford *et al.*, 1997
SCF	T K P F M L P P	Longley *et al.*, 1997
ET-1	V V P Y G L G S	Nakano *et al.*, 1997
Hepatocyte growth factor	I V N L D H P V	Raymond *et al.*, 2006
apoA-I	A T V Y V D V L	Lee *et al.*, 2003a
apoA-I	V S Q F E G S A	Lee *et al.*, 2003a
proIL-18	Q V L F L D Q G	Omoto *et al.*, 2006
rMCP-1		
Ang I	D R V Y I H P F	Le Trong *et al.*, 1987a
Fibronectin	V E V F I T E T	Tchougounova *et al.*, 2001
-''-	T T P F S P L V	Tchougounova *et al.*, 2001
-''-	I T I Y A V E E	Tchougounova *et al.*, 2001
Thrombin	E K T F G A G E	Pejler *et al.*, 1995
-''-	R E T W T T S V	Pejler *et al.*, 1995
-''-	L S D Y I H P V	Pejler *et al.*, 1995
Dog chymase		
Pro-MMP-9	L G K F Q T F E	Fang *et al.*, 1997
-''-	F Q T F E G D L	Fang *et al.*, 1997
mMCP-4		
Ang I	D R V Y I H P F	Caughey *et al.*, 2000b; Lundequist *et al.*, 2004
Ang I	I H P F H L	Caughey *et al.*, 2000b; Lundequist *et al.*, 2004
Hepatocyte growth factor	I V N L D H P V	Raymond *et al.*, 2006

Cleavage sites are indicated using the nomenclature of Schechter and Berger (1967). The scissile bond is indicated by an arrow.

actually may display elastase-like cleavage specificity, that is, cleave after small aliphatic residues rather than after aromatic residues. The basis for this prediction is the presence of a Val (Val199; corresponding to position 216 in chymotrypsin) residue at the primary specificity pocket (S1 subsite), a property that normally confers elastase-like cleavage specificity. In chymotrypsin-like serine proteases, the corresponding residue is occupied by a Gly residue (Solivan *et al.*, 2002). Indeed, the peptide phage display analysis, along with data using chromogenic substrates, clearly indicated that rMCP-5 has elastase-like enzymatic properties with a preference for Ile, Val, or Ala at the P1 position (Karlson *et al.*, 2003). A further examination of the residues flanking the scissile bond revealed a slight preference for hydrophobic residues at the P2 and P3 positions, that is, similarly to other studied chymases (Powers *et al.*, 1985). Further, there was a marked preference for an acidic residue at the P2′ position. The elastase-like properties of rMCP-5 (and also mMCP-5) are also supported by another study (Kunori *et al.*, 2002).

An interesting feature of the human chymase structure is the cationic properties of the S2′ subsite, that is, the binding site for the P2′ residue (Table 3), due to the Lys40 residue (not present in pancreatic chymotrypsin). The cationic nature of S2′ is clearly compatible with accommodation of negatively charged residues at the substrate P2′ position. This, in turn, may explain the productive binding of angiotensin I (Ang I; Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10) to the active site of human chymase, with the carboxyl group of the C-terminal Leu residue of Ang I interacting with the S2′ subsite. Clearly, these findings are well in line with the notion that human chymase preferentially cleaves the Phe8–His9 bond of Ang I (rather than the Tyr4–Ile5 bond which results in Ang I degradation), leading to activation (Section 9.1.1.).

An additional approach to address the cleavage specificity of a protease is to identify the cleavage sites after incubation of the protease with a substrate protein/peptide. Alignment of identified cleavage sites may then reveal consensus sequences for cleavage. Examples of identified cleavage sites for MC chymases in various proteins are displayed in Table 3. An examination of such identified cleavage sites confirms the strict requirements for aromatic residues or Leu at the P1 position. An interesting observation is that Pro is often found at the P2 position of sequences recognized by human chymase, in agreement with that chromogenic substrates with a P2 Pro are good substrates for human chymase (Powers *et al.*, 1985). Aliphatic residues are also frequently found at P2. Moreover, it is clear that the P3–P4 positions show a high degree of variability. Another finding in line with the phage display data reported for rMCP-5 (see above) and the predictions based on the crystal structure of human chymase (see above) is that acidic residues (Asp/Glu) often occupy the P2′ position of sites cleaved by human chymase.

Together, the available data on the fine substrate specificities of chymases suggest that they are relatively promiscuous, except for the strict requirements at the P1 position and the preference for an acidic P2′ residue. It may thus not be possible to determine the physiological targets for the chymases, solely based on identification of preferred cleavage sites. Rather, selection of physiological targets will most likely depend on other factors such as availability of potential substrates at sites of MC degranulation and concentration of substrate. In addition, it is important to note that several of the MC chymases, for example human chymase, mMCP-4, and -5, most likely occur *in vivo* in macromolecular complexes with heparin/chondroitin sulfate (CS) PGs, and there is much evidence suggesting that such association with PGs may profoundly influence the selection of substrates and affect the rate of substrate degradation (Section 8). The actual site of cleavage may also differ depending on the absence or presence of heparin (Le Trong *et al*., 1987a).

7.2. Tryptase

Important knowledge concerning the fine substrate specificity of tryptases has been generated using similar phage display-based systems as those employed for chymases. In one study, Stevens *et al*. examined the specificity of mMCP-7. They found that mMCP-7 showed a remarkable preference for cleaving a consensus sequence of Leu-Ser-Ser-Arg-Gln-Ser (cleavage after the Arg residue), a sequence found within one of the identified tryptase substrates, fibrinogen (Huang *et al*., 1997). Later, the same group examined the cleavage specificity of mMCP-6 using similar techniques. The results indicated a rather broad extended cleavage specificity, with a large degree of variability at the P2–P4 as well as P1′–P3′ positions (Huang *et al*., 1998). Using phage display technique, Harris *et al*. found that βI- and βII-tryptase both displayed a strict requirement for a P1 Arg or Lys. Further, both βI- and βII-tryptase showed a slight preference for Gln at the P2 position, preference for Arg/Lys at P3 and some preference for Pro at P4 (Harris *et al*., 2001). In a parallel study, Huang *et al*. (2001) also found that Pro residues were common components of preferred sequences for human βI-tryptase cleavage. However, Pro residues dominated at P3 rather than P4 (see above), although Pro was also found at P2 and P4. In addition, they found that all of the cleaved sequences had a Lys residue N-terminal of the cleavage site, in particular at the P6 position (Huang *et al*., 2001).

7.3. MC-CPA

The available data on the cleavage properties of MC-CPA have mainly been generated by HPLC analysis of products obtained after cleavage of defined peptides by purified MC-CPA. Using such an approach, it was

shown that human MC carboxypeptidase was able to hydrolyze the C-terminal Leu of Ang I (Table 3), but not the C-terminal His of the generated des-Leu10 Ang I to form Ang II. Further, MC carboxypeptidase was, in contrast to pancreatic CPB, unable to cleave the C-terminal Arg in bradykinin. It was also shown that the MC enzyme hydrolyzed a synthetic dipeptide substrate with a terminal Phe but not the corresponding substrate with a C-terminal Arg (Goldstein *et al.*, 1989). These results suggested that MC carboxypeptidase has cleavage properties similar to that of pancreatic CPA and therefore that the MC carboxypeptidase is a CPA. Also murine MC-CPA has been demonstrated to cleave Ang I, generating des-Leu10 Ang I (Serafin *et al.*, 1987). More recently, it was shown in an *ex vivo* culture of peritoneal MCs that murine MC-CPA in fact may hydrolyze the C-terminal His of des-Leu10 Ang I to form Ang II, and also can hydrolyze the Phe8 C-terminal of Ang II. However, no further hydrolysis was seen, indicating that the Pro residue which is positioned at the C-terminal after cleavage of Phe8 (Table 3) is a poor substrate for MC-CPA (Lundequist *et al.*, 2004). Also in an earlier report, it was noted that Pro, at the penultimate position of dipeptide substrates, prevents hydrolysis by MC-CPA (Everitt and Neurath, 1980).

8. INTERACTION OF MC PROTEASES WITH PGs: IMPLICATIONS FOR STORAGE, ACTIVITY, AND PROCESSING

8.1. Storage

Based on the colocalization of MC proteases with anionic heparin/CS PGs within granules, it was early suggested that these compounds are interacting (Schwartz and Austen, 1980). Evidence for such an interaction between PGs and MC proteases came when it was shown that degranulation of BMMCs caused release of complexes of PGs and serine proteases (Serafin *et al.*, 1986) as well as MC-CPA/PG complexes (Serafin *et al.*, 1987). High concentrations of NaCl disrupted these complexes, demonstrating that the interaction, as expected from the anionic and cationic properties of the MC PGs and proteases, respectively, was of electrostatic nature.

In 1999, two groups simultaneously presented strong evidence supporting an interaction of MC proteases and heparin PG *in vivo* (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). The strategy in both studies was to genetically target the gene for *N*-deacetylase/*N*-sulfotransferase 2 (NDST-2), an enzyme that is required for sulfation of heparin (Forsberg and Kjellen, 2001), but has no role in CS synthesis. NDST-2 appears to be ubiquitously expressed in various tissues and cell types, and is in most tissues coexpressed with an isozyme, NDST-1 (Kusche-Gullberg *et al.*, 1998). However,

CTMCs appear to predominantly express the NDST-2 isoform (Kusche-Gullberg *et al.*, 1998), and with no other compensating NDST isoform present in NDST-2$^{-/-}$ CTMCs, sulfation of heparin is expected to be defective. Accordingly, it was shown that the KO of NDST-2 resulted in profound reductions in the content of sulfated GAGs in CTMCs (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). A striking finding in both studies was that the KO of NDST-2 resulted in an essentially complete absence of stored CTMC proteases, that is, mMCP-4, -5, -6, and MC-CPA (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). Interestingly though, the mRNA levels for the CTMC proteases were unaltered, indicating that the absence of NDST-2 affected the proteases at the level of storage rather than synthesis. Storage of mMCP-6 in BMMCs, in contrast to mMCP-6 storage in CTMCs, was virtually unaffected by the absence of NDST-2 (Henningsson *et al.*, 2002; Humphries *et al.*, 1999). This is presumably due to the presence of CS in these cells (Razin *et al.*, 1982), and that CS can compensate for the lack of heparin in promoting mMCP-6 storage. However, despite the presence of CS, BMMCs were unable to store mMCP-5 and mature MC-CPA (Henningsson *et al.*, 2002; Humphries *et al.*, 1999). Apparently, the latter MC proteases are strictly dependent on GAGs of heparin type for proper storage. In contrast to CTMCs, MMCs were morphologically unaffected by the lack of NDST-2 (Forsberg *et al.*, 1999), a finding that is in good agreement with the predominant presence of CS rather than heparin in MMCs (Enerback *et al.*, 1985).

The NDST-2 KO thus provides strong evidence for a critical role of GAGs of heparin type in storage of several CTMC proteases. However, it does not provide information relating to the core protein that the GAGs are attached to, nor are the NDST-2$^{-/-}$ mice relevant tools for studying the potential role of PGs for granule storage in MMCs. It has been believed for a long time that GAGs in MC granule, irrespective of MC subclass (e.g., CTMC/MMC) or GAG type (heparin/CS), are attached to the same protein core, SG (Kjellén *et al.*, 1989). However, since there are a number of different PG core proteins, it has not been possible to exclude the contribution of GAGs attached to non-SG core proteins to the total pool of PGs stored within MC granules. Therefore, to specifically study the role of serglycin proteoglycan (SG PG) in MC granule homeostasis, the SG core protein gene was targeted (Åbrink *et al.*, 2004). The results revealed, in agreement with the results from the NDST-2 KO, that mMCP-4, -5, -6, and MC-CPA were all critically dependent on SG PG for storage in CTMCs, but not for mRNA expression. Moreover, it was shown that the same proteases, when expressed by BMMCs, were strongly SG-dependent for storage. In contrast, it was noticed both *in vivo* and in BMMCs differentiated into an MMC-like phenotype that mMCP-1 was independent on SG for storage and that mMCP-2 was only partly dependent on SG for storage (Braga *et al.*, 2007). Further, it was

shown that mMCP-7 storage was not affected by the KO of SG (Braga *et al.*, 2007). The latter, together with previous findings (Humphries *et al.*, 1999), supports the notion that mMCP-7 is not dependent on SG PG for storage within MC granules.

Together, the findings derived from the NDST-2 and SG KOs indicate that certain MC proteases are strictly dependent on SG PG for storage, whereas others are non- or only partly SG dependent (Table 4). Although we cannot with certainty explain the differential SG dependence for storage, the most likely explanation lies within the basic charge exposure and corresponding GAG-binding properties of the respective proteases. As displayed in Table 4, mMCP-4, -5, and MC-CPA are all strongly basic proteins and expose defined patches of positive charge on their molecular surface (Section 6). Thus, their strong dependence on SG is not surprising. Also mMCP-6, despite a negative net charge, has been shown to expose defined clusters of positively charged amino acid residues at the molecular surface (most notably His residues; Fig. 4E; Hallgren *et al.*, 2004) and interacts strongly with heparin and other anionic polysaccharides (Hallgren *et al.*, 2001b). mMCP-1, on the other hand, has a much lower net positive charge than mMCP-4/5, well explaining its lack of SG dependence for storage (Braga *et al.*, 2007). Further, the intermediate positive charge of mMCP-2 is well in line with its only partial dependence on SG (Braga *et al.*, 2007). The lack of SG dependence for mMCP-7 storage is in agreement with its independence of NDST-2 (Humphries *et al.*, 1999). On the other hand, it has been shown that recombinant mMCP-7 binds, at low salt conditions, to heparin-Sepharose at acidic pH (Matsumoto *et al.*, 1995).

Although the findings generated through the KO of NDST-2 and the SG core protein firmly establish the importance of SG PGs for storage of CTMC protease, the underlying mechanisms are not fully understood.

TABLE 4 Net charge of MC proteases and their dependence on SG for storage

Protease	Net charge[a] (pH 7.0)	SG dependence for storage	References
mMCP-1	+4.7	−	Braga *et al.*, 2007
mMCP-2	+7.2	(+)	Braga *et al.*, 2007
mMCP-4	+16.7	+	Åbrink *et al.*, 2004
mMCP-5	+13.9	+	Åbrink *et al.*, 2004
mMCP-6	−2.2	+	Åbrink *et al.*, 2004
mMCP-7	−6.2	−	Braga *et al.*, 2007
MC-CPA	+18.5	+	Åbrink *et al.*, 2004

[a] Net charges were calculated for mature proteins, that is, without activation peptide.

An attractive hypothesis would be that SG is necessary for intracellular targeting (sorting) of the SG-dependent proteases into the granule, that is, that SG is sorted to the pathway of regulated secretion and that the various proteases are accompanying SG PG into the granule as cargo. According to such a mechanism, lack of SG would be expected to result in failure to sort MC proteases into granule, accompanied by missorting of MC proteases into the constitutive pathway of secretion. However, the evidence available so far indicate that MC proteases are correctly targeted into MC granule also in $SG^{-/-}$ MCs, and that missorting into the constitutive pathway of secretion is only a partial explanation for the lack of granule-associated MC proteases in $SG^{-/-}$ MCs (Henningsson *et al.*, 2006).

Considering that the interactions between many of the MC proteases and GAGs are of very high affinity, it is likely that the proteases will remain attached to SG PG following MC degranulation. Indeed, such an association has been demonstrated directly by the isolation of MC protease/PG complexes in supernatants obtained from degranulated MCs (Serafin *et al.*, 1986). Such an association may involve a large number of protease units associated with each PG, and there is additionally a potential of cross-linking several PG/protease complexes. For these reasons, high molecular weight aggregates may form with a possible restriction in their ability to diffuse away from the site of MC degranulation. In line with such a scenario, it is known that chymases tend to remain associated with the MC surface after degranulation (Schwartz *et al.*, 1981b; Tchougounova and Pejler, 2001). In fact, chymase activity is present on the surface of MCs even without induction of degranulation (Gervasoni *et al.*, 1986), suggesting that chymase is to some extent constitutively released. In further support, mMCP-6 was reported to remain bound to the extracellular matrix (ECM) following passive systemic anaphylaxis, whereas, in contrast, mMCP-7 is rapidly transported to the blood (Ghildyal *et al.*, 1996). A plausible explanation for these findings is that mMCP-6 remains associated with SG PG, whereas mMCP-7 rapidly diffuses away from the MC surface due to its low affinity for SG PG (see above). Interestingly, it has been shown that mMCP-1 is constitutively released by BMMCs of MMC-like phenotype (Brown *et al.*, 2003), in good agreement with an inability of mMCP-1 to interact with SG, which could result in decreased retention in MMC granules.

The exact organization of the SG PG/MC protease complexes within MC secretory granules is not known. In one potential scenario, individual PG molecules may bind to different proteases, resulting in macromolecular complexes that accommodate both tryptase, chymase, and MC-CPA, with the alternative scenario being that the individual proteases occur in separate complexes with SG PGs. These possibilities have not been addressed so far in rodent systems. However, there is evidence indicating that human MCs actually secrete MC-CPA and chymase in complexes

with PGs which are separate from PG/tryptase complexes (Goldstein *et al.*, 1992). The underlying mechanism behind such a subdivision of protease/PG complexes in the human MC is intriguing, but has not been investigated further. Indeed, it is difficult to envisage how certain PG molecules preferentially ''select'' tryptase over MC-CPA/chymase and vice versa, considering that the interaction between all of the MC proteases and GAGs appears to be of unspecific, electrostatic nature (see below). On a speculative angle, one possibility could be that MCs synthesize tryptase and MC-CPA/chymase with different timing, that is, tryptase/PG complexes are formed during a time frame when tryptase is preferentially synthesized over MC-CPA/chymase and the other way around. Such a mechanism would be in some analogy with the mechanisms behind the formation of granules of discrete contents in neutrophils (Faurschou and Borregaard, 2003). In further support for a separation of tryptase/PG complexes and complexes of PGs with MC-CPA/chymase, it has been shown by ultrastructural analysis that chymase accumulated in electron dense areas of human MC granule, whereas tryptase is found in more electron translucent, crystalline parts of the granule (Whitaker-Menezes *et al.*, 1995). However, such a compartmentalization of granules has not been described for rodent MCs.

It is clear from the above that PGs have a profound impact on the storage of proteases in MC granules. However, it is reasonable to assume that MC granule homeostasis is a dynamic process where each of the granule constituents will affect storage of other granule components. In line with such a notion, it has been shown that lack of mMCP-1 disturbs the crystal intragranular structures in MMCs (Wastling *et al.*, 1998). Further, it was found that lack of MC-CPA affects the solubility of heparin PG in peritoneal MCs (Feyerabend *et al.*, 2005). Another interesting finding is that the KO of MC-CPA caused an impaired ability of CTMCs to store mMCP-5 (Feyerabend *et al.*, 2005). Conversely, it was previously reported that the KO of mMCP-5 resulted in an inability of CTMCs to store MC-CPA (Stevens *et al.*, 1996). These findings, along with a report showing that an increase in MC-CPA storage in cathepsin $C^{-/-}$ BMMCs was accompanied by increased mMCP-5 storage (Henningsson *et al.*, 2003), strongly suggest that mMCP-5 and MC-CPA are dependent not only on SG PG but also on each other for proper storage. Clearly, a plausible explanation would be that MC-CPA and mMCP-5 engage in physical contacts and that such interactions are required for proper sorting into granules or for protection toward proteolytic degradation. In contrast, mMCP-4 and -6 storage was not affected by the KO of MC-CPA (Feyerabend *et al.*, 2005), and the increased storage of MC-CPA in cathepsin $C^{-/-}$ BMMCs was not accompanied by increased mMCP-6 storage (Henningsson *et al.*, 2003), demonstrating that interdependence of MC-CPA and mMCP-5 exhibits a large degree of specificity.

Further support for an interdependence of various granule compounds comes from a study in which the gene for histidine decarboxylase, that is, the enzyme that converts histidine to histamine, was targeted (Ohtsu *et al.*, 2001). It was found that lack of histamine in peritoneal MCs caused reduced metachromatic staining, indicative of a reduced storage of SG PG. Further, it was demonstrated that storage of mMCP-4, -5, -6, and MC-CPA, that is, the SG-dependent CTMC proteases, was defective. A likely explanation for these findings could be that histamine is required for balancing the negative charge of SG PGs in the granules and that the absence of histamine thereby causes defects in PG storage, that in turn may lead to a decrease in the levels of stored proteases.

8.2. Effect of PGs on MC protease activity/activation and processing

Although a major function of SG PG is to promote protease storage, much evidence points to a regulative role of SG PG for MC protease function at various other levels. It has been known for a long time that tryptase tetramers require heparin (or other structurally related polysaccharides) for stability (Schwartz and Bradford, 1986). Further, it has been demonstrated that both mMCP-6 and human β-tryptase are dependent on heparin-like saccharides for tetramerization and enzymatic activation (Hallgren *et al.*, 2001b, 2005; Fig. 5A). Along this line, heparin antagonists have been proven to be potent inhibitors of tryptases (Hallgren *et al.*, 2001a; Lundequist *et al.*, 2003), and one study has suggested that heparin is also needed for processing of protryptase to active enzyme (Sakai *et al.*, 1996b). For a more detailed discussion of various functional aspects of the interaction of tryptase with heparin, see Hallgren and Pejler (2006).

In contrast to the tryptases, chymases do not require GAGs for gaining or maintaining enzymatic activity, but the association of chymase with SG PG may profoundly modulate chymase activity. In early reports, heparin was shown to promote degradation of C3a by rat chymase (Gervasoni *et al.*, 1986) and to enhance the degradation of thrombin by both mMCP-4 (Pejler and Karlstrom, 1993) and rMCP-1 (Pejler *et al.*, 1994). Analysis of the underlying mechanism showed that thrombin, a heparin-binding protein (Sheehan and Sadler, 1994), was attracted to the heparin moiety of the chymase/heparin complex, thus providing an effective presentation of the substrate (thrombin) to the chymase (Pejler and Sadler, 1999; Fig. 5B). As evidence for such a mechanism, it was demonstrated that thrombin in which the heparin-binding region was mutated was degraded to a much lower extent than wild-type thrombin (Pejler and Sadler, 1999). In further support for the proposed mechanism, it has been reported that heparin antagonists efficiently prevent the chymase-dependent degradation of heparin-binding substrates (Tchougounova and Pejler, 2001;

Tchougounova *et al.*, 2001). Together, these findings indicate that the association of chymase with SG PG will favor cleavage of substrates with affinity for heparin. Indeed, several of the known chymase substrates are GAG-binding proteins. However, it should be noted that heparin-binding properties are not absolute prerequisites for being substrates for chymase, as evident by the many nonheparin-binding peptides and proteins (e.g., albumin and Ang I) that are favorable chymase substrates. In fact, it has been shown that cleavage of some substrates, for example, Ang I (Murakami *et al.*, 1995), is actually decreased in the presence of heparin. It should also be stressed that the proposed mechanism is, for obvious reasons, only operative for chymases that interact with anionic GAGs. An important question is if this mechanism is also operative *in vivo*. Certainly, since many chymases most likely remain tightly associated with SG PG also after their release, it is plausible that SG PG will influence the selection of chymase substrates also *in vivo*. In support for this, one of the likely *in vivo* substrates for mMCP-4, fibronectin, is a GAG-binding protein (Maccarana and Lindahl, 1993).

In addition to influencing the selection of substrates, it has been shown that heparin PG offers protection of chymases toward a range of endogenous macromolecular protease inhibitors, including α_1-antichymotrypsin (α_1-AC), α_2-macroglobulin, and α_1-protease inhibitor (α_1-PI) (Lindstedt *et al.*, 2001b; Pejler, 1996; Pejler and Berg, 1995). Thus, SG PG may modulate the activity of GAG-binding chymases at several levels: by promoting selection of certain substrates, by restricting chymase mobility, and by increasing the life span of chymases after their exocytosis.

There are currently no reports describing effects of GAGs/PGs on the enzymatic activities of MC-CPA. However, there is evidence indicating that the processing of pro-MC-CPA into active protease is dependent on PGs. First, it was shown that the processing of pro-MC-CPA was defective in BMMCs lacking NDST-2 (Henningsson *et al.*, 2002) and it has also been demonstrated that BMMCs lacking SG show defects in pro-MC-CPA processing (Henningsson *et al.*, 2006). The underlying mechanism behind these findings is not clear, but it may be speculated that the pro-MC-CPA-processing protease(s) is dependent on SG PG or that the association of pro-MC-CPA with SG PG is required for proper presentation of the cleavage site to the pro-MC-CPA-processing enzyme(s).

8.3. Structural basis for GAG: MC protease interaction

The structural basis for the interaction between GAGs and chymases has been the subject of investigation. It was found that the binding of GAGs to both rMCP-1 (Pejler and Maccarana, 1994) and human chymase (Sayama *et al.*, 1987) was of high affinity and of unspecific electrostatic nature, that is, no specific structural element in the GAG was required for binding.

Moreover, it was determined that the minimal size for binding of heparin to rMCP-1 was a 14-mer oligosaccharide (Pejler and Maccarana, 1994); thus, the minimal binding saccharide was relatively large as compared with minimal oligosaccharides required for binding to other heparin-binding proteins such as antithrombin (Baglin *et al*., 2002). Also the interaction between MC tryptase and GAGs have been shown to be of unspecific electrostatic nature (Alter *et al*., 1987; Hallgren *et al*., 2001b, 2005); see also a review on this subject (Hallgren and Pejler, 2006). To date, there are no reports in which the nature of the interaction between GAGs and MC-CPA is described.

9. SUBSTRATES FOR MC PROTEASES

Given that the MC proteases, with certain exceptions, have a relatively broad cleavage specificity (Section 7), it would be expected that a large number of proteins/peptides could be potential substrates. Indeed, a multitude of proteins and peptides are known to be cleaved by MC proteases (Tables 5–7). Many of these have been identified through incubation of the MC protease with the protein/peptide in a purified system. In other cases, substrates have been identified in cell culture systems to which a MC protease has been added; alternatively, the use of MC protease inhibitors has implicated certain proteins/peptides as substrates. In only a few cases, there is *in vivo* evidence linking an MC protease to degradation of a certain substrate (Table 8).

In order to understand the *in vivo* function of the MC proteases, both in normal physiology as well as in pathological settings, it is imperative to identify their true *in vivo* substrates. Certainly, identification of a given compound as a substrate for an MC protease *in vitro* can give important clues as to this, but one should be careful when attempting to extrapolate such findings into an *in vivo* setting. Most likely, the ''selection'' of substrates by the MC proteases *in vivo* is dependent on a variety of factors. Obvious factors are the availability of potential MC protease substrates at the site of MC degranulation and, importantly, the concentration of a potential substrate in relation to the k_{cat}/K_m ratio of the MC protease for that particular compound. If indeed a potential substrate is present at the site of MC degranulation, and if its concentration is favorable, it is likely that cleavage will occur *in vivo*. It is also very likely that the ''selection'' of substrates by a given MC protease may differ during different phases of a biological process. For example, immediately following the release of a MC protease, for example, in early phases of an inflammatory reaction, only resident potential substrates have the chance of being degraded by the MC protease. Such resident compounds may include various ECM components. In the next phase, compounds that are newly synthesized

TABLE 5 Substrates for MC chymases

	References
Procollagen	Kofford *et al.*, 1997
Procollagenase	Saarinen *et al.*, 1994
Pro-MMP-9	Chen *et al.*, 2002; Fang *et al.*, 1996; Tchougounova *et al.*, 2005
Fibronectin	Banovac and De Forteza, 1992; Lazaar *et al.*, 2002; Tchougounova *et al.*, 2003; Vartio *et al.*, 1981
Vitronectin	Banovac *et al.*, 1993
TIMP-1	Frank *et al.*, 2001
Substance P	Caughey *et al.*, 1988
VIP	Caughey *et al.*, 1988
Bradykinin	Reilly *et al.*, 1985
Kallidin	Reilly *et al.*, 1985
Big-endothelin 1/2	Kido *et al.*, 1998; Nakano *et al.*, 1997; Takai *et al.*, 1998; Wypij *et al.*, 1992
Neurotensin	Goldstein *et al.*, 1991
Hepatocyte growth factor	Raymond *et al.*, 2006
CTAP-III	Schiemann *et al.*, 2006
Pro-IL-18	Omoto *et al.*, 2006
IL-6, IL-13	Zhao *et al.*, 2005
TGF-β1	Taipale *et al.*, 1995
IL-1β-precursor	Mizutani *et al.*, 1991
SCF	de Paulis *et al.*, 1999; Longley *et al.*, 1997
apoE, apoA-I, apoA-II	Lee *et al.*, 2002b, 2003a; Lindstedt *et al.*, 1996
apoB	Kokkonen *et al.*, 1986
Phospholipid transfer protein	Lee *et al.*, 2003b
PAR-1	Schechter *et al.*, 1998
C3a	Gervasoni *et al.*, 1986; Kajita and Hugli, 1991
Albumin	Raymond *et al.*, 2003
Occludin	Scudamore *et al.*, 1998
C1 inhibitor	Schoenberger *et al.*, 1989
Ang I	Urata *et al.*, 1990
Thrombin	Pejler and Karlstrom, 1993

and secreted into the tissue as a consequence of the onset of an inflammatory response, for example, cytokines and chemokines, may be targets for MC proteases. Somewhat later, plasma components that have escaped

TABLE 6 Substrates for MC tryptases

	References
Kininogen	Imamura *et al.*, 1996
Prekallikrein	Imamura *et al.*, 1996
Fibrinogen	Huang *et al.*, 1997; Schwartz *et al.*, 1985
Gelatin	Fajardo and Pejler, 2003b; Raymond *et al.*, 2005
VIP	Caughey *et al.*, 1988
PHM	Tam and Caughey, 1990
CGRP	Tam and Caughey, 1990
Pro-uPA	Stack and Johnson, 1994
Fibronectin	Fajardo and Pejler, 2003a; Hallgren *et al.*, 2001a; Kaminska *et al.*, 1999; Lohi *et al.*, 1992
HDL	Lee *et al.*, 2002c
pro-MMP-3	Gruber *et al.*, 1989; Lees *et al.*, 1994
PAR-2	Cenac *et al.*, 2002; Corvera *et al.*, 1997; Mirza *et al.*, 1997; Molino *et al.*, 1997
Type VI collagen	Kielty *et al.*, 1993
Pre-elafin	Guyot *et al.*, 2005
RANTES	Pang *et al.*, 2006
Eotaxin	Pang *et al.*, 2006

TABLE 7 Substrates for MC-CPA

	References
ET-1	Metsarinne *et al.*, 2002
Sarafotoxin	Metz *et al.*, 2006
Ang I	Goldstein *et al.*, 1989; Lundequist *et al.*, 2004; Serafin *et al.*, 1987
apoB-100	Kokkonen *et al.*, 1986
Neurotensin	Goldstein *et al.*, 1991

into the tissue as a consequence of increased capillary permeability may be cleaved. During this phase of the reaction, it is also likely that protease inhibitors derived from extravasated blood plasma will downregulate the activity of the MC proteases. In a following healing phase of the inflammatory reaction, yet other substrates could be targets for MC proteases. Alternatively, if the acute inflammation proceeds to a chronic phase, additional substrates could be cleaved.

An important consideration regards the consequences of the cleavage of a certain compound by a given MC protease. In many cases, the result

TABLE 8 MC protease KO strains and KO strains in which MC proteases are affected

Knockout	Phenotype	References
mMCP-1	Altered granular organization; delayed expulsion of *T. spiralis*	Knight *et al.*, 2000; Wastling *et al.*, 1997
mMCP-4	Defective pro-MMP-9 processing; skin fibrosis	Tchougounova *et al.*, 2003, 2005
mMCP-5	Defective MC-CPA storage; protection in ischemia reperfusion injury	Abonia *et al.*, 2005
MC-CPA	Altered granular staining; defective mMCP-5 storage	Feyerabend *et al.*, 2005
SG	Severely defective MC protease storage CTMCs, MMCs and BMMCs affected	Åbrink *et al.*, 2004; Braga *et al.*, 2007
NDST-2	Severely defective MC protease storage CTMCs affected	Forsberg *et al.*, 1999; Humphries *et al.*, 1999
Histidine decarboxylase	Reduced MC PG and protease content	Ohtsu *et al.*, 2001

could be degradation of the compound and thereby loss of functional properties. In fact, given that the MC proteases generally have quite broad cleavage specificities, they are indeed well suited to carry out such general degradation processes (Fig. 6). In other situations, the cleavage of a certain compound can lead to its activation, as exemplified by the activation of several proenzymes by different MC proteases (Table 5). It is important to stress that these modes of action are not necessarily mutually exclusive. For example, an initial activating cleavage in a proenzyme may be followed by additional inactivating cleavages.

9.1. Chymase

9.1.1. Angiotensin I

A multitude of substrates has been identified for different MC chymases (Table 5). Among these, Ang I (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10) has by far attracted the largest attention. Early reports

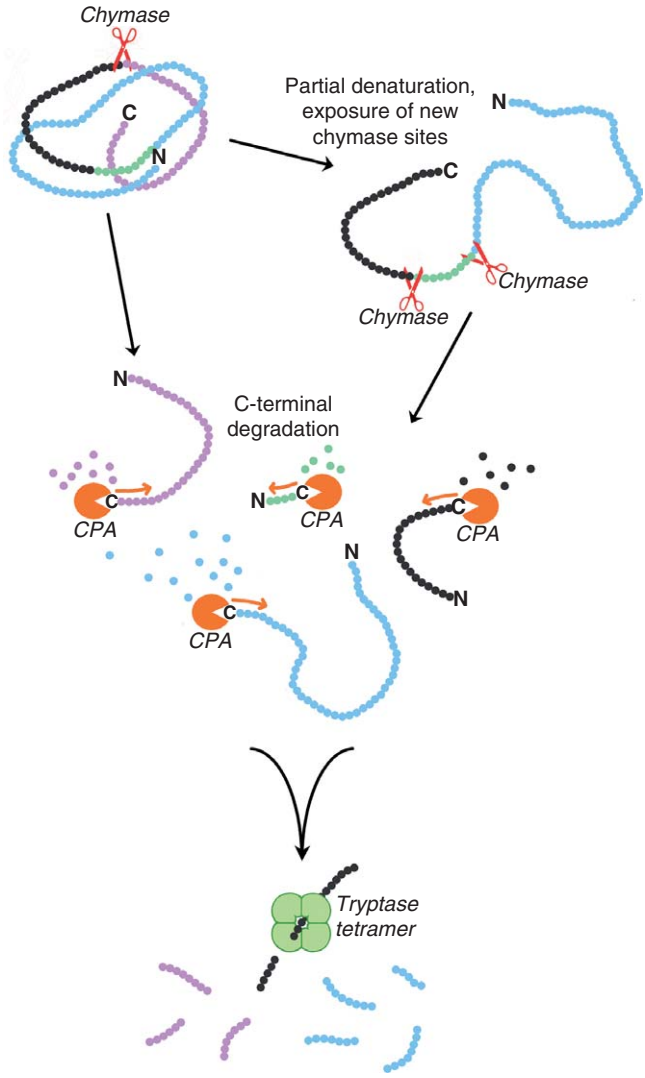


FIGURE 6 Hypothetical model for concerted action of MC proteases in substrate degradation. Initial internal cleavages by chymases expose new C-terminal ends that may be substrates for MC-CPA. Chymase-catalyzed internal cleavages may also lead to partial denaturation and exposure of new chymase cleavage sites. Partially degraded substrates may reach a size compatible with entry into the central pore of tetrameric tryptase, for further degradation.

showed that human chymase can cleave Ang I at the Phe8–His9 bond (Table 3), thereby forming Ang II, a potent vasoconstrictor (Reilly *et al.*, 1982; Urata *et al.*, 1990). It was also demonstrated that whereas human

chymase predominantly cleaves the Phe8–His9 bond, rat chymase (rMCP-1) preferentially cleaves at the Tyr4–Ile5 bond, resulting in degradation of the peptide and destroyed biological activity (Sanker *et al.*, 1997). These findings indicated that the mode of Ang I cleavage, that is, at the Phe8–His9 or Tyr4–Ile5 bond, is a functional parameter that discriminates the α- from the β-chymases, with α-chymases preferentially catalyzing the activation cleavage and β-chymases predominantly causing Ang I degradation. However, it was later shown that certain β-chymases, for example, mMCP-4, in addition to degrading Ang I also can cleave at the Phe8–His9 bond, yielding Ang II (Caughey *et al.*, 2000b). Thus, the ability to activate or degrade Ang I may not be a property that strictly belongs to either the α- or β-chymase family. The possibility that chymase may have a role in the generation of Ang II *in vivo* has been studied intensely (reviewed in Dell'Italia and Husain, 2002). This notion has, for example, been prompted by the findings that Ang-converting enzyme (ACE) inhibitors only produce partial inhibition of Ang II formation (Padmanabhan *et al.*, 1999; Wolny *et al.*, 1997). Further, the KO of ACE only partially prevented Ang II formation (Wei *et al.*, 2002), suggesting that other Ang II-generating mechanisms may be operative, such as chymase catalyzed. Indeed, *in vivo* studies, for example, by using isolated human mammary arteries have shown that chymase accounts for a substantial part of the total Ang II-generating capacity (Richard *et al.*, 2001). Moreover, transgenic overexpression of human chymase in mice indicated a correlation between chymase expression and cardiac content of Ang II (Chen *et al.*, 2002). In further support for a role of MCs in Ang II formation, it was shown that mice lacking MCs (W/W^v mice) showed an impaired ability to generate Ang II (Li *et al.*, 2004). In the latter study, it was suggested that mMCP-5 is a major player in MC-dependent Ang II formation. However, this notion is difficult to reconcile with the elastase-like substrate specificity of mMCP-5 (Section 7), which is not compatible with cleavage of the Phe8–His9 bond in Ang I. To firmly establish a role of MC chymase in Ang II generation *in vivo*, it will be important to investigate Ang II generation in mice lacking appropriate MC chymases. However, this remains to be done. Interestingly, recent evidence suggests that a chymase-dependent pathway for Ang II production, induced by advanced glycation end products (AGEs), contributes to complications in diabetes (Koka *et al.*, 2006).

9.1.2. ECM components

An examination of the identified chymase substrates reveals that many of them are components of the ECM or are involved in regulation of ECM turnover (Table 5). Indeed, the location of MCs within connective tissues and, hence, the release of chymase into connective tissues in association with MC degranulation is well in line with a role for chymase in regulating

ECM composition. It was early shown, in a purified system, that fibronectin is a substrate for human chymase (Vartio *et al.*, 1981). Later it was demonstrated that human chymase released fibronectin fragments from the ECM of cultured airway SMCs (Lazaar *et al.*, 2002) and that fibronectin is also a substrate for murine MC chymases (Tchougounova *et al.*, 2001). More recently, it was reported that the KO of mMCP-4 caused marked accumulation of fibronectin, both in *in vitro*-cultured peritoneal exudate cells and *in vivo* (Tchougounova *et al.*, 2003, 2005). Thus, chymase is likely to be involved in fibronectin turnover by catalyzing its degradation. Also vitronectin, another prominent ECM component, has been shown to be a substrate for MC chymase (Banovac *et al.*, 1993).

Chymase may also regulate ECM composition indirectly, by cleaving and thereby activating compounds that, in turn, can modulate ECM composition. For example, chymase has been found to release latent TGF-β1 bound to ECM, unleashing its stimulating activity on fibroblast ECM production (Taipale *et al.*, 1995). Moreover, rat chymase may activate latent TGF-β1 present within the MC secretory granule (Lindstedt *et al.*, 2001a). Such effects on TGF-β1 would thus promote ECM deposition. Another indirect activity of chymase that would lead to increased ECM deposition, if operative *in vivo*, is the cleavage of procollagen leading to fibril formation (Kofford *et al.*, 1997). Chymase has also been reported to have activities that indirectly can cause ECM degradation. One example is the activating cleavage of procollagenase by human chymase (Saarinen *et al.*, 1994). Another, and well-studied, example is the activation of pro-MMP-9. This activity was first demonstrated for dog chymase, which was shown to cleave pro-MMP-9 released by a mastocytoma cell line (Fang *et al.*, 1996, 1997). Thus, MCs could have the capability of both generating pro-MMP-9 and activating it without involving accessory cells. It has also been shown that mMCP-4 can activate pro-MMP-9 in a purified system (Coussens *et al.*, 1999) and strong evidence for a physiological role of chymase in pro-MMP-9 activation came when it was demonstrated that mice lacking mMCP-4 failed to activate pro-MMP-9 *in vivo* (Tchougounova *et al.*, 2005). Moreover, the lack of mMCP-4 caused exaggerated deposition of collagen in skin, supporting a role for chymase in regulating connective tissue homeostasis *in vivo* (Tchougounova *et al.*, 2005). In further support for a role of chymases in promoting MMP activities, it has been reported that chymase degrades tissue inhibitor of matrix metalloproteases-1 (TIMP-1), an inhibitor of various MMPs (Frank *et al.*, 2001), and that MC chymase can activate pro-MMP-2 (Lundequist *et al.*, 2006).

9.1.3. Lipoproteins

A number of studies from Kovanen and coworkers point to a potential role for chymase in regulating various lipoprotein components. Rat chymase (rMCP-1) was found to degrade lipoproteins containing apolipoprotein

A-I (apoA-I), apoA-II (Lee *et al.*, 2003a; Lindstedt *et al.*, 1996), and apoB (Kokkonen *et al.*, 1986). Moreover, human chymase can degrade apoA-I, -II (Lee *et al.*, 2003a) as well as apoE (Lee *et al.*, 2002b), and phospholipid transfer protein (Lee *et al.*, 2003b). The action of chymase on these apolipoproteins was shown to reduce the transfer of cholesterol from macrophages to lipoprotein particles, thereby promoting foam cell formation. It has therefore been suggested that chymase accordingly may have a role in the formation of atherosclerotic plaques (reviewed in Lee-Rueckert and Kovanen, 2006). However, this notion remains to be proven experimentally *in vivo*, for example, by using animals lacking the appropriate chymase.

9.1.4. Inflammatory substances/bioactive peptides

MC chymases have been implicated in regulating the levels of diverse inflammatory substances and bioactive peptides. One important example is the activation of the IL-1β precursor by human chymase (Mizutani *et al.*, 1991). Other examples include the activation of big-ET-1 and -2 into ET-1 and -2 by human, rat, and monkey chymase (Kido *et al.*, 1998; Nakano *et al.*, 1997; Takai *et al.*, 1998; Wypij *et al.*, 1992); the ability of human chymase to activate connective tissue-activating peptide III (CTAP-III) into neutrophil-activating peptide 2 (NAP-2/CXCL7) (Schiemann *et al.*, 2006); the degradation of TNF by soybean trypsin inhibitor-sensitive proteases (presumably rMCP-1/5) released from rat peritoneal MCs (Alshurafa *et al.*, 2004), and the recently discovered ability of human chymase to activate the IL-18 precursor in a purified system (Omoto *et al.*, 2006). Interestingly, platelet factor 4 (PF-4), a heparin antagonist (Lane *et al.*, 1986), inhibited CTAP-III processing without being cleaved itself. Although not proven, we may speculate that PF-4 acts by binding to the SG PG to which chymase is attached (Section 8; Fig. 5B), thereby affecting chymase activity toward CTAP-III. Chymases have also been shown to degrade substance P (Caughey *et al.*, 1988), vasoactive intestinal peptide (VIP) (Caughey *et al.*, 1988), kallidin (Reilly *et al.*, 1985), bradykinin (Reilly *et al.*, 1985), and C3a (Gervasoni *et al.*, 1986; Kajita and Hugli, 1991). Hence, chymase may play proinflammatory roles by activating certain substances but may also have a dampening effect by degrading others.

9.2. Tryptase

The substrate profile for tryptase exhibits several similarities to that of chymase. First, tryptase has also been reported to act on various ECM compounds or components related to ECM turnover. For example, tryptase can degrade fibronectin (Kaminska *et al.*, 1999; Lohi *et al.*, 1992), although evidence indicates that only the monomeric form of tryptase has this capacity (Fajardo and Pejler, 2003a; Hallgren *et al.*, 2005). Further,

tryptases can degrade denatured collagen (gelatin) (Fajardo and Pejler, 2003b) and type VI collagen (Kielty *et al*., 1993), as well as activate pro-MMP-3, a protease that may regulate ECM composition by subsequently activating pro-MMP-1 (Lees *et al*., 1994). Second, tryptase has, similar to chymase, been demonstrated to degrade a number of bioactive peptides including VIP, peptide histidine-methionine (PHM), and calcitonin gene-related peptide (CGRP) (Caughey *et al*., 1988; Tam and Caughey, 1990). Third, also tryptase has been implicated in lipoprotein degradation, through its ability to cleave the apolipoprotein components of high-density lipoprotein (HDL) (Lee *et al*., 2002c).

9.2.1. Fibrinogen

One of the best-characterized substrates for tryptase is fibrinogen. Schwartz *et al*. (1985) showed that fibrinogen was degraded by human lung tryptase and that fibrinogen thereby lost its clotting activity. Later, Stevens *et al*. expressed recombinant mMCP-7 and when examining its cleavage specificity by phage display technique, they found that mMCP-7 preferentially cleaves a sequence found in fibrinogen (Huang *et al*., 1997; Section 7). They also reported that mMCP-7 prolonged the clotting time when added to plasma (Huang *et al*., 1997), suggesting a role in regulation of blood coagulation. On the other hand, there is to our knowledge no report suggesting that mice deficient in mMCP-7 (the C57BL/6 strain) suffer from clotting disorders. Tryptase may also have the potential of influencing the blood clotting system at another level, by proteolytically activating prourokinase plasminogen activator (Stack and Johnson, 1994). Noteworthy is that also chymase has been reported to have activities of relevance for regulating blood clotting/fibrinolysis, through its ability to degrade both thrombin (Pejler and Karlstrom, 1993) and plasmin (Tchougounova and Pejler, 2001).

9.2.2. Protease-ativated receptor 2

Out of the implicated tryptase substrates, protease-activated receptor 2 (PAR-2) has by far attracted the largest attention. PAR-2 belongs to a group of cell surface receptors (PAR-1–4) that all expose a ''tethered'' ligand which after cleavage binds to its receptor, thereby inducing signaling events that can be recorded by influx of calcium (reviewed in Ossovskaya and Bunnett, 2004). A number of *in vitro* studies have shown that tryptase has the ability to cleave and activate PAR-2 (Corvera *et al*., 1997; Mirza *et al*., 1997; Molino *et al*., 1997), thereby inducing various signaling events such as the MAP kinase pathway (Temkin *et al*., 2002; Weidinger *et al*., 2005a; Yoshii *et al*., 2005), MEK (Yoshii *et al*., 2005), phosphatidylinositol 3-kinase (Brown *et al*., 2005), and ERK1/2 (Brown *et al*., 2005). Lately, evidence has been obtained that PAR-2 may in fact be an *in vivo* substrate for tryptase. Kelso *et al*. demonstrated that injection of human tryptase into mice caused an

arthritis-like joint inflammation. Interestingly, this response was abrogated in animals lacking PAR-2 expression, thus strongly indicating tryptase to cause joint inflammation through activation of PAR-2 (Kelso *et al.*, 2005). Additional evidence for an *in vivo* relevance of tryptase-catalyzed PAR-2 cleavage comes from a study in which tryptase-induced intestinal inflammation was suppressed in PAR-$2^{-/-}$ mice (Cenac *et al.*, 2002).

9.2.3. Other substrates

Tryptase was shown to degrade RANTES and eotaxin, thereby abolishing their chemotactic activities on eosinophils (Pang *et al.*, 2006). Further, a recent study indicated that tryptase may have a role in processing of pre-elafin into active elafin, a serpin protease inhibitor that has been linked to asthma (Guyot *et al.*, 2005).

9.2.4. Effect of the tetrameric organization on substrate cleavage

An important issue when considering possible physiological substrates for tryptase is its tetrameric organization, with the active sites facing a central pore. The narrow entrance of the central pore will most likely cause steric hindrance for entry, and it is therefore difficult to envisage how large proteins, such as fibronectin, fibrinogen, and so on (Table 6), gain access to the tryptase active sites [as discussed by Sommerhoff *et al.* (2000)]. One possibility is that a part of the substrate protein is ''extracted,'' by unknown mechanisms, allowing entry into the central pore and subsequent proteolysis (Sommerhoff *et al.*, 2000). Another possibility is that the tryptase tetramer may dissociate into active monomers, and that monomeric tryptase cleaves at least some of the known tryptase substrates. Indeed, it has been demonstrated that fibronectin is completely resistant to degradation by tetrameric tryptase, but is efficiently cleaved by the respective active monomers (Fajardo and Pejler, 2003a; Hallgren *et al.*, 2005). On the other hand, it is clear that fibrinogen, despite its large size, is degraded equally well by tetrameric and monomeric β-tryptase (Fajardo and Pejler, 2003a). Thus, the mode of tryptase-catalyzed degradation may vary for different protein substrates. We may also speculate that partly unfolded proteins enter the central pore of tryptase more readily than the respective folded counterpart (Fig. 6). In line with the latter notion, it has been shown that denatured collagen (gelatin) is a substrate for tetrameric β-tryptase (Fajardo and Pejler, 2003b) and mastin, a trypsin-like protease secreted by canine MCs (Raymond *et al.*, 2005), whereas intact collagen appears to be resistant (Fajardo and Pejler, 2003b). Clearly, the geometry of the central pore would not impose any constraints for cleavage of smaller peptides. Accordingly, several of the identified tryptase substrates are low molecular weight peptides such as VIP, PHM, and CGRP (Table 6).

9.3. MC-CPA

In comparison with chymase and tryptase, MC-CPA is much less characterized in terms of potential physiological substrates. It has been observed that purified human MC-CPA is able to hydrolyze Ang I to des-Leu10 Ang I (Goldstein *et al.*, 1989). Moreover, it was shown in an *ex vivo* system of peritoneal exudates cells that MC-CPA in fact can convert Ang I into Ang II (Lundequist *et al.*, 2004). Interestingly, when MC-CPA was inhibited, mMCP-4 was shown to compensate for MC-CPA in Ang I conversion. However, when both mMCP-4 and MC-CPA were inhibited, Ang II formation was completely abrogated. Further, not only were MC-CPA and mMCP-4 important players in Ang II formation, both proteases were also shown to be key players in subsequent degradation of generated Ang II (Lundequist *et al.*, 2004). MC-CPA has also been reported to cleave other bioactive peptides, including Leu5-enkephalin, neurotensin, kinetensin (Goldstein *et al.*, 1991), and snake venom sarafotoxin (Metz *et al.*, 2006; Section 10).

Similar to both tryptase and chymase, MC-CPA has been implicated in regulation of lipoproteins, by degrading the apoB component of LDL (Kokkonen *et al.*, 1986). Interestingly, it was suggested that chymase and MC-CPA cooperate in degradation of apoB.

9.4. Concerted action of the MC proteases

Considering that chymases and MC-CPA have been shown to cooperate in the cleavage of several substrates, for example Ang I and LDL, and, furthermore, their possible interdependence in terms of storage (Feyerabend *et al.*, 2005; Stevens *et al.*, 1996; Section 8), it is likely that cooperation between these enzymes is a common mechanism. In fact, we suggest that cooperation between the various MC protease classes can represent a general mechanism leading to efficient substrate degradation (Fig. 6). In this context, it is noteworthy that the MC proteases collectively, at least in rodents, encompass a panel of different proteolytic activities, mimicking those of the pancreatic digestive proteases: trypsin-like (mMCP-6/7), chymotrypsin-like (mMCP-4), elastase-like (mMCP-5), and carboxypeptidase (MC-CPA) activities. A possible scenario is that chymase first performs cleavages at various interior sites in the target protein, thus exposing new C-terminal ends which may be further processed by MC-CPA. Peptides that are released by this process could be small enough to enter the tryptase central pore and be further degraded. This, in turn, may expose new C-terminal targets for MC-CPA and can expose new sites for cleavage by chymase. In addition, the concerted action of chymase and MC-CPA on a given protein target can cause partial denaturation, with the partially denaturated protein being able to enter the

tryptase central pore and become degraded further. According to this suggested scenario, protein targets may thus become extensively degraded (Fig. 6).

10. *IN VIVO* FUNCTION

A multitude of potential biological functions have been ascribed to the various MC proteases (Tables 9 and 10). These functions have been inferred by different approaches. In many cases, the protease has been added to a cell culture system followed by recording of a response. In other approaches, purified protease has been administered into an experimental animal, followed by assessment of, for example, inflammatory parameters. MC protease functions have also been indicated by the correlation between the presence of an MC protease in a tissue and a certain disease. More recently, a number of studies have utilized more or less selective MC protease inhibitors in different *in vivo* models for disease. Finally, functions of MC proteases have in a few cases been inferred using mice lacking individual MC proteases (Table 8) or lacking potential MC protease substrates.

10.1. Chymase

10.1.1. Inflammation

A number of studies have reported various proinflammatory activities of chymases (Table 9). In one pioneering study, injection of purified human chymase into guinea pig skin caused influx of both neutrophils and eosinophils (He and Walls, 1998a). In support of a role for chymase in inflammatory cell recruitment, it has been reported that human chymase is chemotactic *in vitro* for neutrophils and monocytes (Tani *et al.*, 2000; Tomimori *et al.*, 2002a). It has also been demonstrated that intradermal injection of human chymase in mice caused inflammatory cell influx, including eosinophils (Tomimori *et al.*, 2002a,b; Watanabe *et al.*, 2002b). Moreover, it was shown that chymase inhibitors reduced eosinophilic inflammation in various models: (1) a model for atopic dermatitis based on use of the NC/Nga mouse strain (Watanabe *et al.*, 2002b), (2) a model in which contact dermatitis is induced by dinitrofluorobenzene (Tomimori *et al.*, 2002a), and (3) in ascaris extract-induced skin reactions (Tomimori *et al.*, 2002b). A further link between chymase and eosinophil function was provided by the finding that human chymase induces ERK1/2 phosphorylation (Terakawa *et al.*, 2005) and IL-8 production (Terakawa *et al.*, 2006) in eosinophil-like cells. Additional support for a role of chymase in the pathogenesis of allergic skin reactions comes from studies in which presence of chymase correlates with disease progression

TABLE 9 Biological processes in which MC chymase has been implicated

	References
Eosinophil/neutrophil/ monocyte recruitment	He and Walls, 1998a; Watanabe *et al.*, 2002a
Induction of eosinophil migration	Terakawa *et al.*, 2005
Induction of allergic skin reactions	Tomimori *et al.*, 2002b
Degradation of dermal–epidermal junctions	Briggaman *et al.*, 1984
Ischemia reperfusion injury	Abonia *et al.*, 2005
Mucosal permeability	Scudamore *et al.*, 1995, 1998
Decreased barrier function in epithelium	Ebihara *et al.*, 2005a
Induction of vascular permeability	He and Walls, 1998b
Defense toward parasites	Knight *et al.*, 2000
IL-8 induction in eosinophil-like cells	Terakawa *et al.*, 2006
Inhibition of SMC proliferation	Wang *et al.*, 2001
Reversal of VIP-induced SMC relaxation	Franconi *et al.*, 1989
Induction of SMC apoptosis	Leskinen *et al.*, 2001, 2006
Inhibition of SMC collagen synthesis	Wang *et al.*, 2001
Mitogen for fibroblasts	Algermissen *et al.*, 1999; Maruichi *et al.*, 2004; Pemberton *et al.*, 1997
Fibrosis	Jones *et al.*, 2004; Kakizoe *et al.*, 2001; Nakajima and Naya, 2002; Sakaguchi *et al.*, 2004; Satomura *et al.*, 2003; Takai *et al.*, 2003a; Tomimori *et al.*, 2003
Induction of MC degranulation	Kido *et al.*, 1985; Schick and Austen, 1986; Schick *et al.*, 1984
Secretagogue for airway gland serous cells	Sommerhoff *et al.*, 1989
Potentiation of histamine release	Hultsch *et al.*, 1988; Rubinstein *et al.*, 1990
Atopic eczema	Mao *et al.*, 1996, 1998; Tanaka *et al.*, 1999
Atopic dermatitis and nummular eczema	Jarvikallio *et al.*, 1997

(*continued*)

TABLE 9 (*continued*)

	References
Dermatitis	Badertscher *et al.*, 2005; Imada *et al.*, 2002; Watanabe *et al.*, 2002b
Protective role in lung function in severe asthma	Balzar *et al.*, 2005
Stimulation of IgE and IgG1 synthesis	Yoshikawa *et al.*, 2001
Peritoneal adhesion formation	Okamoto *et al.*, 2002b, 2004a,b; Soga *et al.*, 2004
Regulation of blood pressure	Li *et al.*, 2004
Involvement in aneurysm formation	Tsunemi *et al.*, 2002, 2004
Myocardial infarction	Jin *et al.*, 2003; Hoshino *et al.*, 2003
Bypass degeneration	Ortlepp *et al.*, 2001
Congenital heart disease	Hamada *et al.*, 1999
Angiogenesis	Kondo *et al.*, 2006; Muramatsu *et al.*, 2000, 2002; Russo *et al.*, 2005
Wound healing	Nishikori *et al.*, 1998
Lung adenocarcinoma	Nagata *et al.*, 2003
Aortic lipid deposition	Uehara *et al.*, 2002
Regulation of HDL levels	Fukuda *et al.*, 2002
Psoriasis	Harvima *et al.*, 1990
Chronic leg ulcers	Huttunen and Harvima, 2005
Conjunctival epithelial cell apoptosis	Ebihara *et al.*, 2005b
Scleroderma	Shiota *et al.*, 2005
Arteriovenous fistula formation	Jin *et al.*, 2005
Glycogen-induced peritonitis	de Garavilla *et al.*, 2005
Helicobacter pylori-associated gastritis	Matsuo *et al.*, 2003
Intimal hyperplasia after balloon injury	Takai *et al.*, 2003b

(Badertscher *et al.*, 2005) and from studies in which chymase polymorphisms have been correlated with atopic skin disorders (Mao *et al.*, 1996; Tanaka *et al.*, 1999; Weidinger *et al.*, 2005b). On the other hand, studies demonstrating a negative correlation between chymase presence and

TABLE 10 Biological processes in which MC tryptase has been implicated

	References
Airway hyperresponsiveness/ inflammation	Berger *et al.*, 1999; Clark *et al.*, 1995; Jarjour *et al.*, 1991; Molinari *et al.*, 1996; Oh *et al.*, 2002; Rice *et al.*, 2000b; Sylvin *et al.*, 2002; Wright *et al.*, 1999
Neutrophil recruitment	Hallgren *et al.*, 2000; He *et al.*, 1997; Huang *et al.*, 1998
Eosinophil recruitment	He *et al.*, 1997
Vascular permeability increase	He and Walls, 1997
Fibrosis	Kondo *et al.*, 2001
Sepsis	Mallen-St Clair *et al.*, 2004
Ulcerative cholitis	Tremaine *et al.*, 2002
Angiogenesis	Blair *et al.*, 1997; Nico *et al.*, 2004
Arthritis	Buckley *et al.*, 1997, 1998; Tetlow and Woolley, 1995
Multiple sclerosis	Pedotti *et al.*, 2003a; Rozniecki *et al.*, 1995
Sudden infant death syndrome	Buckley *et al.*, 2001
Duchenne muscular dystrophy	Nico *et al.*, 2004
Psoriasis	Namazi, 2005; Naukkarinen *et al.*, 1994
Joint inflammation	Kelso *et al.*, 2005
Intestinal inflammation	Cenac *et al.*, 2002
Atopic dermatitis	Jarvikallio *et al.*, 1997
Itching	Ui *et al.*, 2006
Tumor cell proliferation	Yoshii *et al.*, 2005
Reversal of VIP-induced SMC relaxation	Franconi *et al.*, 1989

atopic dermatitis/nummular eczema have suggested a protective role for chymase in allergic skin disorders (Jarvikallio *et al.*, 1997). Interestingly, a study demonstrated that presence of chymase in small airways showed a positive correlation with preserved lung function in asthmatic patients, suggesting a protective role for chymase in asthma (Balzar *et al.*, 2005). In another correlative study, Harvima *et al.* (1990) found that the number of chymase-positive MCs was clearly decreased in the upper dermis of psoriatic lesions. Although it is not certain if, and how, chymase influences disease progression, it is striking to note that chymase has been linked to such a variety of skin disorders. Other examples in which chymase has been implicated in skin disease include a study where scleroderma in

tight-skin mice was suppressed by a chymase inhibitor (Shiota *et al.*, 2005) and a study suggesting an association of chymase with the pathogenesis of chronic leg ulcers (Huttunen and Harvima, 2005). Chymase presence has also been correlated with other inflammatory disorders, such as Helicobacter pylori-associated gastritis (Matsuo *et al.*, 2003).

The mechanism by which chymase may participate in an inflammatory response is intriguing. In several early studies, it was reported that purified chymase may act in an ''autocrine'' fashion by inducing MC degranulation accompanied by histamine release (Schick *et al.*, 1984; Sommerhoff *et al.*, 1989). Moreover, it has been shown that chymase inhibition may dampen histamine release from MCs (Kido *et al.*, 1985). However, the mechanism by which chymase induces MC degranulation has not been outlined. Another mode of action for chymase could be to degrade epithelial cell–cell or cell-basement membrane contacts, thereby increasing capillary permeability. Indeed, several reports support such a notion. In a hallmark study, Miller *et al*. found that rMCP-2 release strongly correlated with increased mucosal permeability in rats infected with *N. brasiliensis* antigen. Moreover, infusion of purified rMCP-2 induced marked mucosal permeability (Scudamore *et al.*, 1995). Later, the same group reported that the effect of rMCP-2 on epithelial cell integrity may be due to degradation of the tight junction proteins occludin and ZO-1 (Scudamore *et al.*, 1998). The possibility that chymase may act on tight junction proteins is also supported by a study in which human chymase was demonstrated to induce decreased barrier function of human corneal epithelium, an activity that was ascribed to degradation of occludin and fibronectin (Ebihara *et al.*, 2005a). Additional support for a role of chymase in increasing capillary permeability comes from a study in which purified human chymase induced microvascular permeability in guinea pigs (He and Walls, 1998b). In a more recent report, it was shown that MCs were required for increased mucosal permeability during infection with *Trichinella spiralis*, and that this effect could be related to cleavage of occludin by mMCP-1 (McDermott *et al.*, 2003). The collected evidence from these studies strongly suggest that one major function of chymase, in an inflammatory context, may be to promote microvascular permeability by degrading components involved in epithelial cell–cell contacts.

10.1.2. Effect on SMCs

Several reports have implicated MC chymase in regulation of SMC growth and function. Kovanen and coworkers have shown that rat chymase inhibits rat aortic SMC proliferation (Wang *et al.*, 2001). The same group also found that chymase caused apoptosis of SMCs, an effect which was ascribed to cleavage of fibronectin present in the ECM deposited by the SMCs, and proapoptotic activities of released fibronectin fragments (Leskinen *et al.*, 2003). In a follow up study, it was suggested that the SMC

apoptosis induced by chymase was caused by disrupting NF-κB-mediated survival signaling (Leskinen *et al.*, 2006). Of note is that chymase in addition has been demonstrated to be apoptotic for conjunctival epithelial cells, also through a mechanism involving degradation of fibronectin (Ebihara *et al.*, 2005b). Certainly, these findings are well in line with the multitude of evidence suggesting fibronectin to be an *in vivo* substrate for chymase. In another study, human chymase was found to inhibit the proliferative response of airway SMCs to epidermal growth factor (Lazaar *et al.*, 2002). Moreover, chymase has been shown to inhibit collagen synthesis in SMCs (Wang *et al.*, 2001) and to reverse the effect of VIP on SMC relaxation (Franconi *et al.*, 1989). The possibility that chymase may influence SMC function is also supported by the colocalization of MCs and SMCs in human airways (Matin *et al.*, 1992). Considering the strong association between the presence of MCs within the SMC layer and the pathogenesis of asthma (Brightling *et al.*, 2002), it is clear that effects of chymase on SMC function can profoundly influence the pathogenesis. However, whether chymase in this context is harmful or protective (or an ''innocent bystander'') remains to be demonstrated.

10.1.3. Cardiac disorders

Given the discovery that chymase can convert Ang I into Ang II, and the huge potential for this activity in terms of cardiac function, it is not surprising that numerous studies have addressed the role of chymase in heart disease and in models thereof. Thus, correlative evidence has linked chymase to aneurysm formation in humans (Tsunemi *et al.*, 2002) and to congenital heart disease (Hamada *et al.*, 1999), and a chymase polymorphism has been associated with bypass degeneration with implications for atherosclerosis (Ortlepp *et al.*, 2001). Further, studies employing various chymase inhibitors in animal models have supported a role for chymase in formation of abdominal aortic aneurysms (Tsunemi *et al.*, 2004), myocardial infarction (Hoshino *et al.*, 2003; Jin *et al.*, 2003), and intimal hyperplasia after balloon injury (Takai *et al.*, 2003b). The potential of using chymase inhibitors in treatment of heart disease has been reviewed (Takai *et al.*, 2004a). In line with a role for chymase in heart disease, it has also been shown that chymase inhibitors may reduce aortic lipid deposition (Uehara *et al.*, 2002) and that a chymase polymorphism correlates with HDL levels (Fukuda *et al.*, 2002).

10.1.4. Fibrosis

Several lines of evidence point toward a role for chymase in fibrotic conditions. For instance, chymase presence has been correlated with fibrosis in experimental diabetes (Jones *et al.*, 2004) and in autoimmune liver fibrosis (Satomura *et al.*, 2003). Fibrosis progression in sclerotic skin of tight-skin mice has been correlated with a selective upregulation of

mMCP-4, but not mMCP-5, expression (Kakizoe *et al.*, 2001). Further, chymase inhibitors can reduce fibrosis in animal models (Sakaguchi *et al.*, 2004; Takai *et al.*, 2003a; Tomimori *et al.*, 2003). How chymase may contribute to fibrotic conditions is not known but a number of reports indicate that chymase is mitogenic for different types of fibroblasts (Algermissen *et al.*, 1999; Maruichi *et al.*, 2004; Pemberton *et al.*, 1997). On a different angle, the ability of chymase to release ECM-bound TGF-β1 (Taipale *et al.*, 1995) and to activate latent TGF-β1 (Lindstedt *et al.*, 2001a) may contribute to chymase-dependent fibrosis.

10.1.5. Angiogenesis

MCs have been implicated in angiogenesis and, although the exact role of MCs in this process is not clear, several reports suggest a possible involvement of chymase. For example, hamster sponge granuloma models have been used to show inhibition of angiogenesis by a chymase inhibitor (BCEAB) (Muramatsu *et al.*, 2002) and by chymostatin, a broad spectrum inhibitor of chymotrypsin-like proteases (Muramatsu *et al.*, 2000). An effect of chymase on angiogenesis could potentially affect both physiological and pathological processes. Indeed, chymase positivity has been correlated with both wound healing (Nishikori *et al.*, 1998) and tumor angiogenesis (Kondo *et al.*, 2006). Interestingly, the latter report indicated that chymase presence was associated with poor prognosis in gastric cancer. Along the same line, chymase was associated with poor prognosis in lung adenocarcinoma (Nagata *et al.*, 2003). Additional support for a role of chymase in angiogenesis has been obtained in a study in which inhibition of rMCP-5 expression, by antisense nucleotide technology, reduced granuloma-associated angiogenesis in a rat model (Russo *et al.*, 2005). Although the mechanism by which chymase could influence angiogenesis is not known, it may be speculated that chymase can promote angiogenesis by, for example, degrading ECM to facilitate vessel growth, activate proangiogenic pro-MMPs such as pro-MMP-9, or activate latent growth factors.

10.1.6. Peritoneal adhesion

Postoperative intraperitoneal adhesions are common problems during surgery. Several reports have shown that various chymase inhibitors (BCEAB, NK3201, TY-51184; Table 11) reduce the degree of peritoneal adhesion in experimental models, suggesting that chymase inhibitors have the potential of ameliorating this pathological condition (Okamoto *et al.*, 2002a,b, 2004a,b; Soga *et al.*, 2004).

10.1.7. Regulation of immunoglobulin levels

Although it is likely that chymase exerts many of its actions during the acute phase of an inflammatory response, as exemplified by effects on mucosal permeability and histamine release (see above), it cannot be

TABLE 11 Low molecular weight MC chymase inhibitors

	K_i/IC_{50}	Selectivity over cathepsin G	References
Nonpeptide			
NK3201	IC_{50} = 2.5 nM	–	Takai *et al.*, 2001a
SUN-C8257	IC_{50} = 0.31 μM	18	Fukami *et al.*, 2000
SUN-C8077	IC_{50} = 2.5 μM	0.4	Fukami *et al.*, 2000
JNJ-10311795	K_i = 2.3 nM	16.5	de Garavilla *et al.*, 2005
Cpd 41	IC_{50} = 22 nM	>4500	Niwata *et al.*, 1997
BCEAB	IC_{50} = 5.4 nM	ND	Takai *et al.*, 2001b
TY-51184	IC_{50} = 37nM	>2700	Takai *et al.*, 2004b
SPF-32629 A	IC_{50} = 0.25μg/ml	20	Shimatani *et al.*, 2006
TY-51076	IC_{50} = 56 nm	>400	Masaki *et al.*, 2003
TEI-E548	K_i = 6.2 nM	–	Hoshino *et al.*, 2003
Y-40613	K_i = 22.6 nM	32	Imada *et al.*, 2002
Peptide based			
Z-Ile-Glu-Pro-Phe-COOMe	K_i = 1 nM	–	Bastos *et al.*, 1995
Diphenyl N^{α}-benzocarbonyl-l-Arg-Glu-Thr-Phe^{P}-phosphonate	IC_{50}=3.8 nM	2700	Raymond *et al.*, 2003

excluded that chymase also acts at other levels. For example, there is some evidence that chymase (rMCP-1) stimulates IgE and IgG synthesis *in vitro* (Yoshikawa *et al.*, 2001) and, along the same line, there is limited evidence suggesting that a chymase polymorphism correlates with IgE levels in patients suffering from atopic dermatitis (Iwanaga *et al.*, 2004). Clearly, the function of chymase in connection with inflammation may be highly complex and much future work will be needed to fully understand its role in such settings.

10.1.8. Chymase functions implicated from gene KO studies

Only in a few cases have *in vivo* functions of chymases been suggested from studies involving mice deficient in specific chymases (Table 8). In one important study, Miller and coworkers demonstrated that mice lacking mMCP-1 were less effective in clearance of *T. spiralis* than wild-type controls (Knight *et al.*, 2000). This finding is well in line with previous

studies showing that animals lacking MCs altogether have a reduced capacity in *T. spiralis* clearance (Ha *et al.*, 1983), and suggests that the effect of MCs in defense against parasites, at least partly, is dependent on their content of chymase. However, it is not clear to what extent these findings can be extrapolated to humans, considering that mMCP-1 has no obvious functional human homologue. In another study, Gurish and coworkers demonstrated that mice lacking mMCP-5 are protected in an ischemia reperfusion injury model (Abonia *et al.*, 2005). It should be noted that mMCP-5$^{-/-}$ animals additionally lack MC-CPA (Stevens *et al.*, 1996), and it is thus uncertain whether the protection is due to lack of mMCP-5 or due to the secondary defect in MC-CPA storage.

10.1.9. Human chymase polymorphisms

Several studies have been performed in search for correlations between different pathological states and certain polymorphisms in the human chymase gene. The majority of reports focus on two polymorphisms: one located in the promotor region and one in intron 2. It should be noted that both of these polymorphisms are named in various ways in different publications. The promotor polymorphism is presented as, for example, BstXI, G3255A, -1905G/A, rs1800875, or CMA/B. The intron 2 polymorphism is usually referred to as A1625G or CMA/A. As described above, chymase polymorphisms have been correlated with atopic skin disorders, atherosclerosis, and HDL levels. In addition, chymase polymorphisms have been connected to diabetic chronic renal insufficiency (Prasad *et al.*, 2006) and an elevated risk of developing gastric ulcer and gastric cancer (Sugimoto *et al.*, 2006), the latter in specific allele combinations with an ACE polymorphism. Some data also support a possible additive effect of chymase and ACE polymorphisms on hypertrophic cardiomyopathy (Gumprecht *et al.*, 2002; Pfeufer *et al.*, 1996).

10.2. Tryptase

10.2.1. Inflammation

MC tryptase has been linked to a variety of disorders, mainly of inflammatory type (Table 10), but the *in vivo* significances of these findings have in most cases not been verified. Similarly to chymase, a number of studies have reported proinflammatory activities of tryptase. He *et al.* (1997) showed that injection of human tryptase into guinea pig skin induced influx of neutrophils and eosinophils. Using the same model, they also demonstrated that the proinflammatory action of tryptase was associated with increased microvascular leakage (He and Walls, 1997). Subsequently, two independent studies showed that recombinant mMCP-6, when injected intraperitoneally in mice, induced an influx of neutrophils (Hallgren *et al.*, 2000; Huang *et al.*, 1998). Interestingly, when mice instead

were injected with mMCP-7, an influx of eosinophils rather than neutrophils was observed (Huang *et al.*, 2001). Further, it has been reported that instillation of human recombinant β-tryptase into trachea of mice provokes a neutrophilic inflammation (Huang *et al.*, 2001). Using sheep as a model organism, it has been shown that human tryptase induces cutaneous inflammation and that a tryptase inhibitor reduces cutaneous inflammation in allergic animals (Molinari *et al.*, 1995). The mechanisms behind these proinflammatory activities are not known, but it has been suggested that tryptase can stimulate IL-8 and ICAM expression in endothelial cells (Compton *et al.*, 1998; Huang *et al.*, 1998). Similar to chymase, tryptase may induce MC degranulation, and it was reported that tryptase inhibitors can suppress MC degranulation in response to calcium ionophore or IgE receptor cross-linking (He *et al.*, 1998). In addition, tryptase has been reported to provoke degranulation also of eosinophils (Vliagoftis *et al.*, 2004).

10.2.2. Fibrosis

Similarly to chymase, tryptase has been implicated in fibrosis. Several reports have shown that tryptase is a mitogen for fibroblasts and can stimulate collagen synthesis (Abe *et al.*, 1998; Akers *et al.*, 2000; Frungieri *et al.*, 2002; Gruber *et al.*, 1997; Hartmann *et al.*, 1992; Ruoss *et al.*, 1991). Tryptase is also mitogenic for SMCs (Brown *et al.*, 1995, 2002) and myocytes (Corvera *et al.*, 1997). The possibility that tryptase accordingly may have a role in fibrotic conditions, for example in allergic asthma, has been discussed in a previous review (Levi-Schaffer and Piliponsky, 2003).

10.2.3. Airway inflammation

A number of reports suggest a role for MC tryptase in allergic airway inflammation. Strong support for this notion was given when it was demonstrated that two different tryptase inhibitors, APC-366 and BABIM, suppressed inflammatory responses and airway hyperreactivity in allergic sheep provoked by antigen (Clark *et al.*, 1995). APC-366 was later reported to have beneficial effects also in allergic pigs (Sylvin *et al.*, 2002). Further, it was shown that instillation of human tryptase into the trachea of sheep induced bronchoconstriction mediated by histamine release, and that the airway response was suppressed by APC-366 (Molinari *et al.*, 1996). Additional support for a role of MC tryptase in airway responses comes from studies in which human tryptase was found to induce airway reactivity and histamine release in isolated human (Berger *et al.*, 1999) and guinea pig (Barrios *et al.*, 1998) bronchi.

In light of the observed beneficial effects of APC-366 in animal models for allergic airway responses, it subsequently underwent a clinical trial for treatment of asthma (Krishna *et al.*, 2001). However, the outcome of this trial was relatively disappointing, with only a minor beneficial effect

observed. This may thus argue against a major role for MC tryptase in allergic airway responses. On the other hand, APC-366 is a very slow-acting inhibitor, with several hours required for optimal tryptase inhibition (Rice *et al.*, 1998) and, moreover, it is nonselective (Table 12). It can therefore not be ruled out that the poor outcome of the clinical trial is related to these properties and that the contribution of tryptase to airway responses would be more apparent if better tryptase inhibitors would be used. Indeed, more selective and potent tryptase inhibitors have been developed (Table 12), and several of these, including AMG-126737 (Wright *et al.*, 1999) and MOL-6131 (Oh *et al.*, 2002), suppress airway

TABLE 12 Low molecular weight MC tryptase inhibitors

	K_i/IC_{50}	Selectivity over trypsin	References
APC-366	K_i = 0.33–450 μM	0.5	Clark *et al.*, 1995; Hallgren *et al.*, 2001a; Rice *et al.*, 1998
Gabexate mesylate	K_i = 3.4 nM	500	Erbaa *et al.*, 2001
Nafamostat mesilate	IC_{50} = 0.016 nM	–	Sendo *et al.*, 2003
BABIM	K_i = 5 nM	18	Caughey *et al.*, 1993a; Katz *et al.*, 1998
RWJ-56423	K_i = 10 nM	0.8	Costanzo *et al.*, 2003
Cyclotheonamide E4	IC_{50} = 5.1 nM	1.2	Murakami *et al.*, 2002
AMG-126737	K_i = 90 nM	28	Wright *et al.*, 1999
MOL-6131	K_i = 45 nM	24	Oh *et al.*, 2002
BMS-262084	IC_{50} = 4 nM	18	Sutton *et al.*, 2002
BMS-354326	IC_{50} = 1.8 nM	5,600	Bisacchi *et al.*, 2004
BMS-363131	IC_{50} < 1.7 nM	>3,000	Slusarchyk *et al.*, 2002
Compound 27	K_i = 0.07 nM	560,000	Rice *et al.*, 2000a
APC-2059	K_i = 0.1 nM	150,000	Rice *et al.*, 2000b
Diketopiperazine-based	K_i = 10–2400 nM	–	Schaschke *et al.*, 2002
Compound 11b	K_i < 0.01 nM	650,000	Burgess *et al.*, 1999
Benzamidine based	K_i = 1 nM	46,000	Dener *et al.*, 2001

responses in animal models. In many cases, a high selectivity for MC tryptase is obtained by developing dibasic inhibitors, that is, inhibitors that simultaneously interact with two of the active sites in the tryptase tetramer. Examples of such inhibitors include AMG-126737 (Wright *et al.*, 1999), MOL-6131 (Oh *et al.*, 2002), and APC-2059 (Rice *et al.*, 2000b; Tremaine *et al.*, 2002).

10.2.4. Angiogenesis

Tryptase has been shown to promote capillary growth and to be a mitogen for endothelial cells, indicating a potential role for tryptase in angiogenesis (Blair *et al.*, 1997). This is supported by the ability of tryptase to induce proangiogenic, but not antiangiogenic cytokines (Somasundaram *et al.*, 2005). Moreover, a correlation between tryptase-positive neurons and extent of angiogenesis has been reported in a mouse model for Duchenne muscular dystrophy (Nico *et al.*, 2004).

10.2.5. Correlative studies

Correlative studies have indicated potential links between MC tryptase and a variety of disorders, including multiple sclerosis (Pedotti *et al.*, 2003b; Rozniecki *et al.*, 1995), arthritis (Tetlow and Woolley, 1995), sudden infant death syndrome (Buckley *et al.*, 2001), psoriasis (Naukkarinen *et al.*, 1994), fibrosis (Kondo *et al.*, 2001), and atopic dermatitis (Jarvikallio *et al.*, 1997).

10.3. MC-CPA

Until recently, very little information regarding the *in vivo* function of MC-CPA has been available. Few reports have investigated the effect of MC-CPA in cell culture-based systems, although one report suggests that MC-CPA may be a mitogen for human dermal fibroblasts (Abe *et al.*, 2000). Importantly, a mouse strain lacking MC-CPA expression has been generated (Feyerabend *et al.*, 2005), an accomplishment that will greatly facilitate investigations of its *in vivo* function. In the initial report describing the MC-CPA$^{-/-}$ strain, it was demonstrated that MC-CPA did not contribute to passive cutaneous anaphylaxis. Interestingly, secretory granules of MC-CPA$^{-/-}$ MCs showed defective staining with cationic dyes, indicating alterations in storage of PGs, with the latter notion also being supported by an increased solubility of ^{35}S-sulfate labeled PGs in MC-CPA$^{-/-}$ peritoneal MCs (Feyerabend *et al.*, 2005). In an alternative approach, strong evidence for an *in vivo* relevance of MC-CPA-catalyzed degradation of snake venom sarafotoxin was obtained. Using the W/W^{v} strain, Metz *et al.* demonstrated elegantly that MCs have a key role in defense toward sarafotoxin-mediated lethality. Reconstitution of MC-deficient mice with wild-type MCs restored protection toward sarafotoxin

while, strikingly, reconstitution with MCs in which MC-CPA expression was suppressed by RNA interference failed to give protection (Metz *et al.*, 2006). Clearly, these recent and important findings have put MC-CPA on the map of innate immunity, and we anticipate that new and important functions of MC-CPA will be revealed in the near future.

11. MC PROTEASE INHIBITORS

11.1. Synthetic inhibitors

As a consequence of the multitude of pathological settings in which MC proteases have been implicated, much effort has been invested in generating potent and selective MC protease inhibitors. Some of these inhibitors are peptide based, as exemplified by the potent chymase inhibitor Z-Ile-Glu-Pro-Phe-COOMe (Bastos *et al.*, 1995). However, due to a requirement for orally available compounds with favorable pharmacokinetics, recently developed MC protease inhibitors are mainly nonpeptide based and of low molecular weight (Tables 11 and 12). Such inhibitors have been assessed in a variety of animal models for disease and have in many cases proved to be effective in ameliorating the respective condition. Thus, MC proteases are now considered as promising drug targets. However, the recent progress within this area should be taken with some caution. First, several of the used inhibitors show a moderate degree of selectivity for the respective MC protease or, alternatively, the selectivity has to our knowledge not been characterized (Tables 11 and 12). For example, two of the most frequently used MC protease inhibitors, APC-366 and SUN-C8077, do not show any selectivity toward trypsin and cathepsin G, respectively (Tables 11 and 12). Thus, the reported effect in a disease model may not only be a consequence of inhibiting the target MC protease but also to inhibition of related proteases of non-MC origin. Second, most of the inhibitors have been developed for their ability to inhibit human proteases, that is, human chymase or β-tryptase, but are used for targeting an animal counterpart protease. Therefore, it cannot be ruled out that the potency for inhibition of the relevant animal MC protease may differ considerably. For example, we found that APC-366, a potent inhibitor of human β-tryptase (Table 12), was a very weak inhibitor ($IC_{50} \approx 1$ mM) of mMCP-6 (Hallgren *et al.*, 2001a). Hence, in order to fully evaluate the relevance of inhibiting MC proteases in different disease models, it would be useful to develop and assess selective inhibitors of the relevant endogenous MC protease. Nevertheless, studies using various MC protease inhibitors have led to the implication of MC proteases in several novel settings (Section 10).

11.2. *In vivo* regulation

Most likely, the MC proteases are under strict control after their release from degranulated MCs in order to prevent the unleashing of excessive protease activity that could cause damage to the host tissue. However, the *in vivo* mechanisms that control MC protease activities have not been extensively studied. For example, with the exception of the identification of tryptase monomer in complex with the intracellular protease inhibitor PI6 (Strik *et al.*, 2004), *in vivo*-generated complexes of MC proteases and endogenous protease inhibitors have not been isolated to date.

Several studies have shown that α_1-AC, an endogenous protease inhibitor of serpin type, is an efficient chymase inhibitor (Pejler, 1996; Pejler and Berg, 1995; Schechter *et al.*, 1989). Since skin MCs, at least under certain pathological conditions, are positive for α_1-AC (Huttunen and Harvima, 2005), it is likely that α_1-AC indeed is an endogenous inhibitor of chymase. In support for a role of serpins in chymase inhibition, Pemberton *et al.* (2006) showed that mMCP-1, but not mMCP-2, was recovered in complexes with serpins after the protease had been injected into the circulation of mice. Most likely, since α_1-AC is a plasma protein, chymase inhibition by α_1-AC would predominantly occur during inflammatory conditions in which α_1-AC has been extravasated into the tissue. Further, it is of note that chymase, when present in its physiological form, that is, in complex with heparin PG (Section 8), is largely resistant to inhibition by α_1-AC (Pejler and Berg, 1995). Also other plasma protease inhibitors, for example, α_2-macroglobulin (Walter *et al.*, 1999) and α_1-PI (Schechter *et al.*, 1989), have been reported to inhibit chymases, although it has been demonstrated that chymase shows resistance also to these inhibitors when present in complex with heparin PG (Pejler and Berg, 1995). A likely explanation for the protective effect of heparin PG toward plasma protease inhibitors is that these inhibitors do not show affinity for heparin. As a consequence, they may be repelled by the SG PG to which chymase is attached, according to the scheme outlined in Fig. 5B. Another chymase inhibitor of potential physiological significance is secretory leukocyte proteinase inhibitor (SLPI), a protease inhibitor found in mucus secretions. Interestingly, chymase inhibition by SLPI was more effective in the presence of heparin PG, thus in contrast to inhibition by plasma protease inhibitors (Walter *et al.*, 1996). A likely explanation for this finding is that SLPI is a GAG-binding protein (Fath *et al.*, 1998) and would thus be attracted by the SG PG to which chymase is bound (Fig. 5B). Another potential endogenous chymase inhibitor is squamous cell carcinoma antigen 2 (Schick *et al.*, 1997), a serpin that serves as a serological marker for advanced squamous cell tumors. In this context, it is interesting to note that chymase (mMCP-4) has been implicated in squamous carcinoma through its abilities to activate pro-MMP-9 and to induce hyperplastic skin to become angiogenic (Coussens *et al.*, 1999).

The *in vivo* regulation of tryptase is an intriguing issue. To date, no endogenous inhibitor capable of inhibiting tetrameric tryptase has been identified. Therefore, the most likely scenario is that tryptase regulation *in vivo* occurs by other means. A plausible mechanism is that the tryptase tetramer, after exocytosis, dissociates into monomers that rapidly lose activity. This notion is largely based on the known instability of tryptase tetramers in conditions that prevail after its release into the extracellular space, that is, neutral pH and body temperature (Schwartz and Bradford, 1986). However, it should be noted that tryptase monomers generated during such conditions may be active, at least for a short time (Fajardo and Pejler, 2003a). Hence, the active tryptase monomers may become targets for endogenous protease inhibitors that fail to inhibit tetrameric tryptase. In an alternative scenario, released tryptase tetramers may be confronted to various polycationic proteins, for example, neutrophil-derived lactoferrin and myeloperoxidase. Such polycationic compounds will compete with tryptase for binding to tryptase-stabilizing GAGs, leading to tryptase dissociation and inactivation (Cregar *et al.*, 1999; Elrod *et al.*, 1997; Hallgren *et al.*, 2001a).

Very little is known about the control of MC-CPA activity. In one study, Uratani *et al.* (2000) reported that an MC-CPA inhibitory protein-denoted latexin is present intracellularly within rat peritoneal MCs, in granules distinct from those containing MC-CPA and histamine. Latexin was not released following exposure of the MCs to either calcium ionophore or compound 48/80, and it thus remains to be shown whether latexin plays a role in the extracellular control of MC-CPA activity. Another endogenous inhibitor of MC-CPA, denoted tissue carboxypeptidase inhibitor (TCI), was characterized by Normant *et al.* (1995). TCI is expressed in various MC-containing tissues, for example, lung and intestine, and may thus have the potential to regulate MC-CPA *in vivo*.

Clearly, the *in vivo* mechanisms that regulate MC proteases are likely to be of great importance in fine-tuning various MC protease-dependent conditions. Additional insight into this issue is therefore welcome and will hopefully be generated in future research.

12. SUMMARY AND FUTURE PERSPECTIVES

As is evident from this chapter, past research on various MC proteases has gathered large amounts of information regarding their structure, processing mechanisms, expression profiles, cleavage specificity, and potential *in vivo* substrates and functions. However, the critical questions remain in large parts unanswered, that is, what is their true biological function? Since the MC proteases are released during various inflammatory conditions, it is likely that they at least under certain circumstances

may contribute to the pathological condition in which the MC participates, that is, being harmful. On the other hand, it cannot be ruled out that the MC proteases in some cases may actually have a role in dampening the inflammatory response by, for example, degrading inflammatory substances. In fact, we may even envisage a scenario where they may play different roles during various phases of a given response, for example aiding in recruitment of inflammatory cells in the early inflammatory phase and by limiting a too extensive response by degrading inflammatory molecules in subsequent stages of the reaction. Moreover, we cannot rule out that MC proteases also can contribute to tissue homeostasis under normal conditions, that is, in the absence of MC degranulation. To answer these critical questions, it will be imperative to genetically target the various MC protease genes and assess the outcome in different disease models, in particular those in which MCs have been shown to contribute to the pathogenesis. We are now at a stage where KO strains for many of the major MC proteases are available, that is, animals lacking mMCP-1, -4, -5, and MC-CPA (Table 8). With help of these KO strains, and by assessing animals with combined deletions of the respective MC proteases, we anticipate that the next few years will reveal a number of *in vivo* settings in which MC proteases play a role. The next challenge will then be to determine the exact mechanism behind a given finding, with the key question being to determine which substrate that is the *in vivo* target for the protease.

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