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CHAPTER ONE

# Multifarious Determinants of Cytokine Receptor Signaling Specificity

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## Contents



## Abstract

Cytokines play crucial roles in regulating immune homeostasis. Two important characteristics of most cytokines are pleiotropy, defined as the ability of one cytokine to exhibit diverse functionalities, and redundancy, defined as the ability of multiple cytokines to exert overlapping activities. Identifying the determinants for unique cellular responses to cytokines in the face of shared receptor usage, pleiotropy, and redundancy will be essential in order to harness the potential of cytokines as therapeutics. Here, we discuss the biophysical (ligand–receptor geometry and affinity) and cellular (receptor trafficking

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and intracellular abundance of signaling molecules) parameters that contribute to the specificity of cytokine bioactivities. Whereas the role of extracellular ternary complex geometry in cytokine-induced signaling is still not completely elucidated, cytokine-receptor affinity is known to impact signaling through modulation of the stability and kinetics of ternary complex formation. Receptor trafficking also plays an important and likely underappreciated role in the diversification of cytokine bioactivities but it has been challenging to experimentally probe trafficking effects. We also review recent efforts to quantify levels of intracellular signaling components, as second messenger abundance can affect cytokine-induced bioactivities both quantitatively and qualitatively. We conclude by discussing the application of protein engineering to develop therapeutically relevant cytokines with reduced pleiotropy and redirected biological functionalities.

## 1. INTRODUCTION

Four-helical cytokines are secreted proteins that regulate most facets of immune function and numerous other aspects of mammalian physiology (Bazan, 1989, 1990). Cytokines exert their biological activities by inducing cell surface receptor dimerization in either homo- or hetero-oligomeric assemblies (Stroud & Wells, 2004; Wang, Lupardus, Laporte, & Garcia, 2009; Watowich, Hilton, & Lodish, 1994). In the canonical cytokine signaling pathway, assembly of the cytokine-receptor complex activates tyrosine kinases of the Janus Kinase (Jak) and Tyk2 family, which are constitutively bound to receptors (Ihle, Witthuhn, Quelle, Yamamoto, & Silvennoinen, 1995). Jaks, in turn, phosphorylate and activate Signal Transducer and Activator of Transcription (STAT) transcription factors (Levy & Darnell, 2002; Schindler, Hoey, & McKnight, 1996) to modulate gene expression and, ultimately, determine cell fate (Murray, 2007; O'Shea & Plenge, 2012). In addition to their signaling through the Jak/STAT pathway, some cytokines can also activate the Akt and Erk pathways (Platanias, 2005), as well as other signaling networks (Gough, Levy, Johnstone, & Clarke, 2008; Heinrich et al., 2003; Malek, 2008; Schindler, Levy, & Decker, 2007; van Boxel-Dezaire, Rani, & Stark, 2006).

It has been well established that cytokines exhibit two features: (1) pleiotropy, the capacity of one cytokine to elicit a multitude of diverse functional responses; and (2) redundancy, the ability of multiple different cytokines to effect overlapping activities (Ozaki & Leonard, 2002). The properties of pleiotropy and redundancy emanate from the degenerate nature of cytokine complexes. A single cytokine may engage more than one receptor complex to activate distinct sets of Jaks and STATs, leading

to diverse functional effects (Zurawski, Vega, Huyghe, & Zurawski, 1993). Receptor subunits may also be shared between several cytokines, and the limited number of Jak (four) and STAT (seven) proteins results in redundant activation of Jak/STAT combinations by distinct cytokine complexes (Pestka, Krause, Sarkar, et al., 2004; Pestka, Krause, & Walter, 2004; Vignali & Kuchroo, 2012). It is, however, remarkable that despite using such a seemingly constrained set of signaling proteins in a finite number of combinations, cytokines are still able to promote a broad range of activities and to regulate a highly complex immune system (Delgoffe, Murray, & Vignali, 2011). Our understanding of the multifarious mechanisms through which cytokines affect such a diverse range of biological activities remains incomplete, and we have yet to illuminate the details of how functional specificity is achieved given the rampant redundancy and pleiotropy of cytokines. What we do know is that there is no clear correlation between the particular signaling molecule that is activated and the bioactivity that results. For instance, although the cytokines Interleukin (IL)-6 and IL-10 both activate STAT3, their roles in immune regulation are diametrically opposed, with IL-10 eliciting an anti-inflammatory and IL-6 eliciting a proinflammatory response (Hunter & Kastelein, 2008; Mosser & Zhang, 2008; Murray, 2007). Another example of divergent functional outcomes being transmitted through a shared signaling molecule is found in the type I interferon (IFN) system in which more than 16 subtypes engage an identical receptor complex yet elicit distinct biological activities (Borden et al., 2007; Piehler, Thomas, Christopher Garcia, & Schreiber, 2012). Gaining insight into the molecular mechanisms that underlie this observed functional specificity will greatly advance our understanding of cytokine biology and immune regulation. In this review, we summarize findings that demonstrate the importance of biophysical (ligand–receptor binding kinetics and complex stability) and cellular (receptor trafficking and abundance or localization of signaling molecules) parameters in the determination and diversification of cytokine activities. We also discuss recent examples of how these parameters can be used to modulate cytokine activities through implementation of biomolecular engineering techniques.

## 2. LIGAND–RECEPTOR COMPLEX FORMATION: GEOMETRY AND AFFINITY

Signal activation is initiated when cytokines engage the extracellular domains of their cognate receptors. Many receptor subunits are shared by multiple cytokines but paired with different receptor chains to form unique

signaling complexes (Liao, Lin, & Leonard, 2011; Pestka, Krause, & Walter, 2004; Wang et al., 2009). For example, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 share the common γ chain ($\gamma_c$) (Rochman, Spolski, & Leonard, 2009); IL-6, IL-11, leukemia-inducible factor, oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) share the gp130 receptor (Boulanger, Bankovich, Kortemme, Baker, & Garcia, 2003; Silver & Hunter, 2010); and granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and IL-3 share the common β chain ($\beta_c$) (Bagley, Woodcock, Hercus, Shannon, & Lopez, 1995; Tavernier et al., 1991; Wang et al., 2009). In some instances, identical receptor complexes are shared by multiple cytokines which induce distinct biological activities. This is the case for type I IFNs, as well as for the two cytokines IL-4 and IL-13, which signal through heterodimeric complexes composed of IL-4Rα, $\gamma_c$, and IL-13Rα1 (Chomarat & Banchereau, 1998; Junttila et al., 2008; Uze, Schreiber, Piehler, & Pellegrini, 2007). Given their shared usage of receptor subunits, it has been proposed that parameters such as the geometry and affinity of the ligand–receptor complex may account for functional diversity of these cytokines (LaPorte et al., 2008). The role of the inter-subunit geometry of the ligand–receptor complex has been extensively studied but there is still no consensus on whether or not modulation of receptor–ligand architectures can influence downstream signal activation (Ballinger & Wells, 1998). On the other hand, the importance of the ligand–receptor binding affinity in quantitative and possibly qualitative aspects of downstream signaling events has been well established (Pang, Qin, & Zhou, 2011; Piehler et al., 2012). Affinity impacts the kinetics of complex formation as well as the stability of the cytokine-receptor complex, thereby tuning both the strength and duration of signal activation.

## 2.1. Surface receptor complex geometry and signal activation

As more cytokine-receptor ECD complexes have been solved in recent years, it has become apparent that these complexes exhibit a diverse range of molecular architectures, yet all of these structural orientations result in activation of the Jak/STAT pathway (Boulanger, Chow, Brevnova, & Garcia, 2003; de Vos, Ultsch, & Kossiakoff, 1992; Hansen et al., 2008; Jones, Logsdon, & Walter, 2008; LaPorte et al., 2008; Livnah et al., 1996; Livnah et al., 1999; Logsdon, Deshpande, Harris, Rajashankar, & Walter, 2012; McElroy, Dohm, & Walsh, 2009; Rickert, Wang, Boulanger, Goriatcheva, & Garcia, 2005; Ring et al., 2012; Syed et al., 1998; Thomas

et al., 2011, 2012; Walter et al., 1995; Wang, Rickert, & Garcia, 2005). The tolerance of signal activation for variable binding topologies raises the question of whether mere dimerization of receptors is sufficient for the activation of downstream signaling (i.e., dimerization serves as a binary "on/off" switch) or whether particular receptor geometries serve instructive roles in determining the degree and nature of receptor activation.

Several early studies suggested that the structural constraints on dimerization are quite relaxed, since grafting of the extracellular domain (ECDs) of one receptor onto the intracellular domain (ICD) of an unrelated receptor resulted in functional signal activation of the chimeric molecule. As one of many such examples, replacing the ECD of the erythropoietin (Epo) receptor (EpoR) with those of epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), or c-Kit produces an active construct that induces STAT5 phosphorylation and Ba/F3 proliferation in a ligand-dependent manner (Ohashi, Maruyama, Liu, & Yoshimura, 1994). Similarly, fusion of the gp130 ICD to EpoR ECD leads to Epo-mediated activation of STAT1 and STAT3, consistent with activation of the full-length gp130 by its native ligands (Kawahara et al., 2006; Schaeffer et al., 2001). Other examples of receptor ectodomain swapping have been reported for the type I IFN (Pattyn et al., 1999) and IL-4/IL-13 (Fujiwara, Hanissian, Tsytsykova, & Geha, 1997; Heller et al., 2012) systems, and in all cases, the chimeric receptors elicit some extent of activation of the expected Jak/STAT signaling molecules. These results suggest that cytokine signal activation is quite permissive with respect to complex dimer geometry. However, the confounding factor in these signaling studies is that they were performed in cell lines expressing high levels of chimeric receptors and conducted with elevated doses of ligand, thereby limiting their physiological significance. *In vivo*, the consequences of cytokine action are tightly regulated by the relative abundance of various cell types whose sensitivities to stimulation are governed by receptor expression levels. Thus, while *in vitro* signaling of a chimeric receptor may appear to be qualitatively similar to that of wild-type (wt) receptor in terms of STAT activation patterns (which is not surprising given that the ICDs used in the chimeric receptors are identical to those of the wild-type receptors), these studies offer an incomplete perspective of cytokine behavior by failing to account for the finely tuned balance of receptor expression levels maintained by the diverse cell types that exists *in vivo*. In order to paint a clearer picture of the activity of these chimeric receptors, quantitative studies must be undertaken in which the $EC_{50}$ and $E_{max}$ values of induced STAT activation are directly compared

to those of wt receptors for a range of cell types. In addition, sophisticated *in vivo* models must be developed to examine the broader functional consequences of chimeric receptor signaling.

In apparent contrast to the architectural promiscuity observed in chimeric receptor studies, other experimental findings have presented evidence that geometry of the surface complex plays a crucial role in determining the nature and extent of the transmitted signal. It appears that certain orientations of the Epo and thrombopoietin (Tpo) complexes are more likely to activate signaling than others. For these studies, the authors substituted the EpoR ECD with the coiled-coil dimerization domain of the yeast transcription factor *Put3* (Swaminathan, Flynn, Reece, & Marmorstein, 1997), inducing constitutive dimerization of the receptor and thus activating downstream signaling in a ligand-independent manner. The rotational orientation of the EpoR ICD was then systematically modulated by inserting alanine residues into the juxtamembrane region, which was presumed to be helical such that each residue insertion would be predicted to rotate the helix by approximately one-third of a turn. Some alanine insertions are not compatible with signal activation, other insertions lead to wt-like signal activation, and yet another group of insertions preferentially activated one of the two downstream signaling pathways (Constantinescu, Huang, Nam, & Lodish, 2001; Staerk et al., 2011). These findings suggest that there are preferred rotational orientations for signaling, but in the absence of direct structural information these conclusions remain speculative. Nevertheless, the results of these EpoR rotational studies resonate with those obtained from studies investigating the functionality of disulfide-mediated EpoR dimers. Cysteine pairs were introduced in the transmembrane (TM) region of the receptor, forcing constitutive dimerization in the absence of ligand. Among the six disulfide bridges evaluated, only three cysteine–cysteine linkages were capable of promoting robust ligand-independent signal activation, suggesting that specific orientations of the TM domain are required to achieve a productively signaling Epo complex (Constantinescu, Keren, et al., 2001; Kubatzky et al., 2001; Lu, Gross, & Lodish, 2006; Watowich et al., 1992). Moreover, it was reported that the introduction of asparagine mutations to induce changes in the interhelical packing of the EpoR TM segments resulted in complete activation of STAT5, but did not induce the activation of Erk, suggesting that ligand-induced signal diversification could also be regulated by the geometry and packing of the TM dimer interaction (Becker et al., 2008). Here again, in the absence of direct structural information, the precise molecular consequences of these mutations remain

unclear, but evidently alteration of the structural context of the receptor dimer impacts the nature and extent of signaling. As such, these studies on the Epo and Tpo systems provide some of the most compelling evidence to date for a role of receptor–ligand structure in signaling beyond serving as a binary on/off switch. This notion that structural effects can modulate signaling has been corroborated in other cytokine systems such as gp130 (Greiser, Stross, Heinrich, Behrmann, & Hermanns, 2002) and growth hormone (Brown et al., 2005). However, other cytokines such as prolactin do not appear to follow this model, as rotational manipulation of the TM domain of the prolactin receptor does not impact signaling behavior in the absence or presence of ligand (Liu & Brooks, 2011). Overall, studies of the effects of complex rotation on functional outcomes suggest that modifications of the binding mode and receptor architecture impact the signaling events induced by at least some cytokines. However, as with the chimeric receptor studies, the experimental evidence is confounded by overexpression of receptor mutants, making it difficult to draw conclusions about receptor behavior under physiological conditions.

As an alternative to manipulating the signaling complex geometry via direct modification of the receptor, attempts have been made to design ligand variants that alter cytokine-receptor complex structure. A series of studies on Epo peptide mimetics provide some of the most convincing data so far that ligand-mediated alteration of receptor dimer geometry can profoundly influence signaling. Livnah and colleagues demonstrated that a 13-residue Epo mimetic peptide was able to symmetrically dimerize two chains of the EpoR and to agonize receptor activity, inducing activation of STAT5 and proliferation of Ba/F3 cells, albeit less efficiently than the Epo cytokine (Livnah et al., 1996; Remy et al., 1999; Wrighton et al., 1996). Modification of this agonist peptide by adding two bromo groups to Tyr 4 (2–5 dibromotyrosine) abolished the induced signal, rendering it antagonistic (Livnah et al., 1998). In a crystal structure of the nonstimulatory peptide in complex with EpoR, the peptide effected dimerization of two receptors, but the rotational orientation of the dimer differed from that of the agonist peptide-mediated dimer by approximately 15°. The lack of receptor activation was thus attributed to difference in dimer geometry. This result was quite striking, as it implies that a seeming small difference in receptor ECD geometry can be transmitted through the receptor complex and sensed inside the cell. A major caveat to this result, however, is that the binding affinity of the antagonistic peptide for Epo receptor was at least 200-fold lower than that of the agonistic peptide, making it difficult to distinguish

between effects resulting from changes in complex geometry and those resulting from changes in binding affinity. In particular, due to the low affinity of the antagonistic peptide, it is unclear whether it was capable of dimerizing EpoR at the concentrations used in cell signaling studies. A subsequent structure of the EPO cytokine complexed with EpoR again showed a dimeric receptor orientation, but one that was asymmetric and quite distinct from those of the agonistic and antagonistic EPO peptide mimetics (Syed et al., 1998). Here the relative "efficiency" of Epo-induced signaling over the peptide was attributed to this altered dimer geometry (Syed et al., 1998). But again, vast differences in the affinities of the agonist peptide, antagonist peptide, and Epo must be considered when evaluating whether divergence in receptor signaling results from structural effects, kinetic effects, or a combination thereof. Clearly, the role of receptor geometry in determining signal behavior requires further study in the EpoR and other cytokine systems to parse the relative contributions of structural and affinity effects on functional outcomes.

## 2.2. Ligand–receptor affinity: Stability of the ternary complex

Clear correlations have been established between complex stability and signal activation in several cytokine systems. Most cytokines recruit their cognate receptors in a two-step mechanism, first recruiting one receptor chain with high affinity and then forming a ternary complex with a second lower-affinity receptor chain (Cunningham et al., 1991; Junttila et al., 2012; Piehler et al., 2012; Wang et al., 2009). Cytokines form surface complexes with a broad range of stabilities and variations in complex stability can differentially influence the activation of downstream signaling events. The importance of complex stability in determining signal activation is abundantly clear in the type I IFN family, also known as IFN$\alpha/\beta$. IFNs are a large family of cytokines comprising more than 16 different subtypes, all of which bind to a heterodimeric surface receptor complex that consists of the IFNAR1 and IFNAR2 chains (Fig. 1.1, left; Pestka, Krause, & Walter, 2004; Piehler et al., 2012). Through this singular receptor complex, type I IFNs can activate all seven STAT molecules that are present in the cell (van Boxel-Dezaire et al., 2006). A large body of work has shown that different IFN subtypes activate STATs to the same extent despite large discrepancies in binding affinities between the subtypes (Abramovich et al., 1994; Ghislain, Sussman, Goelz, Ling, & Fish, 1995; Jaitin et al., 2006; Marijanovic, Ragimbeau, van der Heyden, Uze, & Pellegrini, 2007;

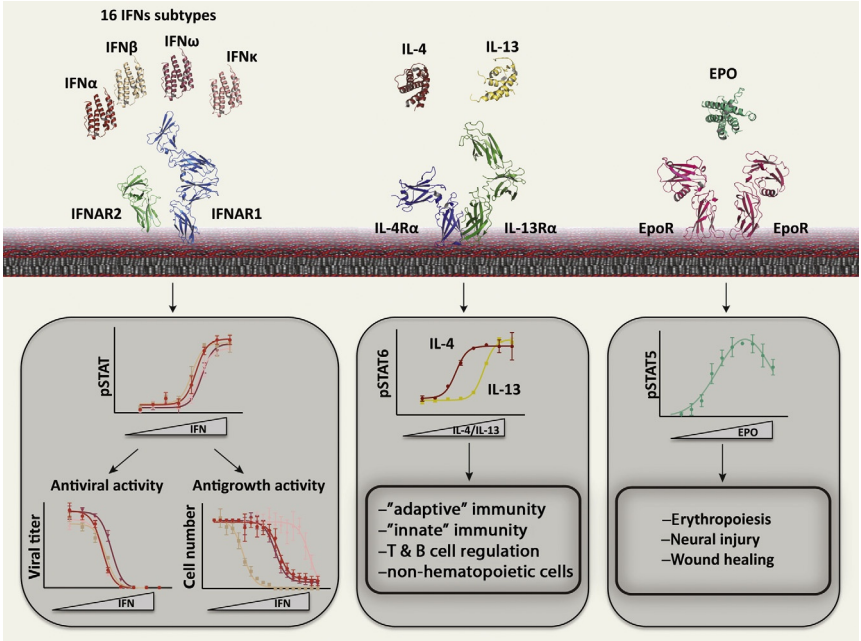


**Figure 1.1** Complex stability and dynamic determination of cytokine-induced bioactivities. Left, extracellular structures of the type I IFN receptor, IFNAR1 and IFNAR2, and four different IFN subtypes are depicted. The four IFN subtypes presented activate STATs to the same extent (as shown by phosphorylated STAT dose–response curves) and exhibit similar antiviral activity (as shown by virus-infected cell abundance, which decreases with increasing IFN doses), but possess starkly different growth arrest potencies (as shown by the number of cells, which decreases with increasing IFN doses). Rather than following STAT activation profiles, the antiproliferative activities of the IFN subtypes closely parallel binding affinities and complex stabilities instead. Center, extracellular structures of IL-4, IL-13, and their shared IL-4 type II receptor comprised of IL-4Rα1 and IL-13Rα1 are shown. IL-4 and IL-13 share a common surface receptor complex, but IL-4 activates STAT6 more efficiently than IL-13 does (as presented in the phosphorylated STAT6 dose–response curve). Greater potency of STAT6 activation by IL-4 is not the result of a more stable complex, since the complex formed by IL-13 is in fact more stable, but rather the result of the differential kinetics of complex formation in response to IL-4 versus IL-13. Right, extracellular structures of Epo and the EpoR homodimer are presented. Epo engages two molecules of EpoR and forms an active surface asymmetric homodimeric complex, leading to STAT5 activation. As is the case for other cytokines that engage homodimeric receptors, Epo-induced STAT5 activation follows a bell-shaped curve, with high doses of Epo activating STAT5 less efficiently than intermediate doses do due to reduced ternary complex formation (our unpublished data).

Piehler et al., 2012; Subramaniam, Khan, Pontzer, & Johnson, 1995). However, when more complex biological activities such as antiproliferative and antiviral effects are studied, a broad range of potencies are found, indicating a lack of connectivity between early signal activation and late biological outcomes (Jaitin et al., 2006; Marijanovic et al., 2007; Moraga, Harari, Schreiber, Uze, & Pellegrini, 2009; Thomas et al., 2011). Furthermore, there does not seem to be a correlation between the potencies of the antiproliferative and antiviral activities; one IFN subtype can have a strong antiproliferative and a medium-strength antiviral effect, whereas another subtype can have a weak antiproliferative but very strong antiviral effect (Brideau-Andersen et al., 2007; Jaitin et al., 2006). How is it possible that a family of ligands that activates the same extent of downstream signaling through the same receptor heterodimer can display these dramatic differences in the potency of induced biological activities?

Several models have emerged over the past 20 years to address this question. One idea that has been proposed is that other non-STAT signaling pathways may be activated in an IFN subtype-specific manner, contributing to the fine-tuning of biological outcomes (Platanias, 2005). There are several reports showing that IFN can activate the PI3K (Pfeffer et al., 1997; Uddin et al., 1995, 1997), MAPK (David et al., 1995; Joshi et al., 2009; Verma et al., 2002), and NFκB pathways (Rani et al., 2001; Wei et al., 2006; Yang et al., 2000) in various cell types, and there is evidence that these pathways play a role in IFN-induced bioactivities (Joshi et al., 2009; Kaur et al., 2008; Rani et al., 2001). However, to date there is no direct and conclusive evidence linking these non-STAT pathways to differences in the intensity of biological activities elicited by different IFN subtypes, and the mechanism through which different IFN subtypes might induce distinct non-STAT signaling events remains unknown. Another proposed explanation for the differential potency of antiviral and antiproliferative effects is that different IFN subtypes could form structurally unique ternary complexes, analogous to the rotationally shifted EpoR complexes formed in the presence of agonistic versus antagonistic peptides (Livnah et al., 1996, 1998). However, this explanation was ruled out by the crystal structures of ternary complexes formed by two different IFN subtypes, an IFNα2 mutant and IFNω, which revealed that despite variation in the ligand, the complex architectures are virtually identical (Thomas et al., 2011).

The most widely accepted rationalization of differential activities in the IFN system at present rests on variation in the stability of the ternary complex (Piehler et al., 2012). Mutations that decrease the IFNα2 binding

affinity for either IFNAR1 or IFNAR2 result in a corresponding decrease in STAT activation and antiviral and antiproliferative potency (Jaitin et al., 2006; Kalie, Jaitin, Podoplelova, Piehler, & Schreiber, 2008; Piehler, Roisman, & Schreiber, 2000; Piehler & Schreiber, 1999). However, the inverse does not hold as an increase in binding affinity for either IFNAR1 or IFNAR2 leads to stronger antiproliferative potency but does not enhance STAT activation or antiviral response, both of which plateau at affinities close to those of wild-type ligands (Jaitin & Schreiber, 2007; Kalie, Jaitin, Abramovich, & Schreiber, 2007; Slutzki, Jaitin, Yehezkel, & Schreiber, 2006). Moreover, any increase in affinity for either one of the IFNAR chains that is accompanied by a concomitant decrease in affinity for the other chain (such that the overall complex stability is unchanged) will result in the same antiproliferative and antiviral potency as the parent IFN, confirming the connection between ternary complex stability and bioactivity potency (Kalie et al., 2008). Taken together, these results suggest that the key factor in determining functional outcome is the half-life of the full ternary complex rather than the independent affinities that a ligand exhibits toward its two independent receptors chains. This finding was confirmed via analysis of STAT activation induced by various IFNs exhibiting a range of complex stabilities (Thomas et al., 2011). Using phospho-flow, a state-of-the-art technique for the quantitative analysis of phosphorylated proteins in a high-throughput manner, the $EC_{50}$ values for activation of all seven STATs were measured in each immune cell type isolated from whole blood (Krutzik & Nolan, 2006). A direct correlation was observed between complex stability and signaling kinetics: IFNs forming a longer lived ternary complex induced faster, although not stronger, STAT3 and STAT5 activation. How this "kinetic proofreading" of signal initiation determines the *in vivo* functional outcomes induced by different IFNs remains to be elucidated, but at this point it seems clear that complex stability allows for a family of ligands sharing the same surface receptor to exhibit differential potencies of biological responses by regulating the kinetics of signal activation. Another case in point is the divergence in the STAT3 activation profiles induced by IL-6 and IL-10. IL-6 induces transient activation of STAT3 whereas IL-10 elicits a more sustained STAT3 response and this difference in activation kinetics was shown to be sufficient to account for the distinct biological outcomes of these two cytokines (Braun, Fribourg, & Sealfon, 2013). In summary, multiple cytokine systems utilize differential stability of the ternary complex to discriminate between redundant ligands in order to affect specific functional outcomes.

## 2.3. Ligand–receptor affinity: Kinetics of complex formation

Another way in which the properties of ligand–receptor binding can modulate cytokine signaling is through differential kinetics of ternary complex formation. An example of this is found in the IL-4/IL-13 system. Both IL-4 and IL-13, which are produced by T helper 2 (Th2) cells, recruit and activate IgE-producing B cells to enhance IgE-mediated responses (Wills-Karp, 2004; Zhu, Yamane, & Paul, 2010). In addition, the IL-4 cytokine plays a critical role in the differentiation of Th2 cells (Wills-Karp, 2004; Zhu et al., 2010). IL-4 and IL-13 exhibit a broad spectrum of common bioactivities, yet there are some pockets of specificity in which the functions of these two cytokines do not overlap (Chomarat & Banchereau, 1998; Junttila et al., 2008; Liang et al., 2011). IL-4 binds to the type I receptor, consisting of the IL-4Rα1 and $\gamma_c$ subunits, which is found exclusively on hematopoietic cells (Kelly-Welch, Hanson, Boothby, & Keegan, 2003; LaPorte et al., 2008; Wang et al., 2009). IL-4 can alternatively bind the type II receptor, composed of the IL-4Rα1 and IL-13Rα1 chains, which also serves as the receptor for IL-13 (Fig. 1.1, center). Both IL-4 and IL-13 activate STAT6 through the type II receptor, with IL-4 inducing more than 10-fold stronger STAT6 activation than IL-13 (Heller et al., 2008; LaPorte et al., 2008; Zhu, Guo, Watson, Hu-Li, & Paul, 2001). Differential activity between the two cytokines cannot be explained by increased stability of the ternary complex, as IL-4 forms a less stable complex than IL-13 (Andrews, Holloway, Holgate, & Davies, 2006; Andrews, Holloway, Puddicombe, Holgate, & Davies, 2002; LaPorte et al., 2008; Zhang, Buehner, & Sebald, 2002; Zhang, Simeonowa, Wang, & Sebald, 2002). In this case, the differences in STAT6 activation are presumably due to the distinct kinetics of complex formation exhibited by these two cytokines, as well as differences in the amounts of IL-4Rα1 and IL-13Rα1 expressed on the surface of immune cells (Junttila et al., 2008, 2012). In type II receptor complex formation, IL-4 first recruits IL-4Rα1 with very high affinity ($K_d \approx 100$ pM) and IL-13Rα1 is subsequently recruited to the binary complex with low affinity. IL-13, on the other hand, follows the reverse scheme, first binding IL-13Rα1 with moderate affinity ($K_d \approx 4$ nM) and then recruiting IL-4Rα1 to the binary complex with low affinity (Andrews et al., 2002, 2006; LaPorte et al., 2008; Zhang, Buehner, et al., 2002; Zhang, Simeonowa, et al., 2002). Due to its high affinity of interaction, formation of the IL-4–IL-4Rα1 binary complex is acutely sensitive to both ligand concentration and surface expression of IL-4Rα1 and cells are responsive to very

low quantities of the IL-4 cytokine (Junttila et al., 2008). The rate-limiting step in this system is engagement of the IL-13Rα1 chain due to the low affinity that the binary complex exhibits for this chain. IL-13, on the other hand, requires higher levels of ligand to form the moderate-affinity IL-13-IL-13Rα1 binary complex, rendering the cells less sensitive to levels of this cytokine. However, due to the higher affinity of the IL-13-IL-13Rα1 binary complex for IL-4Rα1, this system is more resistant to changes in the expression levels of this latter chain. It can therefore be expected that the abundance of IL-13Rα1 will have a strong impact on the ability of IL-4 and IL-13 to induce STAT6 phosphorylation. This hypothesis was confirmed when it was demonstrated that cells expressing high levels of IL-13Rα1 respond more strongly to IL-13 than to IL-4 (Junttila et al., 2008).

There appears to be an interesting structural origin behind the differential complex assembly kinetics for IL-4 and IL-13. The IL-13Rα1 receptor chain appears to have evolved from $\gamma_c$ although it differs in that it contains an additional N-terminal D1 domain (LaPorte et al., 2008). In the structures of the IL-14 and IL-13 ternary complexes, the IL-13Rα1 D1 domain has extensive interactions with both IL-4 and IL-13 (LaPorte et al., 2008). However, mutational and domain deletion studies showed that this contact is only energetically required for the IL-13 interaction; the D1 domain can be removed from IL-13Rα1 without impacting the ability of IL-4 to form the ternary complex, whereas D1 deletion ablates the IL-13–IL-13Rα1 interaction (Ito et al., 2009). Thus, the reversal in assembly order for the type II IL-4 and IL-13 complexes is a result of these structural differences, and ties directly into the "mass action" based mechanism through which cells expressing the type II receptor distinguish IL-4 from IL-13. Clearly, evolutionary pressures have altered the binding energetics between the cytokines and the IL-13Rα1 subunit so as to allow a shared receptor to differentiate between the two stimuli.

The impact of complex formation kinetics and receptor abundance on signal activation is also illustrated in the case of cytokines that engage homodimeric receptors, such as for the cytokines Epo, Tpo, and growth hormone (GH). Epo induces activation of both STAT5 and Erk, leading to the differentiation and maturation of erythrocytes (Constantinescu, Ghaffari, & Lodish, 1999). Although the Epo cytokine recruits two identical EpoR molecules (Fig. 1.1, right), ternary complex formation still follows a two-step assembly scheme similar to those utilized by IFNs and IL-4/IL-13. Epo first recruits one EpoR chain with high affinity and then, using a second

binding interface on the opposite side of the cytokine, recruits a second EpoR chain with low affinity, leading to the formation of an asymmetric complex (Matthews, Topping, Cass, & Giebel, 1996; Philo, Aoki, Arakawa, Narhi, & Wen, 1996; Syed et al., 1998). Complex formation results in the characteristic biphasic Epo activation profile wherein high doses of Epo activate STAT5/Erk less efficiently than do intermediate levels of Epo, generating a bell-shaped curve of activity versus cytokine concentration (Fuh et al., 1992; Wells & de Vos, 1996). The molecular mechanism underlying this unintuitive phenomenon was first described for the growth hormone receptor (GHR) and is applicable to all homodimeric receptors (Fuh et al., 1992; Wells & de Vos, 1996). At low doses of ligand, few ternary complexes are formed as most receptors are unbound or singly bound. As the Epo concentration rises, the number of ternary complexes rises up to the point that Epo saturates the receptors on the cell (specifically, a 2:1 ratio of EpoR:Epo). At super-saturating doses of Epo, the equilibrium will be shifted to the formation of binary complexes as unbound Epo competes with singly bound Epo for EpoR binding, effectively antagonizing the formation of active ternary complexes, and leading to decreased signal activation (Fuh et al., 1992; Wells & de Vos, 1996). As in the IL-4/IL-13 system, the kinetics of Epo complex formation enables modulation of signaling extent through tight regulation of both cytokine concentration and surface receptor levels.

## 3. RECEPTOR DYNAMICS AND ENDOSOMAL TRAFFICKING

Studies of cytokine receptor dynamics and trafficking are complicated by several factors. First, cytokine receptors are expressed at very low levels, generally less than a thousand receptors per cell. IFNAR1 surface levels are estimated to be in the low hundreds (Uze, Lutfalla, & Gresser, 1990) and similar numbers were found for IL-4Rα1 (Lowenthal et al., 1988; Obiri, Debinski, Leonard, & Puri, 1995; Ohara & Paul, 1987). Another complicating factor in examination of receptor trafficking is that surface expression of most cytokine receptors is tightly regulated by their associated Jaks, making it challenging to stably overexpress these receptors at high levels. Nonetheless, there are a few reports suggesting that membrane receptor dynamics and endosomal trafficking play crucial roles in cytokine-induced signal activation and functional diversification. One such report demonstrated the relevance of receptor trafficking in granulocyte colony-stimulating factor (G-CSF) signaling (Avalos et al., 1990; Irandoust et al., 2007). Through

STAT5 and Akt pathway signaling, G-CSF orchestrates both the production of granulocytes by the bone marrow and their subsequent release into the bloodstream, and also regulates the survival, proliferation, and differentiation of neutrophils (Hamilton, 2008). It was reported that truncations of the G-CSF receptor (G-CSFR) ICDs prevent its internalization, causing the receptor to persist on the surface, which in turn promotes the development of myeloid leukemia and severe neutropenia due to sustained G-CSFR signaling (Avalos et al., 1990). A later study established that ICD mutants of G-CSFR that cannot traffic to lysosomes become trapped in early endosomes where they continue to activate STAT5 but not Akt, leading to altered biological activities (Irandoust et al., 2007). This later work not only illustrated the key role of trafficking through the different endosomal compartments in cytokine-induced signal activation but also suggested that regulation of receptor trafficking could impact the extent and diversification of signaling activated by a given cytokine.

The role of receptor trafficking has been extensively studied in the IFN system. As described earlier, IFNs engage a surface receptor composed of the IFNAR1 and IFNAR2 chains. An interesting aspect of this system is that the two chains do not follow parallel paths of downregulation and trafficking (Fig. 1.2). Following activation, IFNAR1 is heavily ubiquitinated and traffics to the lysosomes where it is degraded, regardless of the IFN subtype used for stimulation (Jaitin et al., 2006; Marijanovic et al., 2007). In contrast, the subcellular trafficking of IFNAR2 depends on the binding affinity of the stimulating IFN subtype (Jaitin et al., 2006; Marijanovic et al., 2007; Thomas et al., 2010). While IFNα2, which binds IFNAR1 with low affinity, promotes the recycling of IFNAR2 due to the short half-life of the complex (Marijanovic et al., 2007), IFNβ, which binds IFNAR1 with higher affinity, promotes formation of a more stable ternary complex, leading to the downregulation and lysosomal degradation of both IFNAR1 and IFNAR2 (Jaitin et al., 2006; Marijanovic et al., 2007). Coimmunoprecipitation experiments have revealed that the ternary complex formed by IFNβ remains intact long after STATs have been activated inside the cells. The same is not true, however, for the IFNα2 ternary complex (Croze et al., 1996). This discrepancy is thought to be due to differential pH sensitivity of the ternary complexes formed by the different IFN subtypes. Whereas complexes formed by weaker binders dissociate under the acidic conditions found in early endosomes (pH 6), more stable complexes formed by tighter binding IFN subtypes endure the acidic conditions and continue routing to the lysosomal compartment, where they are degraded

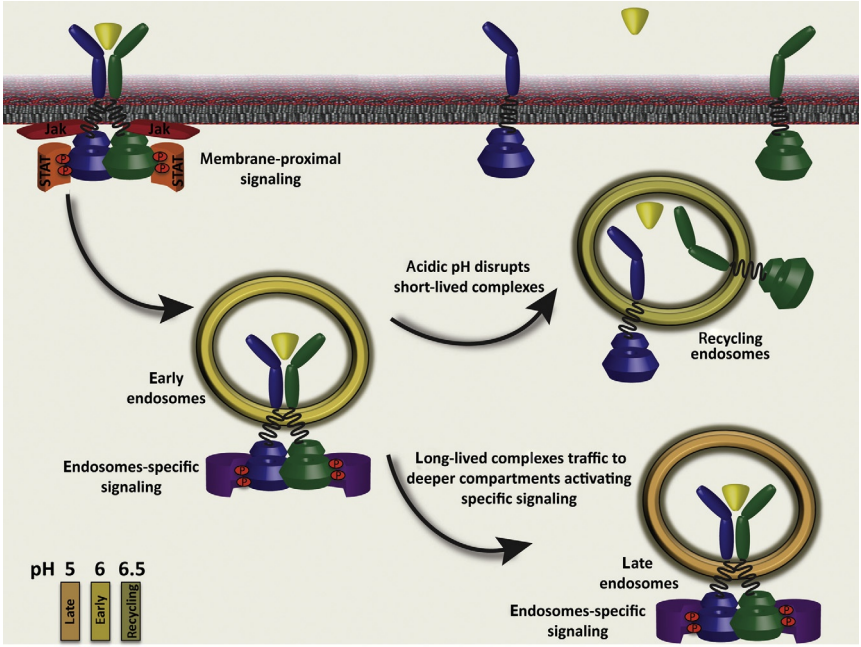


**Figure 1.2** Role of receptor endosomal trafficking in regulating cytokine bioactivities. A canonical cytokine–receptor complex is represented schematically. Upon ligand stimulation the surface complex is formed and leads to the activation of membrane proximal signaling events (e.g., Jak/STAT pathway activity). The complex then traffics to endosomal compartments and follows different subcellular routes depending on its stability. Short-lived complexes dissociate in the acidic pH found in early endosomes and consequently follow a recycling route back to the plasma membrane. Long-lived complexes, on the other hand, persist in early endosomes and continue trafficking through subsequent endosomal compartments where specific signaling molecules are present that help to fine-tune the functional response to a given cytokine.

(Fig. 1.2; Lamken, Lata, Gavutis, & Piehler, 2004; Uze, Bandu, Eid, Grutter, & Mogensen, 1988). It has been established that IFN-activated signaling molecules (Tyk2, STAT1, STAT2) can be found in early endosomes after IFN stimulation, indicating that signaling continues to occur inside the cells following receptor internalization (Payelle-Brogard & Pellegrini, 2010). Moreover, when internalization of the ternary complex is prevented by blocking the formation of endocytic vesicles (via clathrin silencing or overexpression of a dominant-negative form of dynamin), IFN-induced STAT activation along with antiviral and antiproliferative activity is diminished (Marchetti et al., 2006). However, when only downregulation but not endocytosis of IFNAR1 is blocked by mutation of the ubiquitination site

serine 535, IFN induces a stronger antiproliferative response (Kumar, Krolewski, & Fuchs, 2004). These results clearly point to an important role for endosomal trafficking in the determination of the final biological outcomes induced by IFN and also highlight the need for follow-up studies in the IFN system and others to fully elucidate the contributions of endosomal trafficking to signal activation.

Studies of other cytokine systems such as prolactin further support the notion that pH sensitivity of complexes can affect signaling outcomes, as is speculated for the IFNα2 and IFNβ cytokines (Keeler et al., 2007). It was shown that the binding affinity of prolactin to its native receptor is pH sensitive. At acidic pH (such as that of early endosomes), prolactin dissociates from its ternary complex with prolactin receptor and recycles back to the cell surface (Keeler et al., 2007). Prolactin receptor can alternatively be dimerized by growth hormone to form a ternary complex that is not pH sensitive. In this case, GH is transported to the lysosomes and degraded, leading to a completely different set of biological outcomes compared to those evoked by prolactin (Keeler et al., 2007).

Endosomal trafficking also impacts signal activation in the Epo system. Ligand-induced downregulation of murine EpoR was found to require ubiquitination of the receptor, and inhibition of this ubiquitination via mutation of key lysines in the murine EpoR led to a decrease in the phosphorylation of STAT5, Erk, and Akt (Bulut, Sulahian, Ma, Chi, & Huang, 2011). Conversely, a prior study had reported that inhibition of ubiquitination of the human EpoR led to a more sustained level of EpoR on the surface of responsive cells and thus to stronger activation of downstream signaling pathways (Meyer et al., 2007). The discordance between these two studies may be attributable to the differences between mouse and human systems or to the differential sensitivity of the readouts used to detect EpoR degradation and signal activation. What is clear is that receptor dynamics and trafficking significantly impact signaling outcomes induced by Epo and the importance of strictly regulated ligand and receptor availability is magnified in the case of a homodimeric complex that exhibits a biphasic activation profile. Indeed, a recent study showed that ligand depletion and endocytic removal of surface EpoR are hallmarks of Epo-activated signaling and that these events facilitate linear information processing for a broad range of ligand concentrations (Becker et al., 2010).

The role of downregulation and trafficking of cytokine receptors in cytokine-induced signal activation and bioactivities seems universal, as examples have been reported in other systems such as IL-2, IL-4, and

IL-6 (Friedrich et al., 1999; German, Sauer, & Howe, 2011; Rocca, Lamaze, Subtil, & Dautry-Varsat, 2001; Shah et al., 2006; Subtil, Delepierre, & Dautry-Varsat, 1997; Wang & Fuller, 1994). However, we still lack a clear picture of precisely where receptors are localized within cells, which signaling molecules the receptors encounter in various subcellular compartments, and the extent to which signal activation is influenced by receptor trafficking. Further, we know very little about how receptors are organized and distributed on the cell surface, how cytokine-receptor complexes are formed within the cell membrane, and how the mobility of these membrane-associated complexes impacts their availability, trafficking, and ability to activate downstream signaling pathways. Recent developments in super-resolution microscopy and single-particle techniques as well as TIRF microscopy (Huang, Bates, & Zhuang, 2009) have enabled scientists to begin exploring some of these questions by tracking receptors on the surface of living cells.

New methodologies have been employed for multicolor tracking of the IFN receptor complex in living cells. These methodologies are based on the labeling of single proteins with quantum dots (QD) in a 1:1 stoichiometry, thus enabling single particle tracing. To achieve multiplex tracking, proteins of interest are expressed with either an acceptor peptide (AP) tag or a histidine tag. The AP tag is biotinylated by the enzyme BirA and subsequently labeled by streptavidin-coated QDs and the histidine tag is labeled by trisNTA-coated QDs of a different color. This system allows for simultaneous observation of two proteins on the cell surface and analysis of their colocomotion. Using this technique, IFNAR1 and IFNAR2 were found to have two diffusion regimes, one fast and one slow, in the absence of IFN treatment, presumably correlating to the localization of receptors in separate (fast) or common (slow) membrane domains (Roullier et al., 2009). In the presence of IFN$\alpha$2, the diffusion constant of both IFNAR1 and IFNAR2 decreased, presumably due to formation of the constrained ternary complex (You et al., 2010). Triple-color super-resolution imaging of live cells has also been used to study the organization of IFNAR1 and IFNAR2 in the context of the cytoskeleton. IFNAR1 and IFNAR2 were often found in the periphery of actin structures, hinting at the existence of an interaction between these chains and actin or actin-bound adaptor proteins, which is in accord with previous reports implicating involvement of actin-associated adaptors in IFN-induced signaling (Pfeffer, Stebbing, & Donner, 1987; Usacheva et al., 2001; Wilmes et al., 2012).

Super-resolution microscopy has also been applied to the study of other cytokine systems. The use of AlexaFluor-647-labeled IL-4 for the study of type I and type II IL-4 receptor complex formation revealed that while the engagement of the type II IL-4 receptor (IL-4Rα1–IL-4–IL-13Rα1) by IL-4 follows the aforementioned canonical ligand-induced receptor dimerization model, the type I receptor complex (IL-4Rα1–IL-4-$\gamma_c$) does not (Weidemann et al., 2011). In contrast with the recruitment of the IL-13Rα1 chain to the IL-4–IL-4Rα1 binary complex, recruitment of the $\gamma_c$ chain could not be detected, suggesting that the affinities of the binary complex for the two coreceptors, IL-13Rα1 and $\gamma_c$, differ dramatically. This result conflicts with biochemical studies, performed in solution, which concluded that the affinities of the binary IL-4–IL-4Rα1 complex to IL-13Rα1 and $\gamma_c$ were similar (Weidemann et al., 2011). The discrepancy in these findings could be explained by the presence of the cytoplasmic portion of the receptor in conjunction with the membrane environment in live-cell imaging studies compared to the biochemical studies performed with receptor extracellular domains in solution.

The integration of highly sensitive and quantitative microscopy techniques into the study of cytokine-receptor binding and cytokine-induced signal activation will enable us to capture the dynamics of ligand–receptor interaction at the single-molecule level, providing details that are obscured when using more traditional indirect methods. These new technologies will further our understanding of dimerization kinetics, receptor trafficking and mobility, and the role of membrane structures in regulating signal transduction, enabling the fine-tuning of cytokine systems as well as the design of new and more efficient immunological therapies.

## 4. INTRACELLULAR PROTEIN LEVELS AND SIGNALING ACTIVATION

Studies of signaling pathways activated by cytokines have historically focused on how the binding of the cytokine to its cognate receptor induces activation of a limited number of downstream pathways and how modulation of that binding event impacts signaling events. More recently, scientists have begun to appreciate the critical role that intracellular signaling proteins play in defining the extent of activation and the nature of the bioactivities induced by each cytokine.

### 4.1. Jaks and receptor expression and affinity

The surface levels of most cytokine receptors are determined by the abundance of Jak proteins present in the cell (Huang, Constantinescu, & Lodish, 2001; Radtke et al., 2002; Ragimbeau et al., 2003). Manipulation of the levels of Jaks will therefore impact the biological outcomes induced by any cytokine that utilizes a receptor associated with that Jak, making it difficult to distinguish between effects resulting from altered Jak levels versus those arising from altered receptor levels. Moreover in some cases, Jaks also play a role in modulating receptor binding affinity. Early studies showed that certain mutations of the Tyk2 protein, while not affecting surface levels of IFNAR1, lead to a decrease in its affinity for IFNα2 (Gauzzi et al., 1997). It has been shown that binding of the protease USP18 (a negative regulator of IFN signaling) to IFNAR2 ICD also leads to a decrease in its binding affinity for IFN, again without altering the surface levels of the receptor (Francois-Newton et al., 2011). These examples suggest an interplay between the signal-mediating intracellular compartment and the ligand-binding extracellular domains of cytokine receptors in determining the affinity of binding and, therefore, the cellular responses elicited by different cytokines.

### 4.2. Crosstalk between STATs

It was discovered more than a decade ago that there are networks of communication between the different STATs activated by a cytokine. An example of one such network is observed in the IFN system. All the IFN-induced STATs, with the exception of STAT2, bind to the same two phosphotyrosine (P-Tyr) sites on the ICD of IFNAR2 and the mutation of either of those two tyrosines impairs the receptor's ability to promote full STAT activation (Wagner et al., 2002; Zhao et al., 2008). It is therefore possible that there is a competition between different STATs for the binding to these tyrosine residues, as most STATs exhibit similar kinetics of activation.

Using phospho-flow barcoding, we recently showed that the ratios of activated STATs in a given cell differs not only between treatments with different IFN subtypes, but also between different cell types treated with the same IFN, presumably due to their differing amounts of STATs (Thomas et al., 2011). These differences in STAT activation ratios play an important role in signal modulation. The potency of IFN was shown to be determined by STAT1/STAT3 ratio, as activated STAT3 was able to sequester activated STAT1 and in turn decrease the antiviral and antiproliferative potency of IFN (Ho & Ivashkiv, 2006). Thus, the treatment of

cells with any cytokine or other agent that upregulates STAT3 levels negatively impacts the potency of a future response to IFN (Ho & Ivashkiv, 2006). Similarly, a treatment that upregulates STAT1 results in a more potent IFN response, clearly demonstrating that the balance between the expression levels of different STATs in a given cell will greatly impact its response to a cytokine stimulation (Tassiulas et al., 2004). Another example of the importance of STAT levels in determining signal activation is found in the Epo system. Erythroblasts modulate the level of STAT5 during erythroid maturation and, consequently, different stages of maturation induce different levels of maximal Epo-induced STAT5 activation (Porpiglia, Hidalgo, Koulnis, Tzafriri, & Socolovsky, 2012). These results demonstrate that the dynamics of STAT5 protein levels convey specific information to a cell, allowing that cell to transduce Epo stimuli with high fidelity over its entire physiological and stress range.

Changes in intracellular STAT protein levels can impact the activity of a given cytokine not only quantitatively, but also qualitatively. For example, IL-6 primarily signals via STAT3, but in murine embryonal macrophages that lack expression of STAT3, IL-6 completely alters its signaling behavior by predominantly activating STAT1, evoking biological responses nearly identical to those induced by the STAT1-activating cytokine IFNγ (Costa-Pereira et al., 2002; Fig. 1.3). These data suggest that while IL-6 preferentially activates STAT3, it can activate STAT1 as well, and the intracellular ratio between these two STATs will determine the nature of IL-6 response.

Another example of the crosstalk between STAT molecules can be found in the IL-10 system. IL-10 induces the activation of STAT3, which mediates the anti-inflammatory response induced by this cytokine (Williams, Bradley, Smith, & Foxwell, 2004). However, if macrophages are stimulated with type I or type II IFN prior to IL-10 exposure, STAT1 protein levels become upregulated, and IL-10 will primarily promote the activation of STAT1 to induce proinflammatory behavior. This aberrant IL-10-induced proinflammatory behavior has been implicated in the pathology of immune disorders such as lupus and endotoxemia (Herrero et al., 2003; Sharif et al., 2004).

The exquisite balance that exists between different STATs in order to regulate functional outcomes is not restricted to STAT1 and STAT3; other STAT proteins are also susceptible to tight regulation to modulate biological activities. For many years it was not known why type I IFN promotes the proliferation of activated T cells, as it potently inhibits the proliferation of all

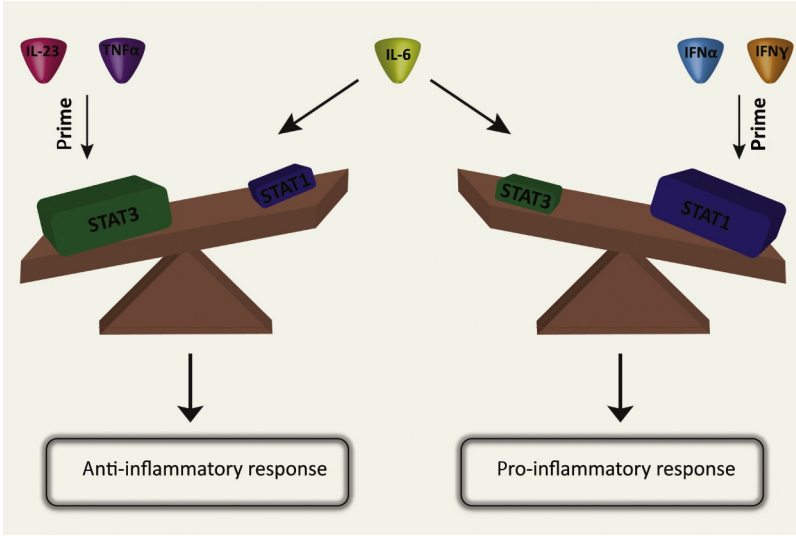


**Figure 1.3** The levels of intracellular signaling proteins impact the nature of the bioactivities induced by cytokines. The cytokine environment surrounding a cell contributes critically to the nature of that cell's response to a given stimulus. As an example, the IL-6 cytokine complex signals through both STAT1 and STAT3 activation. If a cell has previously been exposed to IL-23 or TNFα, its levels of STAT3 will be upregulated. In this case, stimulation with IL-6 will promote a strong activation of STAT3 and will lead to a potent anti-inflammatory response (left). Conversely, if a cell is primed with type I or II IFNs, its levels of STAT1 will instead be upregulated and IL-6 stimulation will therefore induce STAT1 activation, leading to a potent proinflammatory response.

other cell types (Dondi, Rogge, Lutfalla, Uze, & Pellegrini, 2003; Dondi, Roue, Yuste, Susin, & Pellegrini, 2004; Gil, Salomon, Louten, & Biron, 2006; Kolumam, Thomas, Thompson, Sprent, & Murali-Krishna, 2005; Kurche, Haluszczak, McWilliams, Sanchez, & Kedl, 2012). Recently, an elegant study showed that STAT1 is the most abundant STAT molecule in naïve T cells, and, consequently, treatment of these cells with IFN activates STAT1, leading to inhibition of cell proliferation (Gil et al., 2012). Upon activation via T cell receptor (TCR), STAT4 is upregulated within the T cell. In consequence, IFN preferentially activates STAT4 in activated T cells, leading to their proliferative response (Gil et al., 2012). In summary, there is compelling evidence over a range of cytokine systems of the importance of the absolute and relative abundance of STATs in determining signaling behavior and functional outcomes.

## 5. TUNING CYTOKINE SIGNALING VIA PROTEIN ENGINEERING

Cytokines control virtually every function of the immune response and thus deregulation of these secreted factors can lead to the development of immunological diseases such as cancer, lupus, allergy/asthma, and multiple sclerosis (O'Shea & Plenge, 2012). Although several instances of cytokines being used as therapeutic drugs can be found (e.g., Epo, IL-2, IFNβ), their usage in the clinical setting is not as widespread as one might imagine. One of the main impediments to therapeutic use of cytokines is their pleiotropic nature, which results in off-target effects and toxicity. Therapeutics that target signaling pathways activated downstream of cytokines have also experienced only limited success in the clinic. For instance, the inhibition of a hyperactivated Jak can be used to treat diseases (e.g., Jak2 in polycythemia vera), but given that Jaks and STATs are activated by all cytokines, these drugs elicit nonspecific and toxic side effects (O'Shea & Plenge, 2012).

One way to circumvent the complications of pleiotropy is to generate cytokines skewed toward one given bioactivity, so-called cytokine "tuning." Not only would cytokine tuning help treat immune disorders by enhancing the desired activity while minimizing side effects, it would also help enrich our fundamental understanding of cytokine receptor activation and signaling.

Most attempts to modify cytokine signaling so far have focused on decreasing ligand–receptor affinity for one of the two receptor chains in the ternary complex, thus generating antagonistic cytokine mimics that impede formation of the signaling dimer (Debinski, Gibo, Obiri, Kealiher, & Puri, 1998; Kang, Bondensgaard, Li, Hartmann, & Hjorth, 2010; Kruse, Tony, & Sebald, 1992; Liu, Maier, Hafler, & Wittrup, 2009). The reason for this focus on antagonism is that we can synthesize structural and biochemical information to predict with reasonable accuracy which amino acid substitutions will promote a decrease of binding affinity. In addition to antagonizing binding, decreasing the complex affinity can also result in modulation of receptor trafficking, leading to unexpected effects on signaling (Berndt, Chang, Smith, & Ciardelli, 1994; Reddy, Niyogi, Wells, Wiley, & Lauffenburger, 1996).

Modulation of cytokine signaling through increasing ligand affinity has proven to be a more difficult objective to achieve, as we cannot accurately

predict amino acid changes that will increase affinity. Combinatorial mutant libraries provide the most effective and comprehensive means of selecting for high-affinity binders. Genentech pioneered the use of cytokine engineering and phage display in the hGH system to demonstrate the power of this approach (Cunningham et al., 1991; Cunningham & Wells, 1997; Fellouse et al., 2007; Fuh, Colosi, Wood, & Wells, 1993; Fuh et al., 1992; Pal, Kossiakoff, & Sidhu, 2003; Sidhu & Kossiakoff, 2007). We will present examples of the use of combinatorial libraries to increase the affinity of the cytokine for one receptor chain, leading to modifications in biological outcomes. Another strategy for overcoming the complications of pleiotropy is through development of cytokine-based drugs with preferential receptor specificity, for example, engineering cytokines with higher affinity to one and lower affinity to another receptor complex. The use of large combinatorial libraries of random mutants has enabled the engineering of specificity by allowing for sophisticated selection schemes that stringently isolate variants with customized binding properties. We will discuss a few examples in which cytokine engineering has gone beyond the straightforward maturation of cytokine binding affinity and evolved receptor specificity, leading to interesting, informative, and therapeutically relevant results.

## 5.1. Modulation of trafficking: IL-2 and EGF

Two decades ago, an IL-2 mutant was generated in which leucines 18 and 19 were mutated to methionine and glycine, respectively, resulting in a cytokine variant that exhibited similar binding affinities for its cognate receptor chains (IL-2Rα, IL-2Rβ, and $\gamma_c$) compared with the wt protein, yet evoked a fivefold increase in the stimulation of T cell proliferation. The authors showed that the increase in activity was the result of an increase in the recycling rate of the IL-2–IL-2Rβ complex, which led to a decrease in IL-2 depletion. The authors speculated that this mutant could dissociate from the signaling complex at the acidic pH found in endosomes and thus follow a different endosomal trafficking route than the wt complex, leading to increased recycling to the cell surface. This increased recycling would help to sustain levels of the cytokine in the media in addition to maintaining the levels of IL-2 receptor components on the surface of cells (Berndt et al., 1994).

Another example of complex binding kinetics modulating receptor trafficking can be found in the EGF system. An EGF mutant, EGF Y316G, exhibits a 50-fold decrease in receptor-binding affinity compared to the

wt ligand, yet elicits potent signaling activity that surpasses that of wt EGF. As in the case of IL-2, it was shown that this mutant promotes recycling of the ligand–receptor complex, leading to more sustained and stronger activation of downstream signaling (Reddy et al., 1996).

Collectively, these two studies suggest that controlling the endosomal trafficking of cytokine-receptor complexes through affinity modulation can lead to alternative functional outcomes, introducing novel strategies for the manipulation of cytokine signaling. For instance, it is conceivable that designed cytokine mutants with a high receptor affinity at extracellular pH (7) but low receptor affinity at endosomal pH (5–6) could promote recycling and lead to increased signal activation. Additionally, faster ternary complex dissociation in endosomes could qualitatively alter signaling outcomes by reducing the activation of signaling pathways that are primarily activated in endosomes. Thus, by integrating affinity engineering with knowledge of trafficking dynamics, we can design cytokine variants with new and potentially advantageous signaling properties.

## 5.2. Modulation of ligand affinity and activities: IFN and IL-3

In addition to modulating the trafficking behavior of cytokine-receptor complexes, altering complex affinity and stability can also directly impact signal responses. Recently, an IFNα2 mutant was isolated from phage library selection that binds to the IFNAR1 chain more than 100-fold tighter than the wt protein. In agreement with its enhanced binding affinity, this mutant induces more than 100-fold stronger inhibition of cell proliferation than the wt protein. However, the antiviral responses induced by the high-affinity and wt protein are very similar (Jaitin et al., 2006; Kalie et al., 2007). These data suggest that the two activities induced by IFN require different degrees of complex stability and introduce the possibility for development of new IFN subtypes that specifically regulate either of these two bioactivities. There have also been reports of IFN mutants obtained by DNA shuffling that induce stronger antiviral responses but weaker antiproliferative activity than the wt cytokine (Brideau-Andersen et al., 2007). However, it should be noted that these antiproliferative assays were performed on Daudi B cells, a cell line known to be extremely sensitive to IFN and which exhibits atypical behavior with respect to cell growth.

In addition to providing an opportunity to generate activity-specific mutants (antiviral vs. antiproliferative), the IFN system also allows for the potential development of virus-specific cytokine mutants. It was found that

IFN may employ distinct strategies to inhibit different types of viruses through activation of unique subsets of genes that target specific viral types (Schoggins et al., 2011).

Selective alteration of particular functionalities through cytokine manipulation has also been achieved in the IL-3 system. IL-3 stimulates the differentiation of multipotent hematopoietic stem cells into lymphoid progenitor cells and promotes the proliferation of cells of the myeloid lineage through engagement of a surface receptor comprised of IL-3Rα and the common β chain. An IL-3 mutant was generated that exhibited stronger binding to IL-3Rα, which led to a 20-fold stronger proliferation response of hematopoietic cells compared to wt IL-3 stimulation. However, this mutant only marginally increased the release of histamine from peripheral blood leukocytes relative to the wt cytokine, demonstrating yet again that an increase in binding affinity for one of the receptor chains can lead to selective increase of some bioactivities but not others (Thomas et al., 1995).

## 5.3. Increasing ligand specificity: IL-2 and IL-4

Recently, our laboratory has reported two examples of cytokine tuning in which the objectives went beyond basic receptor affinity maturation to improve specificity of the cytokines for a particular receptor complex. In these studies, we took advantage of yeast surface display-mediated directed evolution, a robust platform for expressing and selecting many binding variants of a protein of interest (Boder & Wittrup, 1997). The principle underlying the yeast display methodology is analogous to that of phage display, but the advantage of this system is that yeast have a robust secretory apparatus and can therefore express complex proteins such as cytokines, which possess intricate glycosylation patterns and disulfide bonds (Boder & Wittrup, 1997).

The first of our recently reported cytokine tuning efforts focused on the IL-2 system (Levin et al., 2012). IL-2 requires the IL-2Rα, IL-2Rβ, and $\gamma_c$ chains to form the high-affinity and fully active heterotrimeric surface receptor (Wang et al., 2005), which activates the STAT5, Erk, and Akt pathways (Liao et al., 2011). The high-affinity IL-2Rα chain is upregulated in activated T cells and is also constitutively expressed in regulatory T cells ($T_{regs}$) (Depper et al., 1985; Zheng & Rudensky, 2007). However, naïve T cells lack the expression of this chain and therefore respond very poorly to IL-2. Regulation of IL-2Rα expression levels thus determines the IL-2 sensitivity of different T cell subsets and, consequently, the extent and nature

of the subsequent immune response. Using yeast surface-displayed combinatorial libraries, our laboratory isolated an IL-2 mutant, termed super-2, that had lost dependency on the IL-2Rα chain for formation of the functional IL-2 signaling complex. This was achieved through a series of core cytokine mutations that stabilize the active conformation of the IL-2 C helix, which is stabilized by engagement of the IL-2Rα chain in the wt quaternary complex (Wang et al., 2005). Super-2, by activating IL-2-responsive cells independently of their IL-2Rα expression levels, induces a stronger proliferation of cytotoxic CD8+ T cells but has little effect on the growth of $T_{regs}$. *In vivo* experiments with super-2 demonstrate that it lowers the occurrence of the IL-2-associated toxic side effect edema compared to the wt cytokine (Levin et al., 2012). Moreover, super-2 is more potent than IL-2 therapy and as potent as IL-2/anti-IL-2 antibody therapy (Boyman, Kovar, Rubinstein, Surh, & Sprent, 2006; Krieg, Letourneau, Pantaleo, & Boyman, 2010; Letourneau et al., 2010) in reducing the growth of B16F10 melanoma, as well as murine colon carcinoma and Lewis lung carcinoma in mouse tumor models. Thus, by modulating receptor chain affinity and the dynamics of complex formation, super-2 appears to enhance the therapeutic properties of IL-2 in cancer while decreasing its toxic side effects (Levin et al., 2012).

Our second recent cytokine engineering report focused on IL-4 signaling (Junttila et al., 2012). Through activation of its type I and type II receptors, IL-4 plays a critical role in the differentiation of Th2 and Th9 T cells as well as in B cell maturation, macrophage activation, and dendritic cell (DC) differentiation. Whereas the type I receptor is mainly expressed on cells of hematopoietic origin, the type II receptor is more abundant in cells of epithelial origin (Wills-Karp, 2004; Zhu et al., 2010). To better define the activities downstream of both IL-4 receptors, we engineered a series of IL-4 mutants with different specificities for the type I and type II receptor complexes. We found one mutant, termed super-4, that exhibits more than a 1000-fold increase in affinity for the $\gamma_c$ and more than a 10-fold decrease in affinity for IL-13Rα1, rendering this variant highly specific for type I receptor complexes. Another mutant, termed KFR, exhibits higher affinity for IL-13Rα1 and somewhat lower affinity for $\gamma_c$, making it selective for type II receptor. With these two mutants in hand, we explored the relative contributions of type I versus type II receptors to different IL-4-induced bioactivities. Super-4 induces stronger activation of STAT6 in naïve CD4+ T cells (which express only the type I receptor) compared to wt IL-4 and promotes their differentiation into Th9 cells. However, Super-4

is unable to support the differentiation of monocytes into DCs despite activating STAT6 in monocytes to the same extent as IL-4, suggesting that type II receptor-induced pathways are required for monocyte differentiation into DCs. Consistent with this hypothesis, IL-4 variant KFR, which preferentially engages the type II receptor, is fully capable of inducing DC differentiation at levels comparable to those achieved by the wt cytokine. Collectively, our results shed light on the biology of the type I and type II IL-4 receptor complexes and introduce a new IL-4 mutant, super-4, that could potentially be a safer alternative to the wt cytokine in the clinic as it increases the anti-inflammatory Th2 response without activating innate immunity (Junttila et al., 2012). In sum, the recent IL-2 and IL-4 tuning examples from our laboratory provide a glimpse into the bountiful opportunities that cytokine engineering offers for enhancing our understanding of basic scientific questions regarding the specificity and diversification of cytokine signaling, and for applying this knowledge to the design of novel therapeutics for the treatment of immune disorders.

## 6. PERSPECTIVES

The first cytokine (IFN) was discovered in 1957 (Isaacs & Lindenmann, 1957) and cloned in 1980 (Taniguchi, Fujii-Kuriyama, & Muramatsu, 1980; Weissenbach et al., 1980). Twelve years later, the first cytokine-activated Jak kinase, Tyk2, was isolated and demonstrated to be required for type I IFN signaling (Velazquez, Fellous, Stark, & Pellegrini, 1992). Since then, more than 30 cytokines have been identified and the signaling pathways they activate and share have been extensively characterized. We have also acquired deeper insight into the immune processes controlled by cytokines as well as the pathologies that result from deregulation of their activities. However, there are still many fundamental questions in cytokine biology that remain unanswered, hindering our ability to modulate cytokine activities and to develop treatments for cytokine-dependent immune disorders. Why is Jak3 associated only with $\gamma_c$? Is there a dominant Jak in a given pair or can any Jak do the job? Can the geometry of the ternary complex affect not only the quantity, but also the quality of induced signaling? What role does receptor localization within the membrane play in cytokine signaling behavior?

The development of more sophisticated imaging techniques that will allow us to follow in real time the cytokine-induced surface complexes within the cell membrane as well as in subcellular compartments, together

with the engineering of new cytokines variants with precisely tuned properties, will enable us to probe cytokine systems in a much more controlled manner. This will in turn provide us with a more profound understanding of how cytokine surface complex formation is transmitted intracellularly to induce signal activation and the upregulation of specific gene expression programs, leading to the broad range of immunological activities that are elicited by cytokine signaling.

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CHAPTER TWO

# Pathogenic Mechanisms of Bradykinin Mediated Diseases: Dysregulation of an Innate Inflammatory Pathway

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## Contents



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## Abstract

Binding of negatively charged macromolecules to factor XII induces a conformational change such that it becomes a substrate for trace amounts of activated factor present in plasma (less than 0.01%). As activated factor XII (factor XIIa or factor XIIf ) forms, it converts prekallikrein (PK) to kallikrein and kallikrein cleaves high molecular weight kininogen (HK) to release bradykinin. A far more rapid activation of the remaining unactivated factor XII occurs by enzymatic cleavage by kallikrein (kallikrein-feedback) and sequential cleavage yields two forms of activated factor XII; namely, factor XIIa followed by factor XII fragment (factor XIIf ). PK circulates bound to HK and binding induces a conformational change in PK so that it acquires enzymatic activity and can stoichiometrically cleave HK to produce bradykinin. This reaction is prevented from occurring in plasma by the presence of C1 inhibitor (C1 INH). The same active site leads to autoactivation of the PK–HK complex to generate kallikrein if a phosphate containing buffer is used. Theoretically, formation of kallikrein by this factor XII-independent route can activate surface-bound factor XII to generate factor XIIa resulting in a marked increase in the rate of bradykinin formation as stoichiometric reactions are replaced by Michaelis–Menton, enzyme–substrate, kinetics.

Zinc-dependent binding of the constituents of the bradykinin-forming cascade to the surface of endothelial cells is mediated by gC1qR and bimolecular complexes of gC1qR-cytokeratin 1 and cytokeratin 1-u-PAR (urokinase plasminogen activator receptor). Factor XII and HK compete for binding to free gC1qR (present in excess) while cytokeratin 1-u-PAR preferentially binds factor XII and gC1qR-cytokeratin 1 preferentially binds HK. Autoactivation of factor XII can be initiated as a result of binding to gC1qR but is prevented by C1 INH. Yet stoichiometric activation of PK–HK to yield kallikrein in the absence of factor XII can be initiated by heat shock protein 90 (HSP-90) which forms a zinc-dependent trimolecular complex by binding to HK. Thus, endothelial cell-dependent activation can be initiated by activation of factor XII or by activation of PK–HK.

Hereditary angioedema (HAE), types I and II, are due to autosomal dominant mutations of the C1 INH gene. In type I disease, the level of C1 INH protein and function is proportionately low, while type II disease has a normal protein level but diminished function. There is trans-inhibition of the one normal gene so that functional levels are 30% or less and severe angioedema affecting peripheral structures, the gastrointestinal tract, and the larynx results. Prolonged incubation of plasma of HAE patients (but not normal controls) leads to bradykinin formation and conversion of PK to kallikrein which is reversed by reconstitution with C1 INH. The disorder can be treated by C1 INH replacement, inhibition of plasma kallikrein, or blockade at the bradykinin B-2 receptor. A recently described HAE with normal C1 INH (based on inhibition of activated C1s) presents similarly; the defect is not yet clear, however one-third of patients have a mutant factor XII gene. We have shown that this HAE has a defect in bradykinin overproduction whether the factor XII mutation is present or not, that patients' C1 INH is capable of inhibiting factor XIIa and kallikrein (and not just activated C1) but the functional level is approximately 40–60% of normal, and that $\alpha_2$ macroglobulin protein levels are normal. *In vitro* abnormalities can be suppressed by raising C1 INH to twice normal levels.

Finally, aggregated proteins have been shown to activate the bradykinin-forming pathway by catalyzing factor XII autoactivation. Those include the amyloid β protein of Alzheimer's disease and cryoglobulins. This may represent a new avenue for kinin-dependent research in human disease. In allergy (anaphylaxis; perhaps other mast cell-dependent reactions), the oversulfated proteoglycan of mast cells, liberated along with histamine, also catalyze factor XII autoactivation.

## 1. INTRODUCTION

Since our original review (Joseph & Kaplan, 2005) of the plasma bradykinin-forming pathway as one of many contributors to the "innate" inflammatory response, there have been many developments that we wish to emphasize in this updated review. The first is that coagulation factor XII, long considered to be the initiating protein for the production of bradykinin, need not be. It can be shown that an active site is induced in prekallikrein (PK) upon binding to high molecular weight kininogen (HK) which, although not usually evident because of the presence of C1 inhibitor (C1 INH) (the inhibitor of the activated first component of complement), can initiate bradykinin formation in the absence of factor XII and without conversion to kallikrein (Joseph & Kaplan, 2013). This, in turn, relates to the disease hereditary angioedema (HAE) types I and II which are characterized by absence or dysfunction of C1 INH resulting in severe, potentially fatal, angioedema (swelling) due to bradykinin overproduction (Zuraw, 2008). In addition, a new form of HAE (type 3) has been described (Binkley & Davis, 2000; Bork, Barnstedt, Koch, & Traupe, 2000) in which C1 INH is normal yet bradykinin overproduction is suggested, particularly since mutations in factor XII have been associated with it (Bork, Wulff, Hardt, Witzke, & Staubach, 2009). These findings have led to the development of drugs for the treatment of diseases characterized by bradykinin overproduction, including ecallantide (Cicardi, Levy, & McNeil, 2010) an inhibitor of plasma kallikrein, and icatibant (Cicardi, Banerji, & Bracho, 2010) an inhibitor of bradykinin by virtue of binding to the B-2 receptor.

The "classical" pathway for the production of bradykinin in human plasma consists of three proteins, namely, factor XII (Hageman factor), PK, and HK (Joseph & Kaplan, 2005). PK normally circulates as a 1:1 bimolecular complex with HK (Mandle, Colman, & Kaplan, 1976). Activation of factor XII is followed by conversion of PK to kallikrein, and kallikrein digests HK to release bradykinin. These proteins are bound to vascular endothelial

cells and activation *in vivo* may be dependent on endothelial cell activation/perturbation. Key cell surface proteins are gC1qR (the receptor for the globular heads of complement C1q) which binds factor XII and HK equally (Joseph, Ghebrehiwet, Peerschke, Reid, & Kaplan, 1996; Reddigari, Shibayama, Brunnee, & Kaplan, 1993); a complex of gC1qR and cytokeratin 1 (Joseph, Tholanikunnel, Ghebrehiwet, & Kaplan, 2004), which preferentially binds HK (Joseph, Ghebrehiwet, & Kaplan, 1999); and a complex of cytokeratin 1 and u-PAR (urokinase plasminogen activator receptor) (Joseph et al., 2004) which preferentially binds factor XII (Colman et al., 1997; Mahdi, Madar, Figueroa, & Schmaier, 2002). All binding is zinc ion dependent and is demonstrable utilizing human umbilical vein endothelial cells (HUVECs) (Joseph et al., 1996), microvascular endothelial cells of the skin and lungs (Fernando, Natesan, Joseph, & Kaplan, 2003), as well as astrocytes. HK binding to neutrophils has been shown to be dependent on MAC-1 (CD11b/CD18) (Wachtfogel et al., 1994) whereas HK binding to platelets requires interaction with glycoprotein 1b (Bradford et al., 1997; Joseph, Nakazawa, Bahou, Ghebrehiwet, & Kaplan, 1999). Some of the first clues regarding the possibility of bradykinin formation in the absence of factor XII were provided by studies of bradykinin formation along the surface of HUVECs that is dependent on cell-derived proteins such as heat shock protein 90 (HSP-90) (Joseph, Tholanikunnel, & Kaplan, 2002a) or prolylcarboxypeptidase (Shariat-Madar, Mahdi, & Schmaier, 2002).

Although factor XII is always a major contributor to bradykinin formation (except in congenital factor XII deficiency), we will contrast initiating mechanisms that are directly dependent on factor XII with those in which factor XII is recruited secondarily to kallikrein formation, and discuss these possibilities in relation to the pathogenesis of HAE. Finally we will describe protein–protein or protein–proteoglycan interactions that can initiate activation of the bradykinin-forming cascade in disorders or diseases such as Alzheimer's disease, cryoglobulinemia, or systemic anaphylaxis.

## 2. CONTACT ACTIVATION: FACTOR XII-DEPENDENT INITIATION OF BRADYKININ FORMATION

This is the traditional way of describing the plasma kinin-forming cascade which originated when addition of blood to a glass tube was found to initiate blood coagulation, and simultaneously, release of the nonapeptide, bradykinin (arg pro pro gly phe ser pro phe arg). A diagrammatic representation of the reactions is shown in Fig. 2.1. A brief summary of the proteins

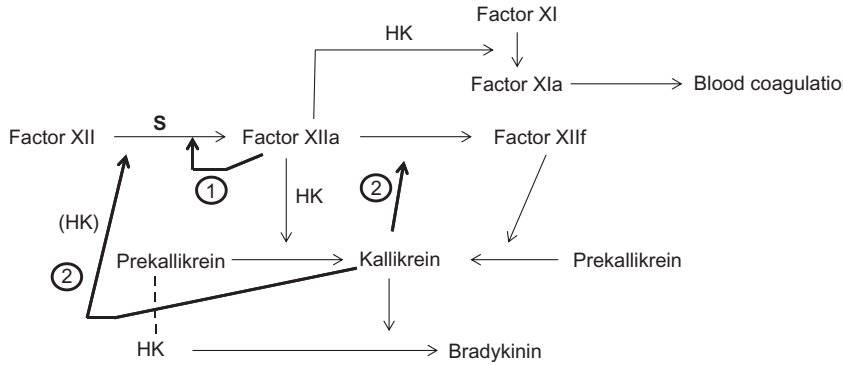


**Figure 2.1** Activation of the plasma bradykinin forming cascade upon binding to initiating surfaces (S). Factor XII autoactivation (reaction ①) yields sufficient factor XIIa to convert prekallikrein to kallikrein and factor XI to factor XIa. A positive kallikrein feedback (reaction ②) rapidly converts factor XII to factor XIIa and then factor XIIf. Factor XIIf lacks the surface binding site and is ineffective as a factor XI activator compared to factor XIIa, but can continue to convert prekallikrein to kallikrein in the fluid phase. HK along arrows emanating from factor XIIa indicates that the binding of prekallikrein and factor XI to HK (circulating complexes) has a kinetic effect in which HK augments the rate of conversion of prekallikrein to kallikrein and factor XI to factor XIa. The (HK) indicates that HK augmentation of factor XII activation by kallikrein is indirect as described in the text.

involved follows: Factor XII (Hageman factor) circulates as a β globulin, of molecular weight 80 kDa, which autoactivates on binding to surfaces bearing negative charges (Silverberg & Kaplan, 1982). Although a conformational change occurs upon binding, an active enzymatic site is not expressed—rather factor XII becomes a substrate (Griffin, 1978) for traces of circulating enzymes. For example, if plasma had as little as $10^{-13}$ molar factor XIIa, contact activation could proceed such that 50% activation would occur in 13 s (Tankersley & Finlayson, 1984). Cleavage of factor XII (80 kDa) within a disulfide bond converts it to factor XIIa (82 kDa) in which a heavy chain (50 kDa) is disulfide linked to a light chain (32 kDa) and the active site is located within the light chain (Que & Davie, 1986). A second cleavage converts factor XIIa to factor XIIf (Dunn & Kaplan, 1982) (28–30 kDa) which actually consists of a mixture of two closely related forms (factor $XIIf_1$, and factor $XIIf_2$; kDa 30 and 28, respectively) resulting from two cleavages external to the disulfide bridge (see diagrammatic representation—Fig. 2.2) such that the light chain of factor XIIa becomes the heavy chain of factor $XIIf_1$. Conversion of factor $XIIf_1$ to factor $XIIf_2$ removes a 1.5 kDa small peptide from the C-terminus of the factor XIIa heavy chain (Dunn & Kaplan, 1982; Fujikawa & McMullen, 1983).

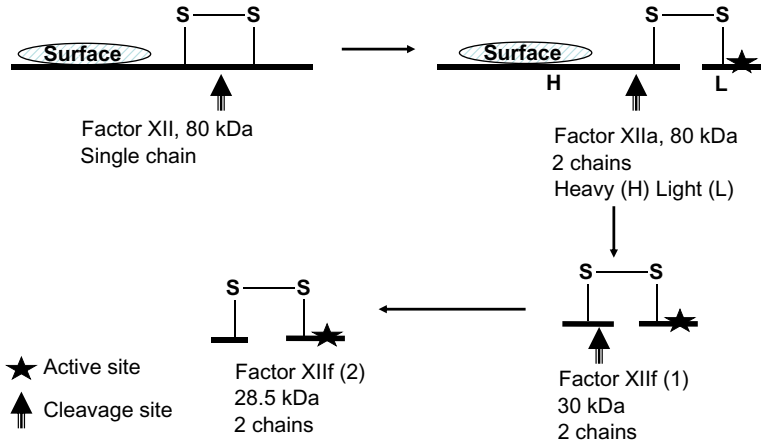


**Figure 2.2** *Diagrammatic representation of the cleavage of factor XII leading to formation of factor XIIa and then factor XIIf (two closely related forms).* Cleavage of surface bound native factor XII within the disulfide bridge shown converts it to factor XIIa (80 kDa) in which the active site (star) is in the light chain. Further cleavage at the distal end of the heavy chain produces factor XII fragment (1) (XIIf, 30 kDa); the active site is the same but the surface binding site is lost. A third cleavage shortens the heavy chain remnant further to yield the final factor XIIf (2) at 28.5 kDa. Typically a mixture of factor $XIIf_1$ and factor $XIIf_2$ is obtained seen as a doublet in SOS gels.

PK is converted to kallikrein (Fig. 2.1; Mandle et al., 1976) by factor XIIa (or factor XIIf). It is a γ globulin of 84 kDa and on SDS gel electrophoresis has two closely spaced bands that differ in carbohydrate content. The protein sequence of the two forms is identical. Activation occurs by cleavage within a disulfide bond (Chung, Fujikawa, McMullen, & Davie, 1986) such that a heavy chain of 50 kDa is disulfide linked to a light chain of either 33 kDa or 36 kDa. The carbohydrate heterogeneity therefore resides within the light chain, and the active enzymatic site is within the light chain (Mandle et al., 1976).

PK and coagulation factor XI each circulate as a complex with HK (Mandle et al., 1976; Thompson, Mandle, & Kaplan, 1977) with stoichiometry of 1:1 and 1:2, respectively (factor XI is a dimer). The binding sites for PK and factor XI on HK overlap (Tait & Fujikawa, 1986, 1987) to such a degree that HK can bind only one molecule of each, but never both. HK, however, is present in considerable molar excess. Thus, we have separate complexes of PK–HK and factor XI-HK and the percent bound to HK in each case is 85% and 95% based upon equilibrium considerations (Scott & Colman, 1980).

HK is a key factor which regulates contact activation. It is also the link protein that allows assembly of the kinin-forming cascade along the surface of cells and we will therefore consider its structural features in some detail. Human plasma has two kininogens (Jacobsen, 1966) that are designated HK and low molecular weight kininogen (LK). They are assembled by alternative splicing of the terminal exons such that a large portion of their amino acid sequence is identical (Kitamura et al., 1985). The domain structure of the protein HK is shown in Fig. 2.3 and it consists of six domains. At the N-terminus are three domains (encoded by exons 1–9) that are homologous to cystatins and stefans (Kellermann, Lottspeich, Henschen, & Muller-Esterl, 1986a) including sulfhydryl proteases such as cathepsin B, H, and L, and domains 2 and 3 actually retain cysteine protease inhibitory activity (Gounaris, Brown, & Barrett, 1984; Higashiyama et al., 1986; Ishiguro, Higashiyama, Ohkubo, & Sasaki, 1987; Muller-Esterl, Fritz, et al., 1985). Domain 4 contains the bradykinin sequence plus the next 12 amino acids. Up to this point LK and HK have identical amino acid sequences. Then exon 10, which includes bradykinin plus domains 5 and 6, is added for HK (Fig. 2.4), whereas exon 11 is added for LK with the attachment at the C terminus of domain 4. When HK is cleaved by plasma kallikrein to release bradykinin (the first cleavage occurs at a C-terminal Arg-Ser bond, followed by cleavage at an N-terminal Lys-Arg bond) (Mori & Nagasawa, 1981; Mori, Sakamoto, & Nagasawa, 1981), and the kinin-free HK is

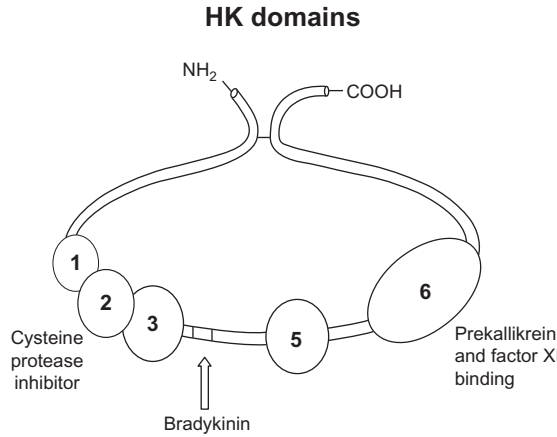


**Figure 2.3** *The structure of HK.* The heavy chain region consists of three homologous domains (1–3) of which the latter two are sulfhydryl protease inhibition sites. Domain 4 contains the bradykinin moiety. The light chain region contains the surface binding site (domain 5) and overlapping binding sites for PK and factor XI (domain 6).

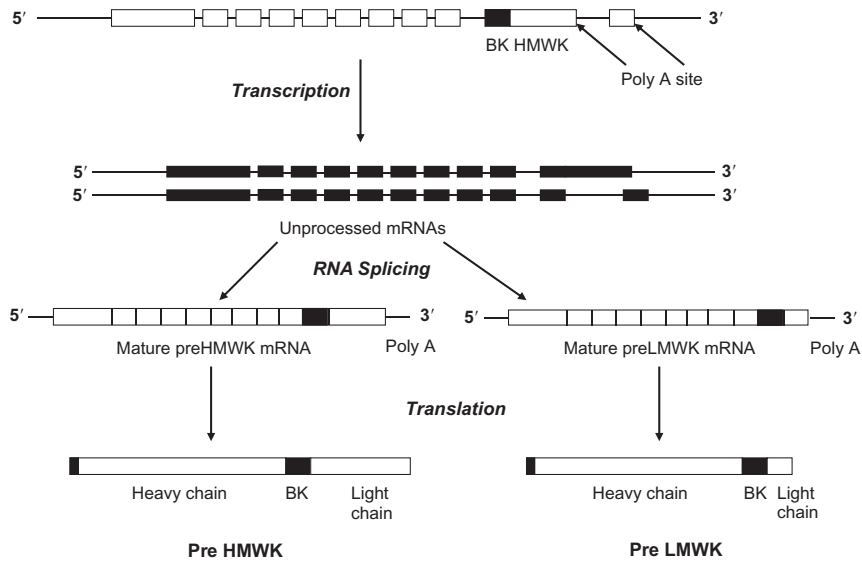


**Figure 2.4** *The gene for HK*. The boxes 1–9 represent the exon coding for the heavy chain of both HK and LK. Exon 10 codes for the bradykinin sequence and the light chain of HK, whereas exon 11 codes for the light chain of LK. The mature mRNAs are assembled by alternative splicing events in which the light chain sequences are attached to the 3′ end of the 13 amino acid common sequence C terminal to bradykinin.

reduced and alkylated, one can isolate a heavy chain (domains 1–3) and a light chain (the C-terminal 12 amino acids of domain 4 plus domains 5 and 6) (Thompson, Mandle, & Kaplan, 1979). Thus, the light chain of HK and LK is quite different (Kellermann, Lottspeich, Henschen, & Muller-Esterl, 1986b) and this accounts for the difference in molecular weight and many of the functional properties of HK that are not shared by LK. It should be noted that plasma kallikrein preferentially cleaves HK (Reddigari & Kaplan, 1988, 1989) whereas tissue kallikrein (encoded by a separate gene from that of plasma kallikrein) cleaves both HK and LK, but with more favorable kinetics if LK is the substrate (Lottspeich, Kellermann, Henschen, Rauth, & Muller-Esterl, 1984; Muller-Esterl, Rauth, Lottspeich, Kellermann, & Henschen, 1985).

The function of HK in contact activation, as depicted in Fig. 2.1, is multiple. First, it accelerates the conversion of PK and factor XI to kallikrein and factor XIa, respectively (Griffin & Cochrane, 1976; Meier, Pierce, Colman, & Kaplan, 1977; Wiggins, Bouma, Cochrane, & Griffin, 1977). This acceleration appears due to the ability of PK and factor XI to bind

to HK; as a result each of them is in a more favorable conformation for activation than when they are tested unbound. In addition, HK provides the attachment to initiating surfaces and brings both PK and factor XI to the surface as a complex. If PK and factor XI bind to the surface in the absence of HK, activation by factor XIIa is markedly inhibited, even when compared with activation in the fluid phase. Thus, the conformational effects of binding of PK and factor XI to HK are even more evident when activation along the surface is compared to fluid-phase activation (since factor XII is activated along surfaces, this comparison is made by adding preformed factor XIIa to PK or PK–HK either in solution or bound to a surface).

Figure 2.1 also depicts a positive feedback in which kallikrein activates surface-bound factor XII to form factor XIIa (Cochrane, Revak, & Wuepper, 1973; Dunn, Silverberg, & Kaplan, 1982; Meier et al., 1977; Silverberg, Nicoll, & Kaplan, 1980) and then factor XIIf (Dunn et al., 1982). Autoactivation of factor XII can initiate the cascade once sufficient factor XIIa forms to overcome plasma inhibitors (Silverberg et al.,., 1980; Tankersley & Finlayson, 1984) and only a few percent conversion to factor XIIa is required. Then the kallikrein formed activates the remaining surface bound factor XII at a much more rapid rate. This positive feedback is also accelerated by the presence of HK (Griffin & Cochrane, 1976; Meier et al., 1977; Silverberg et al., 1980). The factor XIIa formed remains attached to the initiating surface; further digestion of factor XIIa by kallikrein yields factor XIIf (Dunn & Kaplan, 1982; Kaplan & Austen, 1970, 1971) that lacks the binding site to the surface. It can continue to activate PK in the fluid phase until inactivated by C1 INH. Factor XI is activated by factor XIIa in the presence of HK, but factor XIIf possesses only 2–4% of the coagulant activity of factor XIIa (Kaplan & Austen, 1970) and HK does not augment its reaction rate. Thus, factor XIIf can be important for bradykinin formation, but not for intrinsic coagulation.

The mechanism by which HK accelerates factor XII activation is multifactorial and indirect, since HK does not increase the enzymatic activity of kallikrein, nor does it interact with factor XII to render it a better substrate (Silverberg et al., 1980). Its main effect is to allow dissociation of kallikrein from its complex with HK so that it can enzymatically activate factor XII along the cell surface (Cochrane & Revak, 1980). Since HK is required for the formation of kallikrein, that is, activation of PK, the amount of kallikrein is increased when HK is present. Thus, the effective ratio of kallikrein/factor XII in this enzymatic reaction is significantly augmented when HK is present.

A detailed discussion of the structure of each protein; transcription and translational events involved in the synthesis of each protein; and mechanistic details regarding activation of factor XII, PK, and factor XI have been presented in this series previously (Kaplan et al., 1997) and the reader is referred to that publication for further details.

## 3. ASSEMBLY ON CELL SURFACES: BINDING OF HK TO HUVEC

The initial studies of the interaction of proteins of the kinin-forming cascade with cells were performed with platelets (Greengard & Griffin, 1984; Gustafson, Schutsky, Knight, & Schmaier, 1986) and then HUVECs (Schmaier, Kuo, Lundberg, Murray, & Cines, 1988; van Iwaarden, de Groot, & Bouma, 1988). In each instance, HK was shown to bind to each cell type on a zinc-dependent fashion. The binding was saturable and reversible, however, binding was found to be dependent on domains 3 and 5 (Hasan, Zhang, Samuel, & Schmaier, 1995; Herwald, Hasan, Godovac-Zimmermann, Schmaier, & Muller-Esterl, 1995; Reddigari, Shibayama, et al., 1993) so that both heavy chain and light chain were capable of similar ion-dependent interactions (Reddigari, Kuna, et al., 1993) (Fig. 2.5). Factor XII interacts with HUVECs in a similar fashion to HK; the interaction requires zinc ion and factor XII, and HK can compete for binding to the cell surface (Reddigari, Shibayama, et al., 1993). The latter observation suggests that they bind to similar cell surface proteins with comparable affinity.

The next step was to purify and characterize this binding protein; the results (Joseph et al., 1996) are summarized below and correspond to a p33 endothelial cell protein isolated also by Herwald, Dedio, Kellner, Loos, and Muller-Esterl (1996) and identified to be gC1qR, the receptor for the globular heads of C1q discovered by Ghebrehiwet, Lim, Peerschke, Willis, and Reid (1994). A solubilized endothelial cell membrane preparation was passed over an HK affinity column in the presence or absence of zinc ion and eluted with glycine–HCl (0.1 M, pH 2.5), and the fractions were neutralized. Positive fractions were pooled, concentrated, and analyzed by SDS-PAGE. The main feature was the appearance of a new prominent band at 33 kDa. Ligand blot experiments demonstrated that biotinylated HK bound only to the 33 kDa band. Based on this information, the 33 kDa protein was subjected for N-terminal amino acid sequence analysis and the first 13 amino acids were found to be identical to the known $NH_2$ terminus of gC1qR (Ghebrehiwet et al., 1994). A Western blot using

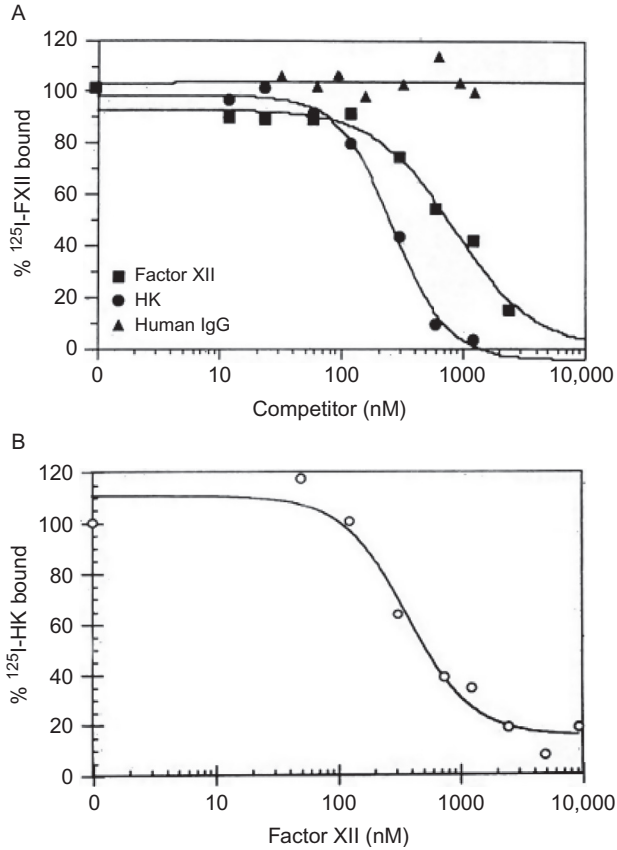


**Figure 2.5** *High molecular weight kininogen competes with factor XII for the same binding sites of HUVEC.* (A) HUVECs were incubated with 1 μg/ml [$^{125}$I]FXII in triplicate in the presence of increment concentrations of unlabeled factor XII, HK, or normal human IgG for 120 min and bound ligand was determined. The percentage bound in the presence of a competitor is plotted against the concentration of the competitor. (B) HUVECs were incubated with 1 μg/ml [$^{125}$I]HK in triplicate in the presence of increasing concentration of unlabeled factor XII and bound ligand was determined.

anti-gC1qR monoclonal antibody 60.11 was performed to further assess the identity of these two proteins. Monoclonal antibody 60.11, which interacts with an epitope at the N-terminus of gC1qR, identified the 33 kDa HUVEC-derived membrane binding protein.

Factor XII could also bind to gC1qR. HUVEC membrane-purified gC1qR or recombinant gC1q-R (rgC1qR) at 1.0–2.0 μg was applied to nitrocellulose membranes and blotted with biotinylated HK or factor XII in the presence or absence of 50 μM zinc. We found that both HK and factor

XII bind to either purified or rgC1qR in the presence of zinc. Addition of excess unlabeled HK reversed the ability of factor XII to bind to gC1qR by over 90% which suggests interaction with a common domain within the protein. These data completely paralleled those observed on binding of factor XII and HK to endothelial cells.

Demonstration that the interaction with gC1qR is indeed responsible for binding to the cell surface was addressed by inhibition experiments. HUVECs were incubated for 30 min with HK ($8.7 \times 10^{-8}$ M), or anti-gC1qR mAbs 74.5.2 and 60.11. Then, in the presence of 50 μM zinc, [$^{125}$I] HK was added and incubated for 60 min at 37 °C, conditions known to saturate the binding sites (Hasan et al., 1995). For each condition, the percentage inhibition of [$^{125}$I] HK binding was determined. Whereas a 100-molar excess of nonradiolabeled HK inhibited subsequent [$^{125}$I] HK binding, a comparable concentration of C1q did not, indicating that the binding sites of gC1qR for C1q and for HK do not overlap. Furthermore, although mAb 60.11 did not efficiently inhibit [$^{125}$I] HK binding to HUVECs, mAb 74.5.2 did. Herwald et al. (1996) also demonstrated that gC1qR is a major endothelial cell-binding protein for HK, using a domain 5-derived peptide from the light chain rather than whole HK as the ligand. In aggregate, these data indicate that gC1qR serves as a zinc-dependent binding protein for factor XII as well as for HK, and that binding to HK occurs via the light chain moiety. The specific location within HK for binding to endothelial cells is within domain 5 (Hasan et al., 1995; Schmaier, 2003) and this also appears to be the site of interaction with gC1qR. It is also clear that the HK heavy chain also binds to endothelial cells. This interaction has been shown to require domain 3.

The methods employed to identify the cell surface protein that interacts with heavy chain are analogous to those described earlier for isolation of gC1qR. Later, a second HK-binding protein was identified in HUVECs by affinity chromatography employing HK as ligand (Hasan, Zisman, & Schmaier, 1998; Shariat-Madar, Mahdi, & Schmaier, 1999) and was identified as cytokeratin 1. This protein could contribute to heavy chain interaction with cells and an affinity column was therefore prepared by covalently coupling peptide LDC27 sequence to the matrix; this is a 27-amino acid peptide derived from domain 3, which has been identified as an HK-binding site (Herwald et al., 1995). When membrane extracts derived from HUVECs were applied to the column in the presence and absence of 50 μM zinc and each was eluted with 0.1 M glycine–HCl, pH 2.5, a band was noted at molecular weight 68 kDa in the zinc-containing eluate.

A ligand blot with HK confirmed binding to this band. When we attempted to sequence it, the N-terminus was blocked. The protein was therefore digested with cyanogen bromide and the mixture was subjected to mass spectrometry. A major peptide at molecular weight 2721.7 was identified, and its sequence was determined and shown to correspond to an internal peptide derived from cytokeratin 1 (Joseph, Ghebrehiwet, et al., 1999; Joseph, Shibayama, et al., 1999). Thus, HK binding to HUVECs appeared to depend on interaction with two proteins; cytokeratin 1 and gC1qR, with binding to each by domains 3 and 5 of HK, respectively (i.e., binding of heavy chain to cytokeratin 1 and light chain to gC1qR). gC1qR could not bind heavy chain at all, whereas cytokeratin 1, when tested as a purified protein could bind both heavy and light chain, however binding to heavy chain clearly predominates. Factor XII is capable of binding to both proteins. To confirm that these proteins are important for binding to endothelial cells, we performed inhibition experiments in which antibody to gC1qR and antibody to cytokeratin 1 were employed. As can be seen in Fig. 2.6 antibody to gC1qR inhibited zinc-dependent binding by 65%, antibody to cytokeratin 1 inhibited binding by 30%, whereas a combination of antisera inhibited binding by 85%. Since 15% binding corresponds to zinc-independent binding, our data suggest that we account for most, if not all, HK binding to endothelial cells by these two proteins.

A third protein reported to be important for HK binding to HUVECs was identified to be u-PAR (the urokinase plasminogen activator receptor) by inhibition of HK binding with antisera to domain 2/3 of u-PAR (Colman et al., 1997). However, we had not been able to isolate u-PAR from cell membranes (confirmed to contain considerable u-PAR) by HK affinity chromatography. One difference in the experiments is that the studies by Colman et al. employed cleaved HK lacking the bradykinin moiety; it was later demonstrated that cleaved HK binds far more avidly to u-PAR than does native HK while native HK binds more avidly to gC1qR and cytokeratin 1 than it does to u-PAR.

## 4. BINDING OF FACTOR XII TO HUVEC

Early studies demonstrated that factor XII binds to both gC1qR and cytokeratin 1 and that it competes for the same binding sites as does HK (Fig. 2.5). The first study to attempt to identify the binding site on HUVECs (rather than testing purified proteins shown to bind HK) was a study by Mahdi et al. in which antisera to u-PAR, cytokeratin 1, and gC1qR were

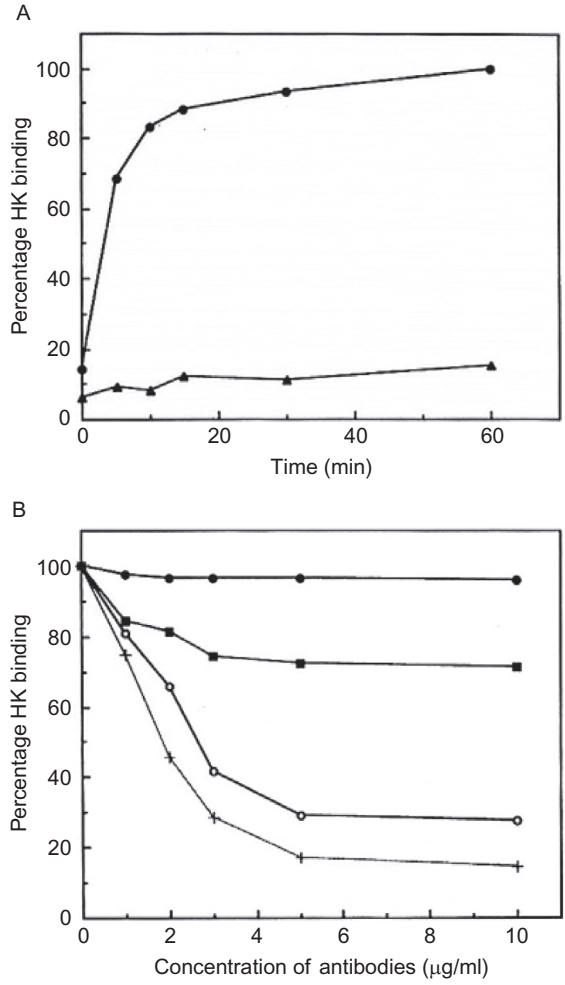


**Figure 2.6** *$^{125}$I HK binding to HUVEC and its inhibition by monoclonal antibodies.* (A) HUVECs were incubated with $^{125}$I HK (20 nM) in the presence (•) or absence (▲) of 50 μM zinc. After each indicated times, the cells were washed and counted for bound radioactivity. (B) For inhibition studies, cells were preincubated with monoclonal antibodies or nonimmune mouse IgG for 30 min. After 30 min, $^{125}$I HK was added and further incubated for 1 h at room temperature. The lines represent HK binding values after treatment with a monoclonal antibody to gC1qR (○), monoclonal antibody to cytokeratin 1 (◆), a combination of monoclonal antibodies to gC1qR and cytokeratin 1 (+), and a control mouse IgG (•). Antibody to gp1b showed similar results to the control mouse IgG. Each point is a mean of three different experiments performed in triplicate.

employed to inhibit cell binding of factor XII (Mahdi et al., 2002). A surprising result was that antibody to u-PAR inhibited best although the other antisera were contributory. We therefore sought to corroborate the observation by isolation of factor XII-binding proteins directly from HUVEC-derived cell membrane preparations by affinity chromatography employing factor XII as ligand. The major zinc-dependent binding protein was clearly u-PAR; gC1qR was also isolated as well as small amounts of cytokeratin 1 (our unpublished data). We could also demonstrate more avid binding of factor XII to u-PAR than to either gC1qR or cytokeratin 1 by competitive displacement of factor XII bound to one protein, employing increasing quantities of either of the other two. Thus, we began to develop a model for assembly of the kinin-forming cascade on endothelial cells in which zinc-dependent binding of factor XII is associated predominantly with u-PAR, and HK binds to gC1qR as well as cytokeratin 1 while PK is bound to the HK. How that binding occurs, particularly for HK, depends on the way these proteins are distributed within the cell membrane of HUVECs as well as their molar ratio.

## 5. INTERACTION OF gC1qR, CYTOKERATIN 1, AND u-PAR WITHIN HUVEC CELL MEMBRANES

One dilemma posed by antibody inhibition studies in which all three antisera were employed was that the total percent inhibition obtained when the three percentages were added exceeded 100%. One possible explanation was that these proteins might interact in some fashion within the cell membrane and a trimolecular complex containing all three was proposed (Colman & Schmaier, 1997). Thus, antisera to one protein might sterically interfere with binding to the others and falsely influence the percent inhibition observed. A second, different alternative was proposed by another group, who reported that HK binding to HUVECs was not due to interaction with proteins at all, but that proteoglycans such as syndican and glypican were in fact responsible (Renne, Dedio, David, & Muller-Esterl, 2000) and questioned whether gC1qR is truly demonstrable along the cell membrane surface (Dedio, Jahnen-Dechent, Bachmann, & Muller-Esterl, 1998; Dedio & Muller-Esterl, 1996). It should be noted that u-PAR is known to be linked to cell membrane constituents by a phosphatidylinositol bond, but that gC1qR and cytokeratin 1 are not, and the latter two proteins lack typical transmembrane domains. Thus, if they are present within cell membranes, the mode of attachment is unknown. Finally, the number of binding

sites reported for gC1qR on the cell membrane varied from just under 1 million to 10 million (Motta, Rojkjaer, Hasan, Cines, & Schmaier, 1998; Reddigari, Shibayama, et al., 1993; van Iwaarden et al., 1988); questions were raised regarding such a high figure, although binding to a cell membrane proteoglycan could achieve such levels.

We (and others) addressed each of these issues. Employing high-titer, monospecific antisera to gC1qR, the protein was clearly demonstrated to be at the HUVEC surface (Joseph, Ghebrehiwet, et al., 1999). Mahdi et al. then demonstrated the presence of all these proteins within cell membranes by immunoelectron microscopy (Mahdi et al., 2002; Mahdi, Shariat-Madar, Todd, Figueroa, & Schmaier, 2001). Cytokeratin 1 and u-PAR appeared to be colocalized while gC1qR was present throughout the cell membrane. We addressed the question of binding to proteoglycan by employing heparanases, which remove all heparan sulfate-containing structures from cell membranes and demonstrated that HUVECs treated so as to become unreactive to antisera to heparin sulfate (the major sulfate source associated with cell surface proteoglycans) bound HK normally, and the binding was then inhibited with antisera to gC1qR and cytokeratin 1 (Fernando et al., 2003). We next solubilized purified cell membranes from HUVECs and demonstrated that gC1qR, cytokeratin 1, and u-PAR are all present by immunoblot analysis. There was no significant contamination by other cell constituents, particularly mitochondria, which are known to contain large amounts of gC1qR (Dedio et al., 1998). We then addressed the interactions of these proteins with each other. First, we could show that gC1qR binds to cytokeratin 1 but not u-PAR while u-PAR also binds to cytokeratin 1, but not to gC1qR. Thus, a trimolecular complex is not possible, but two bimolecular complexes seemed feasible. We then precipitated gC1qR and u-PAR from cell membrane preparations and analyzed the composition of the precipitated materials. Cytokeratin 1 was precipitated with both anti-gC1qR and anti-u-PAR; however, the gC1qR-cytokeratin 1 containing fraction had no u-PAR while the cytokeratin 1-u-PAR fraction contained no gC1qR. One current view of the assembly of the proteins of the kinin-forming cascade on HUVECs envisions factor XII bound to a complex of u-PAR-cytokeratin 1 while HK binds to a complex of gC1qR-cytokeratin 1. We do not know whether HK binds to the complex by both domain 3 and domain 5 simultaneously or whether binding to one site affects binding to the other. Complicating this assessment is the fact that the number of gC1qR sites within the cell membrane is at least three times that of u-PAR or cytokeratin 1, and thus gC1qR unassociated with either

**Table 2.1** Endothelial cell binding proteins for HK and factor XII

| | |
|---|---|
| *Binding proteins* | |
| gC1qR | Binds HK light chain and factor XII |
| Cytokeratin 1 | Binds HK heavy chain |
| u-PAR | Binds factor XII and cleaved HK |
| *Complexes* | |
| gC1qR–cytokeratin 1 | Preferentially binds HK |
| u-PAR–cytokeratin 1 | Preferentially binds factor XII |
| *Affinity purification* | |
| Antibody to gC1qR | gC1qR and cytokeratin 1 |
| Antibody to u-PAR | u-PAR and cytokeratin 1 |

cytokeratin 1 or u-PAR is likely present and can bind either HK or factor XII. Given the relative affinities of factor XII, HK heavy chain, and HK light chain for gC1qR, we would anticipate preferential binding of light chain (domain 5) of HK to gC1qR. Consistent with this is the very prominent inhibition of HK binding to the cell when employing peptide HKH20 derived from domain 5 of the light chain (Nakazawa, Joseph, & Kaplan, 2002). A summary of these binding moieties is given in Table 2.1. The available data, in aggregate, suggest that the binding moieties are gC1qR (present in excess) and the complexes of gC1qR-cytokeratin 1 and u-PAR-cytokeratin 1.

## 6. BINDING TO OTHER CELLS

The interaction of factor XII and HK with other cell types resembles that seen in HUVECs, although there are differences in number of binding sites, the affinity of binding, and the nature of the binding proteins. We reported studies of microvascular endothelial cells derived from skin and lung, and compared binding to that seen in HUVECs, since these cells are more likely to be "physiological" (Fernando et al., 2003). We also compared binding to HUVECs with that seen with astrocytes, because upregulation of bradykinin B2 receptors has been demonstrated within the central nervous system of patients with Alzheimer's disease (Jong, Dalemar, Seehra, & Baenziger, 2002), and aggregated Aβ protein of

Alzheimer's disease is a potent activator of the kinin cascade (Shibayama et al., 1999). It should be noted that a very large number of HK-binding sites was demonstrated for HUVECs by two separate methods, including a fluid-phase based assay in which 850,000 sites per cell were documented. It has been suggested that values in the 10 million range (Motta et al., 1998) may have been due to ligand binding to the plates used rather than to the cells coating the plates, and thus the cell surface represented only a fraction of the total binding seen (Baird & Walsh, 2002, 2003). However, the inhibition of such binding, employing antisera to the cell surface ligands, suggests some (Mahdi, Shariat-Madar, & Schmaier, 2003) other interpretation and our values in the fluid phase are in close agreement with binding studies performed with microtiter plates, for example, for HUVECs, $669{,}427 \pm 123{,}497$ or $771{,}666 \pm 175{,}000$ on 96-well plates or on suspension, respectively. Studies of binding to neutrophils and platelets are reviewed in Joseph and Kaplan (2005) and involve binding to MAC-1 (C36 receptor) and glycoprotein 1b, respectively.

## 7. ACTIVATION OF THE KININ CASCADE: THE ROLE OF ENDOTHELIAL CELLS

### 7.1. Activation by binding to the cell surface

We have demonstrated that factor XII can slowly autoactivate when bound to endothelial cells and that addition of kallikrein can digest bound HK to liberate bradykinin at a rate proportional to the kallikrein concentration and with a final bradykinin level dependent on the amount of bound HK (Nishikawa et al., 1992). Bradykinin then interacts with the $B_2$ receptor to increase vascular permeability. Bradykinin can also stimulate cultured endothelial cells to secrete tissue plasminogen activator (Smith, Gilbert, & Owen, 1985), prostaglandin $I_2$ (prostacyclin), thromboxane $A_2$ (Crutchley, Ryan, Ryan, & Fisher, 1983), and nitric oxide and can thereby modulate platelet function and stimulate fibrinolysis. We next questioned whether factor XII binding to gC1qR is capable of initiating this cascade. We therefore incubated purified factor XII with a wide concentration of gC1qR (0–100ug/μl) for a 30-min time period and replicate samples were incubated in the absence of zinc ion. As shown in Fig. 2.7, the rate of PK conversion to kallikrein increased as the concentration of gC1qR increased (Joseph, Ghebrehiwet, & Kaplan, 2001; Joseph, Shibayama, Ghebrehiwet, & Kaplan, 2001) and there was no activation if zinc is eliminated from the reaction mixture. Purified cell membrane (native) gC1qR

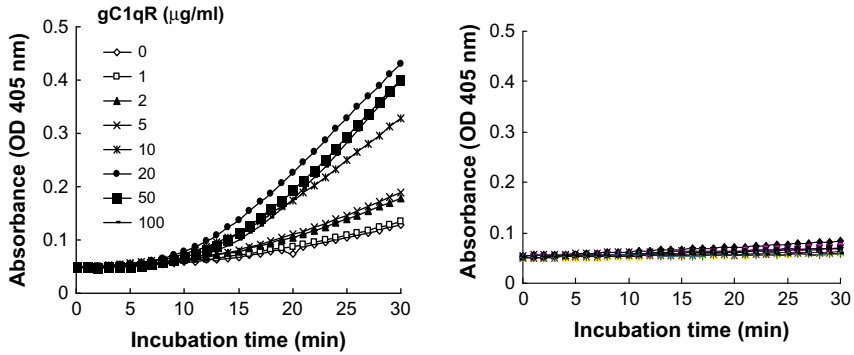


**Figure 2.7** *Effect of gC1qR and zinc ion on factor XII-dependent conversion of prekallikrein to kallikrein.* Each reaction mixture contained 1 μg/ml of factor XII, 1 μg/ml HK, 1 μg/ml prekallikrein and 0.6 mM S2302 and 0–100 μg/ml recombinant gC1qR in HEPES buffered saline with 50 μM zinc chloride (left) or without zinc chloride (right) at 25 °C. The rate of conversion of prekallikrein to kallikrein was monitored at 405 nm.

yields a response that is indistinguishable from a recombinant protein, indicating that gC1qR glycosylation does not affect its "surface" properties. If gC1qR is incubated directly with PK or with PK plus HK, there is no conversion of PK to kallikrein, again emphasizing the requirement for factor XII. This appears to be a physiologic phenomenon which is controlled by C1 INH and $\alpha_2$-macroglobulin. This may be one source of the minute quantities of factor XIIa that escape inhibition that are requisite for contact activation in plasma or during pathologic processes, although additional possibilities will be discussed subsequently. Other data employing endothelial cells corroborate the aforementioned effect of gC1qR when endothelial cells are incubated with normal plasma, and the rate of kallikrein formation is compared with that seen with plasma deficient in factor XII, PK, or HK. There was no detectable activation in any plasma except normal plasma (Fig. 2.8A), and the activation was inhibited by antisera to gC1qR and cytokeratin I (Fig. 2.8B). When the reaction proceeds beyond 2 h, the factor XII-deficient plasma activates, but the HK-deficient plasma and PK-deficient plasma do not, thus a cell-dependent activation of the PK in the presence of HK but absence of factor XII appeared possible.

## 7.2. Factor XII-independent activation of the PK–HK complex on endothelial cells

Studies have demonstrated that binding of the PK–HK complex to endothelial cells leads to activation in the absence of factor XII (Rojkjaer, Hasan,

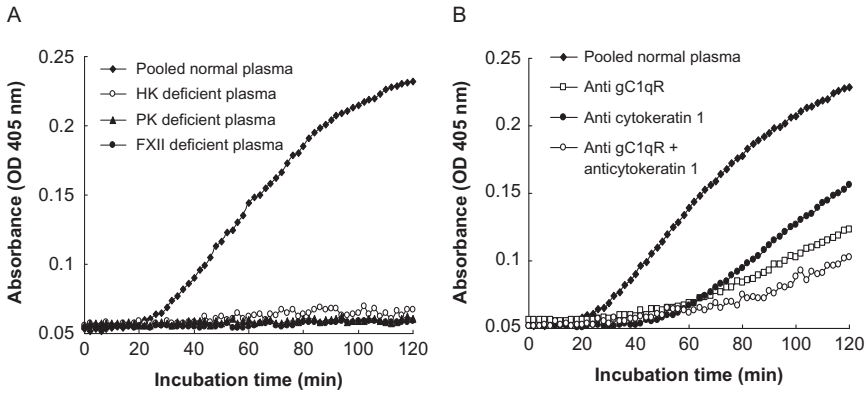


**Figure 2.8** *Prekallikrein activation on endothelial cells*. (A) Endothelial cells were incubated with normal, prekallikrein deficient, factor XII deficient or HK deficient plasmas for 1 h at 37 °C. After incubation, the cells were washed with HEPES buffered saline containing 50 mM zinc chloride and prekallikrein activation was monitored by the cleavage of a kallikrein specific substrate, S2302 (0.6 mM) at 405 nm. In (B), endothelial cells were preincubated with antibodies to cytokeratin 1, gC1qR, or a combination of both for 30 min before addition of normal plasma.

Motta, Schousboe, & Schmaier, 1998; Rojkjaer & Schmaier, 1999a, 1999b) and that the kallikrein which forms can digest HK to liberate bradykinin and also initiate fibrinolysis (Lin et al., 1997). The latter reaction is dependent on kallikrein activating prourokinase (bound to cell membrane u-PAR) to urokinase which in turn converts plasminogen to the fibrinolytic enzyme plasmin. Once such a reaction is set in motion, the addition of factor XII leads to a marked increase in reaction kinetics as a result of the conversion of factor XII to factor XIIa by kallikrein. These observations raise two important questions: (1) What is the nature of the PK activator? (2) When factor XII is present (the normal circumstance), is the cascade initiated by factor XII autoactivation, or is the PK first activated by some cell-derived factor and kallikrein then activates the factor XII?

We next sought to purify and characterize the cell-derived protein(s) responsible for PK activation in the absence of factor XII. We first noted the activity was present in both cell membrane fraction as well as the cytosol derived from endothelial cells and chose to isolate it from the cytosol. We took advantage of the fact that the PK-activating moiety appeared to be inhibitable by corn trypsin inhibitor (CTI) (as is factor XIIa) and a CTI affinity column was shown to bind the activity and it was recoverable by eluting from the column. A single step purification, followed by sequence analysis of suspect bands seen upon SDS gel electrophoresis, ultimately determined that

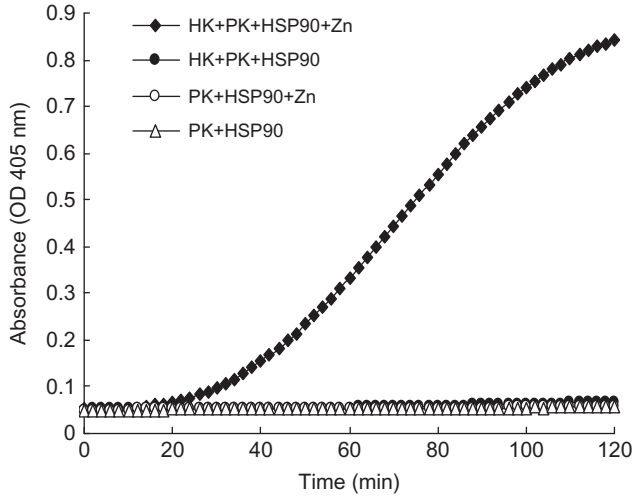


**Figure 2.9** *Prekallikrein activation on HSP-90.* Purified HSP-90 (2 μg) was incubated with prekallikrein (20 nM), HK (20 nM), zinc (50 μM), and S2302 (0.6 mM) and chromogenic activity was monitored. Controls were either in the absence of zinc or HK or both.

HSP-90 is responsible for the activity seen. Thus, when cloned HSP-90 was incubated with PK and HK, the PK was converted to kallikrein and HK was cleaved to liberate bradykinin (Fig. 2.9; Joseph et al., 2002a; Joseph, Tholanikunnel, & Kaplan, 2002b). This is also demonstrable by binding PK and HK to endothelial cells and assessing the rate of conversion of PK to kallikrein. Both HK and zinc ion are requisite and the reaction rate is fast. However, this is in contrast to the slow and factor XII-dependent activation seen when whole plasma (Fig. 2.8) is employed. The reaction is readily demonstrable in the fluid phase as well as by assembly of components along the cell surface; however, it differs strikingly from that seen with factor XIIa. The most critical difference is that PK is not activated unless HK is present. Factor XIIa readily activates PK although the presence of HK does augment the reaction rate. Second, the reaction is stoichiometric, that is, the amount of PK activated has a 1:1 molar ratio to the amount of HSP-90 present. When we tried to determine the structural features of HK that are required, we found that individual heavy and light chains were inactive, and cleaved HK, with bradykinin removed (two chain HK rather than single chain) lost about 70% of the activity. Thus, native HK is required. Addition of a peptide that prevents the interaction of PK with HK also completely inhibits the effect of adding HSP-90. HSP-90 is therefore a stoichiometric activator of the PK–HK complex and is not a PK activator, as is factor XIIa.

Other studies have isolated yet another protein with very similar functional capability. Shariat-Mader et al. isolated a membrane protein that converts PK to kallikrein within the PK–HK complex, and identified it to be prolylcarboxypeptidase (Motta, Shariat-Madar, Mahdi, Sampaio, & Schmaier, 2001; Shariat-Madar et al., 2002). This is an exopeptidase that, if its enzymatic capability is relevant, is behaving as an endopeptidase. It is said to be active along the cell surface, but not in the fluid phase, which differs from HSP-90 but its mechanism of action is otherwise strikingly similar. Both require the presence of HK and zinc ion, the reaction in each case is stoichiometric. Although the prolylcarboxypeptidase is assumed to be the enzyme that activates PK within the PK–HK complex, we suspect that some other mechanism maybe operative, as addressed below. The prolylcarboxypeptidase provides an interesting link of the kinin-forming cascade to the biology of angiotensin since its function, when originally isolated, was to inactivate angiotensin II by conversion to angiotensin III. Thus, a molecule that can generate bradykinin, a vasodilator, inhibits another that is a vasoconstrictor. HSP-90 is also of particular interest since this is a protein that is constitutively present yet upregulated with tissue stress such as hypoxia or during an inflammatory response. In fact, we speculate that its release by activation of endothelial cells can be an important initiating mechanism for the bradykinin-forming cascade.

## 8. INHIBITION OF CONTACT ACTIVATION

Regulation of factor XII-dependent pathways occurs by both intrinsic and extrinsic controls. Cleavage of factor XIIa to XIIf (Fig. 2.2) is one example of an intrinsic control. The factor XIIf produced is not surface bound and is a very poor activator of factor XIa. At the same time, the heavy chain moiety, which has no enzymatic activity, retains the surface-binding site and can compete with factor XII and HK for binding to the surface. Thus, the conversion of factor XIIa to factor XIIf will reduce the rate of the surface-dependent reactions of coagulation, whereas bradykinin generation via fluid-phase activation of PK continues.

Extrinsic controls are provided by plasma inhibitors for each enzyme. Table 2.2 indicates the major inhibitors of each active enzyme and, where known, their relative contributions to the total inhibition in plasma. Inhibition of the contact activation proteases is clearly different from that of the rest of the coagulation pathways in that antithrombin III (ATIII) appears to play only a minor role. Instead, contact activation appears to be limited

**Table 2.2** Plasma inhibitors of enzymes of contact activation: Relative percent contributions to inhibition in normal human plasma

| | Enzyme | | | |
|---|---|---|---|---|
| **Inhibitor** | **Factor XIIa** | **Factor XIIf** | **Kallikrein** | **Factor XIa**[a] |
| C1 inhibitor | 91.3 | 93 | 52 (84)[b] | 8 (47) |
| Antithrombin III[c] | 1.5 | 4 | nd | 16 (5) |
| $\alpha_2$-Macroglobulin | 4.3 | – | 35 (16)[b] | – |
| $\alpha_1$-Protease inhibitor | – | – | nd | 68 (23.5) |
| $\alpha_2$-Antiplasmin | 3.0 | 3 | nd | 8c (24.5) |

[a]Data given are from kinetic studies and irreversible complexes in plasma are given in parenthesis.
[b]Data obtained from generation of Kallikrein *in situ*.
[c]Data are for results obtained in the absence of added heparin.
nd, Not determined separately.

mainly by C1 INH, which is not active against any of the other clotting factors except for inhibition of factor XI.

C1 INH is the only major plasma inhibitor of factor XIIa and factor XIIf (de Agostini, Lijnen, Pixley, Colman, & Schapira, 1984; Pixley, Schapira, & Colman, 1985). Although ATIII can inhibit activated factor XII (Cameron, Fisslthaler, Sherman, Reddigari, & Silverberg, 1989; de Agostini et al., 1984), its contribution to factor XIIa inhibition in plasma is apparently only a few percent of that due to C1 INH (de Agostini et al., 1984; Pixley et al., 1985). Disagreement exists over the effect of heparin on the inhibition of activated factor XII by ATIII. Some investigators have observed little enhancement of the rate of factor XIIa inhibition (Pixley, Cassello, De La Cadena, Kaufman, & Colman, 1991), whereas others have observed a significant increase (Cameron et al., 1989). Alpha-2-macroglobulin, although thought of as a "universal" protease inhibitor, does not significantly inhibit either form of activated factor XII.

The two major inhibitors of plasma kallikrein are C1 INH and $\alpha_2$-macroglobulin (Gigli, Mason, Colman, & Austen, 1970; Harpel, 1973; Harpel, Lewin, & Kaplan, 1985). Together they account for over 90% of the kallikrein inhibitory activity of plasma, with the remainder contributed by ATIII (Schapira, Scott, & Colman, 1982; van der Graaf, Koedam, & Bouma, 1983). When kallikrein is added to plasma, approximately one-half is bound to C1 INH and one-half to $\alpha_2$-macroglobulin (Harpel et al., 1985). $\alpha_2$-Macroglobulin does not bind to the active site of kallikrein but appears to trap the protease within its structure so as to sterically interfere with its ability

to cleave large protein substrates. The degree of inhibition is greater than 95%, but the residual activity is detectable when assayed for lengthy incubation periods. In contrast, digestion of small synthetic substrates is much less affected, and approximately one-third of the starting activity is retained. When a surface such as kaolin is added to plasma so that kallikrein is generated *in situ* close to 70% or 80% of it is bound to C1 INH (Harpel et al., 1985). The reason for the difference between the patterns of inhibition of added kallikrein and of endogenously produced kallikrein is unknown. Interestingly, at low temperatures, most of the inhibition of added kallikrein is accounted for by $\alpha_2$-macroglobulin (Harpel et al., 1985); C1 INH appears to be ineffective in the cold (Cameron et al., 1989), and this may underlie the phenomenon of "cold activation" of plasma. The inhibition of kallikrein by ATIII is also enhanced by heparin and may therefore become significant in heparinized plasma.

By contrast, when C1 INH inhibits an enzyme, it covalently binds to the active site serine and the enzyme is inactivated regardless of assay employed. C1 INH is an activatable inhibitor: The enzyme destined to be inactivated cleaves a bond within the C1 INH ("bait sequence"). A major conformational change in C1 INH ensues and an active inhibitor site is exposed which covalently binds to the active serine of the enzyme (159, 160).

The predominant role of C1 INH in the regulation of contact activation in human plasma is underscored by the fact that it alone is an efficient inhibitor of activated factor XII, kallikrein, and factor XIa. In plasma from patients with HAE, in which C1 INH is absent, the amount of dextran sulfate required to produce activation is reduced 10-fold compared to normal plasma (Cameron et al., 1989); similar results are obtained in cold plasma. Because some surface was still required for activation under these conditions, we may surmise that the other inhibitors that are active against the contact factors do serve to limit their reactions, but that in normal plasma it is inhibition by C1 INH that forms the barrier to the initiation of contact activation. The plasma concentration of C1 INH is approximately 2 μM, and it is remarkable that its inhibition is ever overcome. That surfaces are able to induce activation must reflect the protection of the proteases at the surface from inhibition. It has also been proposed that kallikrein bound to HK is protected from inactivation by C1 INH (Schapira, Scott, & Colman, 1981, 1982) and $\alpha_2$-macroglobulin (Schapira, Scott, James, et al., 1982; van der Graaf, Koedam, & Bouma, 1983; van der Graaf, Koedam, Griffin, & Bouma, 1983) and that factor XIa is similarly protected from α1-antiproteinase inhibitor (Scott, Schapira, James, Cohen, & Colman,

1982); this mechanism, however, has been ruled out in the case of kallikrein and C1 INH (Silverberg, Longo, & Kaplan, 1986; van der Graaf, Koedam, & Bouma, 1983).

## 9. INACTIVATION OF BRADYKININ

Bradykinin is an exceedingly potent vasoactive peptide that can cause venular dilatation, activation of arterial endothelial cells, increased vascular permeability, hypotension, constriction of uterine and gastrointestinal smooth muscle, constriction of the coronary and pulmonary vasculature, bronchoconstriction, and activation of phospholipase $A_2$ to augment arachidonic acid metabolism. Its regulation is of prime importance, and a variety of enzymes in plasma contribute to kinin degradation. Carboxypeptidase N (Erdos & Sloane, 1962) removes the C-terminal Arg from bradykinin to leave an octapeptide, des-Arg$^9$ bradykinin (Sheikh & Kaplan, 1986b), which is then digested by angiotensin-converting enzyme (ACE), acting as tripeptidase, to separate the tripeptide, Ser-Pro-Phe, from the pentapeptide Arg-Pro-Pro-Gly-Phe (Sheikh & Kaplan, 1986a). Enzymes that have not been characterized rapidly digest Ser-Pro-Phe to individual amino acids and more slowly convert the pentapeptide to Arg-Pro-Pro plus Gly and Phe. The final products of bradykinin degradation are the peptide Arg-Pro-Pro, plus 1 mol each of Gly, Ser, Pro, and Arg, and 2 mol of Phe (Sheikh & Kaplan, 1989a, 1989b). The initial change of bradykinin to des-Arg$^9$ bradykinin formed by this initial cleavage retains some but not all the various activities of bradykinin (Marceau & Bachvarov, 1998). It can, for example, interact with $B_1$ receptors (Regoli & Barabe, 1980) induced by inflammation (e.g., interleukin-1 and tumor necrosis factor $\alpha$) in the vasculature and cause hypotension, but the activities of bradykinin on the skin and the contraction of other smooth muscles are abolished. Bradykinin interacts with constitutively expressed $B_2$ receptors to mediate all its functions. Selective $B_2$ and $B_1$ receptor antagonists have been synthesized and one of these will be discussed subsequently as therapy for acute attacks of angioedema in HAE patients (Beierwaltes, Carretero, Scicli, Vavrek, & Stewart, 1987; Stewart, Gera, York, Chan, & Bunn, 1999; Vavrek & Stewart, 1985).

When blood is clotted and serum is studied, all of the reactions for bradykinin degradation occur as described, but the rate of the initial Arg removal is accelerated fivefold compared to plasma (Sheikh & Kaplan, 1989a). This is probably due to the action of a plasma carboxypeptidase that is distinct from carboxypeptidase N and is expressed (activated) as a result of

blood coagulation. One such carboxypeptidase is the thrombin-activated fibrinolysis inhibitor (Bajzar, Manuel, & Nesheim, 1995; Bajzar, Morser, & Nesheim, 1996). It should also be noted that bradykinin degradation *in vivo* occurs largely along the pulmonary vasculature and that endothelial cells there have a separate carboxypeptidase (carboxypeptidase M) as well as ACE activities. In the pulmonary circulation, the initial cleavage may occur by ACE acting as a dipeptidase to first remove Phe-Arg and then Ser-Pro (each of which is next cleaved to free amino acids), leaving the pentapeptide Arg-Pro-Pro-Gly-Phe. This is then metabolized further. The angioedema sometimes associated with use of ACE inhibitors for treatment of hypertension or heart failure is likely due to inhibition of kinin metabolism leading to increased levels of bradykinin (Nussberger et al., 1998). Because bradykinin is a peripheral vasodilator, it has been considered to be a counterbalance to the vasopressor effects of angiotensin II. It is clear that the two peptides are also related in terms of metabolism, because ACE cleaves His-Leu from the C-terminal of angiotensin I, a decapeptide, to leave the octapeptide angiotensin II. Thus, ACE creates a vasoconstrictor and inactivates a vasodilator. Additional enzymes with a less well-defined role in bradykinin metabolism include neutral endopeptidase, aminopeptidase P, and dipeptidylpeptidase IV (180–182), however these become critical when ACE is inhibited by antihypertensive medication. Figure 2.10 shows an amino acid sequence of bradykinin depicting the cleavage site of each enzyme capable of inactivating it.

## 10. FURTHER CONSIDERATIONS OF THE INITIATING SITE FOR BRADYKININ FORMATION: EVIDENCE THAT THE PK–HK COMPLEX HAS ENZYMATIC ACTIVITY

Studies of the interaction of HSP-90 with the PK–HK complex were perplexing because some of the constituents are not known to have proteolytic (enzymatic) activity yet there is bond cleavage of PK to convert it to kallikrein. Assuming that HSP-90 is not an enzyme, we considered the possibility that PK can autoactivate within the trimolecular complex of HSP-90–PK–HK or even that HK could possess enzymatic activity. In the course of performing those experiments, one control is to incubate PK–HK (1:1 molar ratio) in absence of HSP-90. PK is not converted to kallikrein, but surprisingly, the HK is cleaved to liberate bradykinin. Our initial assumption was that the PK must be contaminated with a small amount of kallikrein. However, prolonged incubation with a synthetic substrate (pro-phe-arg p-nitroamalide) that is cleaved by kallikrein but not PK indicated no

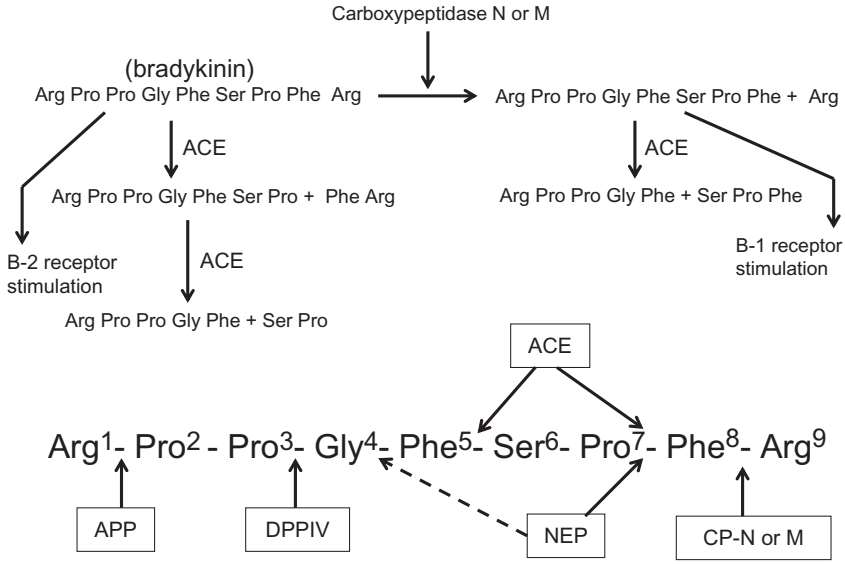


**Figure 2.10** *Top*: Cleavage of bradykinin, a B-2 receptor agonist, by carboxypeptidase N (plasma) or M (endothelial cells) to yield des-arg$^9$ bradykinin, a B-1 receptor agonist. Inactivation of bradykinin and des-arg$^9$ bradykinin by angiotensin converting enzyme (ACE). *Bottom*: Sites of cleavage by additional enzymes including aminopeptidase P (APP), neutral endopeptidase (NEP), and dipeptidyl peptidase IV (DPPIV).

detectable kallikrein. Further, we found that the peptide used earlier in the HSP-90 studies which prevents stoichiometric binding of PK to HK prevents HK cleavage yet this peptide has no effect on kallikrein cleavage of HK. Finally, addition of CTI or C1 INH stops the HK cleavage and CTI, in particular, has no effect on kallikrein. All the evidence suggests that it is PK that is cleaving HK and not kallikrein. Further, since the HK cleavage is inhibited by C1 INH, plasma C1 INH might prevent this reaction from occurring in normal plasma.

These observations were soon followed by a serendipitous discovery that the reaction observed depends on the buffer system used. In Tris–chloride buffer, PK cleaves HK to liberate bradykinin, but in phosphate-containing buffer, the final products of the reaction are kallikrein and cleaved HK. In other words, the PK appears to autoactivate (autodigest) to kallikrein in the absence of any activated factor XII or HSP-90. If PK is incubated in phosphate buffer in the absence of HK, no kallikrein is seen. Thus, it appears that binding of PK to HK (which represents the normal circulating complex) induces a conformational change in PK and exposes an enzymatic site that

is inhibitable by CTI. In chloride buffer, the PK cleaves HK while in phosphate buffer the PK autoactivates to kallikrein and the HK is cleaved by both PK and kallikrein. Since CTI inhibits PK–HK autoactivation to kallikrein, the initiating active site is within PK thus it is likely that PK–HK complexes attack each other to yield kallikrein-HK because if kallikrein activates either PK or PK–HK, it would not be inhibited by CTI.

Since CTI was originally described as an inhibitor of factor XIIa and not kallikrein, its use as an inhibitor of bradykinin formation does not necessarily imply a role for factor XIIa since the aforementioned reactions involving PK are also inhibited. If phosphate buffers are employed so that kallikrein is the product, addition of HSP-90 is additive, and increases the rate of conversion of PK to kallikrein. It is important to note that the HSP-90 reaction requires addition of zinc ion, which does not affect PK cleavage of HK or PK–HK autoactivation and the HSP-90 is effective in chloride buffer (plus zinc) in which the PK–HK complex will not autoactivate.

These reactions are summarized in Fig. 2.11. Since factor XII is normally present and major activation of factor XII, quantitatively, is due to the kallikrein feedback (see Fig. 2.1), factor XII is recruited even in circumstances in which kallikrein is present before factor XIIa. On the other hand, reference to factor XII-dependent activation in a plasma milieu means that the first active site is factor XIIa which is required to generate the first few molecules of kallikrein. An example is the recently described activation of

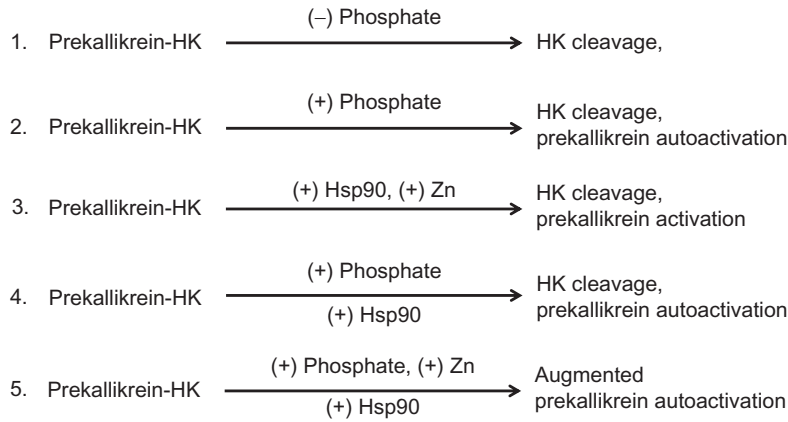


**Figure 2.11** *A summary of reactions in the absence of factor XII.* Digestion of HK by prekallikrein (reaction 1) and conditions for conversion of prekallikrein to kallikrein as well as HK cleavage (reactions 2–5) to produce bradykinin. Specific requirements are shown along the arrow.

the bradykinin forming cascade by infusion of heparin contaminated with oversulfated species (Kishimoto et al., 2008). These oversulfated molecules bind factor XII and catalyze factor XII autoactivation. In the absence of factor XII, there is no activation, that is, no bradykinin is generated and the PK–HK complex in plasma, in the presence of C1 INH is stable. Furthermore, plasma that is congenitally deficient in PK (Fletcher trait) cannot generate any bradykinin by contact activation, but addition of "initiating" negatively charged surfaces lead to slow factor XII autoactivation and activation of the intrinsic coagulation cascade, that is, factor XIIa activation of factor XI-HK to yield factor XIa. This represents a proof that factor XII is the initiating protein of the bradykinin forming cascade initiated by negatively charged surfaces or macromolecules (the classical "contact" activation), even though the very rapid activation of factor XII by the kallikrein formed is kinetically critical. However, there is another alternative. If endothelial cell activation were to liberate either HSP-90 or the prolylcarboxypeptidase to stoichiometrically convert PK to kallikrein within the PK–HK complex, the kallikrein thus formed could activate surface bound factor XII. The resulting factor XIIa then changes the kinetics thousands of fold and a burst of kallikrein formation and bradykinin release ensues. Thus, the discovery that PK (or kallikrein) can be the initiating active site and not factor XIIa is of great interest, yet circumstances in which this might occur need to be delineated experimentally. The most likely possibility, at present, is the disease HAE types I and II due to deficiency of C1 INH. Such patients are missing the key control protein for inhibition of factor XIIa (or factor XIIf) and the protein that stabilizes the PK–HK complex. The new inhibitory function of C1 INH defined for the latter processes includes inhibition of PK cleavage of HK, inhibition of PK–HK autoactivation in phosphate, and inhibition of HSP-90 conversion of PK to kallikrein within the PK–HK complex. These may be exceptions in which C1 INH is not acting as an irreversible inhibitor, that is, it may not form a covalent bond with the ligand, as it does with kallikrein or factor XIIa.

## 11. HEREDITARY ANGIOEDEMA (TYPES I AND II = C1 INHIBITOR DEFICIENCY)

This is an autosomal dominant disorder in which the gene for C1 INH is mutated (Cugno, Zanichelli, Foieni, Caccia, & Cicardi, 2009; Pappalardo et al., 2008). The diagnosis is suspected when there are symptoms of

recurrent angioedema in the absence of urticaria (hives) and it is typically diagnosed by measuring complement C4 levels and determination of C1 INH by protein and function. Since this is inherited as a dominant disorder, with one normal gene (there are two exceptional cases in the literature with homozygous mutations), the C1 INH level should theoretically be 50%. However, there is "trans" inhibition of the one normal gene (Kramer, Rosen, Colten, Rajczy, & Strunk, 1993) and excess utilization of the normal C1 INH product such that levels are frequently less than 35% of normal (i.e., vary from close to zero to 35%) and attacks of angioedema tend to occur when functional C1 INH levels are within this range (Zuraw, 2008). Attacks of swelling are generally of three types: (1) peripheral; that is, hands, feet, face, genitals; (2) gastrointestinal with edema of the bowel wall causing symptoms reminiscent of a partial bowel obstruction such as severe cramps, vomiting, and occasional diarrhea; symptoms increase in intensity and are severe enough to lead to emergency room visits; (3) laryngeal edema which can cause asphyxiation and is a major cause of death in patients with HAE. One must be prepared to intubate patients or perform a tracheostomy in the E.R. and patients need to seek help quickly once such an attack is evident. Episodes have a duration of 2–5 days with 3 days as the mean.

The first component of complement has three subcomponents; namely C1q, C1r, and C1s. In the absence of "sufficient" C1 INH, C1r autoactivates (Ziccardi, 1982), activated C1r activates C1s, and activated C1s cleaves and activates C4 (the more sensitive substrate) and C2. Apparently, patients with HAE always have a small percentage of activated C1s present so that C4 levels are depressed in 95% of patients even when they are feeling well. Thus, quantitation of C4 is a good screening test for angioedema of this sort. However, there are other causes of a low C4 and 5% of HAE patients will have a normal value. Thus, determination of C1 INH is done in all instances. Type I disease (85% of patients) has a mutation that leads to no circulating product, due, for example, to stop codons, insertion or deletions, producing a nonviable m-RNA and/or protein that is degraded intracellularly (Cicardi, Igarashi, Rosen, & Davis, 1987; Pappalardo et al., 2008; Tosi, 1998). Such mutations are spread throughout all eight exons of the C1 INH gene. Type II disease (15%) usually is due to a mutation that causes an amino acid substitution in exon 8, where the C1 INH "active site" resides (Patston, Gettins, Beechem, & Schapira, 1991). The product is produced and circulates, but is inactive. Thus, type II disease has normal, or even occasionally elevated, C1 INH protein levels but abnormal function while type I disease has low C1 INH protein and function.

Phenotypically, type I and II disease are indistinguishable and the specific level of functional C1 INH (e.g., 2% or 10% or 25%) cannot be used to predict severity, in general, or presence of any particular manifestation. When an attack of angioedema occurs, C4 levels are further depressed (a low C4 level is then present in 100% of patients and approaches zero in some) and C2 levels are decreased (Austen & Sheffer, 1965). Thus during an attack of swelling, there is evidence of increased activation of C1. The vasoactive substance originally thought to cause the angioedema was a kinin-like molecule derived from C2 by digestion with plasmin (Donaldson, Rosen, & Bing, 1977). Augmented skin reactivity of individuals with HAE or guinea pigs to activated C1 (Klemperer, Donaldson, & Rosen, 1968; Strang, Auerbach, & Rosen, 1986) seemed consistent with this idea. This was shown to be incorrect by Fields, Ghebrehiwet, and Kaplan (1983), perhaps due to an artifact (Kaplan & Ghebrehiwet, 2004), and the mediator of the swelling has now been clearly identified as bradykinin. Observations include: (1) instability of HAE plasma which seemingly spontaneously evolves bradykinin without addition of an initiating surface (Fields et al., 1983); (2) augmented kallikrein formation in induced blisters in HAE patients (Curd, Prograis, & Cochrane, 1980); (3) C2 depleted HAE plasma generated bradykinin, and no other kinin (Shoemaker, Schurman, Donaldson, & Davis, 1994); (4) rare patients with a mutant C1 INH that fails to inhibit complement but functions normally in factor XII-dependent bradykinin formation have no family members with angioedema (Zahedi, Bissler, Davis, Andreadis, & Wisnieski, 1995); (5) a mouse model in which spontaneously occurring vascular leakage can be observed if the C1 INH gene is "knocked out," has no increase in vascular permeability if the bradykinin B-2 receptor is also knocked out (Han, MacFarlane, Mulligan, Scafidi, & Davis, 2002). Finally, it has been shown that bradykinin formation can be localized to the site of swelling in patients who are symptomatic with HAE (Nussberger, Cugno, Cicardi, & Agostoni, 1999).

## 12. BRADYKININ FORMATION WHEN C1 INH IS ABSENT/DYSFUNCTIONAL

The first demonstration that HAE plasma is unstable and "spontaneously" generates bradykinin was performed by prolonged incubation of HAE plasma (in EDTA) in polystyrene plastic tubes (Fields et al., 1983). It was assumed that trace quantities of uninhibited activated factor XII would be sufficient to initiate bradykinin formation, yet it was also evident

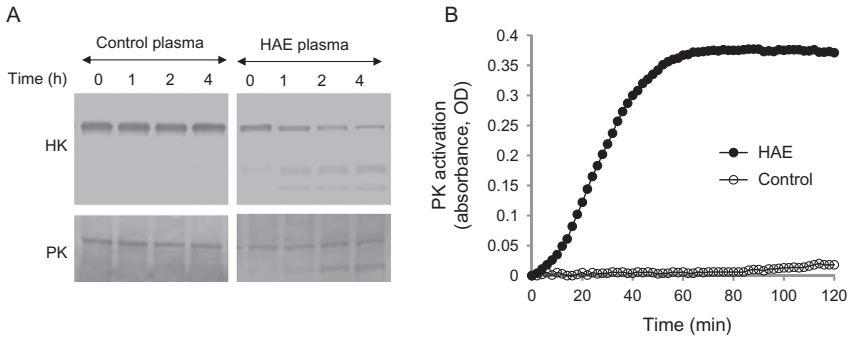


**Figure 2.12** *Activation of plasma bradykinin-forming pathway by prolonged incubation*: Plasma were incubated in PEG-coated 96-well polystyrene plates at 37 °C. Samples were withdrawn at the indicated time points and the reaction was stopped by the addition of SDS sample buffer. (A) Western blot of samples from control plasma (left) and HAE plasma (right) using antibody to light chain of HK (upper panel) or antibody to prekallikrein (lower panel). Progressive HK cleavage with time is evident in HAE plasma but not in normal plasma. Formation of kallikrein is also evident in HAE plasma and not in control plasma. (B) Assay of kallikrein activity in HAE and control plasma employing a specific synthetic substrate (pro-phe-arg p-nitroanilide).

that no initiator of factor XII autoactivation had been added. Incubation of normal plasma, under the same conditions, does not generate bradykinin, HK is not cleaved, and PK is not converted to kallikrein. A time course comparing incubation of normal plasma vs HAE plasma is shown in Fig. 2.12. It is clear that HK is progressively digested in HAE plasma and bradykinin is released (not shown). We considered the possibility that PK is activating the HK in the absence of C1 INH but a time course of cleavage of pro phe-arg p. nitroanalide also indicated progressive conversion of PK to kallikrein. This could represent PK autoactivation within PK–HK since plasma is a phosphate milieu but even a trace of activated factor XII in the plasma could account for all the observations.

We next tested factor XII-deficient plasma and tried to activate it with typical initiators of factor XII autoactivation, for example, kaolin, dextran sulfate, etc. There was no activation. We next removed C1 INH by immunoadsorption and incubated factor XII-deficient plasma vs factor XII-deficient plasma minus C1 INH for 4 h in polystyrene microtiter wells and checked a time course of kallikrein formation. As shown in Fig. 2.13, once C1 INH is absent, the PK–HK complex is unstable, kallikrein evolves, and there is no factor XII that can account for this. Thus, for the first time, we demonstrated in a plasma system, activation can occur with no factor

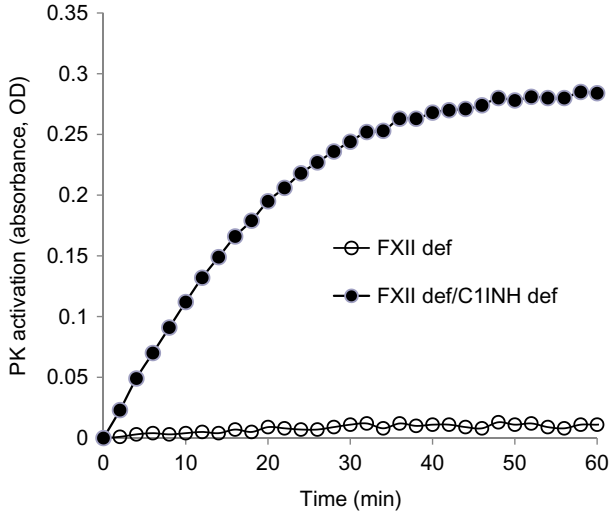


**Figure 2.13** *Factor XII independent prekallikrein activation in plasma*. Factor XII-deficient plasma was passed over a C1-INH antibody affinity column to remove C1 inhibitor. Plasma were incubated in PEG-coated 96-well polystyrene plates at 37 °C and kallikrein activity was measured using a synthetic substrate.

XII, but only if C1 INH is absent (Type I HAE) or dysfunctional (Type II HAE). Thus, the inherent instability of HAE plasma does not require factor XII. However, the rapid kinetics needed to activate the plasma requires factor XII and a key issue for understanding the initiating event(s) in HAE is whether an attack of angioedema is started by factor XII activation or secondary recruitment of factor XII upon activation of the PK–HK complex.

## 13. TYPE III HAE

A new form of HAE has been described (Binkley & Davis, 2000; Bork et al., 2000) which is also a dominantly inherited disorder but the C1 INH is normal. The underlying defect leading to angioedema has not yet been elucidated. It appears to be strongly estrogen dependent so that most patients are women and attacks often occur concurrently with use of estrogen-containing birth control tablets or hormonal replacement therapy. There is a lesser incidence of gastrointestinal attacks compared to types I and II HAE and facial swelling is particularly prominent. Since C4 and C1 INH determinations are normal, we do not as yet have a screening assay for this form of HAE and it cannot therefore be diagnosed with certainty unless

there is a positive family history and the propoitus has recurrent angioedema without urticaria. Suspicion is raised, even without a family history if documented laryngeal edema has occurred or if swelling peaks at about 2–3 days. But even then, distinction from a severe case of "idiopathic" angioedema becomes very difficult.

Approximately 30% of patients with type 3 HAE have a mutant factor XII (Bork, Wulff, et al., 2009; Dewald & Bork, 2006) with an amino acid substitute of threonine 309 with either "lys" or less commonly "arg." This has been characterized as an "overactive" factor XIIa because activators of factor XII appeared to generate more kallikrein than is seen in normals (Cichon et al., 2006). However, an attempt to further characterize the functional status of the mutant factor XII compared to normal factor XII failed to do so (Bork, Kleist, Hardt, & Witzke, 2009). Clearly the mutation segregates with type 3 HAE, but its role in causing bradykinin overproduction (if that is actually the case) is not known. Then there is the other 70% of type 3 patients, presenting identically, without the factor XII mutation. We have sought evidence for instability of the plasma of type 3 HAE patients compared to normals and types I and II HAE patients, since this instability seems to correlate with bradykinin overproduction. However, a clear distinction from normal was not observed. Perhaps the defect lies with a mutant enzyme which renders it poorly inhibitable by C1 INH (whether factor XIIa or something else), which is different from postulating an enzyme with greater intrinsic activity. We also considered an inherited abnormality of kininases so that angioedema might result from impaired degradation. In that case the angioedema would resemble angioedema due to ACE inhibitors (Kaplan & Greaves, 2005). However, the rate of destruction of bradykinin when added to serum of type 3 HAE patients was not different from normal indicating that angioedema in this condition is more likely due to overproduction of bradykinin rather than abnormal degradation. Levels of other inhibitors are normal including $\alpha_2$ macroglobulin, $\beta_2$ glycoprotein I (which has inhibitory activity for activated factor XII), as well as inter-trypsin inhibitor and the protein $C_a$ inhibitor. Recently, we developed an assay for diagnosis of types I and II HAE by determination of functional C1 INH levels based on inhibition of either factor XIIa or kallikrein. When applied to type III HAE, C1 INH functional levels in eight such patients were 40–60% of normal and type III HAE plasma diluted 1:2 would autoactivate on prolonged incubation as does types I and II HAE plasma that are undiluted while normal plasma at 1:2 dilution was still stable. It is not clear whether these low levels in asymptomatic patients are due to consumption or represent an underlying functional abnormality.

## 14. NEW MODALITIES FOR TREATMENT OF HAE

Prior therapies available for HAE have been divided into "acute" and "prophylactic." It is important to note that HAE is unresponsive to antihistamines or corticosteroids and even epinephrine is unreliable. Until recent years, we in the US have had no specific therapy for an acute episode. Peripheral attacks were observed but not treated, gastrointestinal attacks, often necessitating an ER visit, were treated with IV fluids, medications for nausea and pain (often resulting in narcotic addiction for those with frequent episodes), and throat/laryngeal symptoms approached by careful observation with preparedness to do an endotracheal intubation or tracheostomy. In Europe, replacement C1 INH has been available for intravenous administration for over 25 years yet was more often used to treat acute episodes, even though it could be administered on a more routinized base for prophylaxis. Although dramatic changes in the course of the disease could be effected by administration of C1 INH, even twice/week prophylaxis, now used in the US (and elsewhere), does not completely eliminate attacks of angioedema, presumably because a sufficiently high steady-state level of C1 INH is not sustained. Some percentage of patients may need to sustain blood levels above the 50% of normal mark although this level would suffice for most. Prophylaxis in the US had employed attenuated androgen compounds such as danazol (Gelfand, Sherins, Alling, & Frank, 1976; Hosea et al., 1980) or stanazolol (Sheffer, Fearon, & Austen, 1981; Sloane, Lee, & Sheffer, 2007) which stimulate C1 INH synthesis by the one normal gene, and raise C4 levels as a consequence. Another reported effects include increasing kinin degradation by kininases (Drouet et al., 2008). Given that some dose of androgen is employed for a lifetime, significant cumulative side effects can occur (virilization, hypertension, increased cholesterol, hepatoma, etc.) so that other modalities were sought (Szeplaki et al., 2005). Antifibrinolytic agents, which prevent conversion of plasminogen to plasmin, are less effective than androgens, but in the past had a role for prophylaxis. Epsilonaminocaproic acid and tranexamic acid were the choices (Frank, Sergent, Kane, & Alling, 1972; Sheffer, Austen, & Rosen, 1972); the latter is still considered to have particular utility in children (Boyle, Nikpour, & Tang, 2005).

Now two intravenous preparations of purified C1 INH are available in the US, and expanding worldwide, for therapy of acute attacks or prophylaxis known as "Berinert and Cinryze," respectively (Farkas et al., 2007; Zuraw, Busse, & White, 2010). Now that it is clear that bradykinin is the mediator

of HAE, therapies specifically targeting this cascade have been developed. Subcutaneous ecallantide, a plasma kallikrein inhibitor (Cicardi, Levy, et al., 2010; Sheffer, MacGinnitie, Casapion, Stolz, & Pullman, 2013), and icatibant, a bradykinin B-2 receptor antagonist (Cicardi, Banerji, et al., 2010; Lumry et al., 2011) are available for therapy of any type of acute episodes or for breakthrough episodes for those receiving prophylactic C1 INH (although additional C1 INH can also be used) which are very effective (Cicardi et al., 2012).

The molecular defect in type III HAE has not yet been found, although bradykinin overproduction appears to be the cause of attacks of swelling. All of the above modalities have been reported to be effective (Bork, Wulff, et al., 2009; Bouillet et al., 2009; Cronin & Maples, 2012), but controlled studies are lacking. We have recently reported that an *in vitro* abnormality in kallikrein formation observed or prolonged incubation of citrated type III HAE plasma diluted 1:2 can be reversed by doubling the plasma C1 INH level, but it remains to be seen whether this relates to the underling abnormality.

## 15. PROTEOGLYCAN-DEPENDENT ACTIVATION OF FACTOR XII: THEORETICAL MECHANISMS FOR BRADYKININ PRODUCTION IN "ALLERGIC" DISEASES; PARTICULARLY ANAPHYLAXIS, CHRONIC SPONTANEOUS URTICARIA, AND RHINITIS/ASTHMA

Among the physiologic initiators of factor XII activation are proteoglycan (Hojima, Cochrane, Wiggins, Austen, & Stevens, 1984; Kishimoto et al., 2008; Noga, Brunnee, Schaper, & Kunkel, 1999), endotoxin (Morrison & Cochrane, 1974), uric acid and pyrophosphate crystals (Ginsberg, Jaques, Cochrane, & Griffin, 1980), cryoglobulins (Maas, Govers-Riemslay, Bouma, & Schiks, 2008), and the aggregated amyloid β protein responsible for plaques in Alzheimer's disease (Shibayama et al., 1999). For allergic disorders, focus is on the proteoglycan present in mast cells (mast cell heparin) which is oversulfated relative to the heparin used for anticoagulation (Hojima et al., 1984). Thus, mast cell degranulation in allergic or allergic-like disorders can release mast cell heparin, interact with factor XII present in plasma interstitial fluid, and lead to bradykinin formation. Although not proven for any of these disorders, all are amenable to study. It is clear from animal models that elevated bradykinin levels on the arterial, rather than venous, side of the circulation results in hypotension. Thus, the "shock-state" associated with anaphylaxis may be a bradykinin mediated rather than being due to histamine or platelet activating factor. Certainly it is known that HK is completely cleaved when

assessed in blood samples obtained from postanaphylactic shock (Smith et al., 1980). Further, although epinephrine administration is key to interrupting anaphylaxis once it begins, there is evidence that hypotension, in the case of insect sting anaphylaxis, can be resistant to epinephrine (Smith et al., 1980), a feature consistent with a role for bradykinin. The mechanism for mast cell degranulation in chronic spontaneous urticaria is not yet completely clear, but approximately 40% of patients appear to have an autoimmune disorder (Kaplan & Finn, 1999) with IgG anti-$F_c$eRI$\alpha$ (Hide et al., 1993; Kikuchi & Kaplan, 2001) so that cross-linking of $\alpha$ subunits of the IgE receptor activates the cells. Complement-derived C5a, an anaphylatoxin and chemotactic factor (Kikuchi & Kaplan, 2002), augments the process. Thus, there is prominent cutaneous mast cell degranulation leading to severe urticaria which is accompanied by angioedema in about 40% of patients. Half the patients (with or without angioedema) are refractory to antihistamines. Therapy for this subpopulation includes steroids (Kaplan, 2004), cyclosporine (Grattan et al., 2000), or omalizumab (Kaplan, Joseph, Maykut, Geba, & Zeldin, 2008; Maurer, Casale, Hsieh, & Canvin, 2013; Saini et al., 2011), and although bradykinin-mediated symptoms are steroid resistant, the possibility that bradykinin is a contributing factor requires further study. Finally, activation of the bradykinin-forming cascade in allergic asthma (Christiansen et al., 1992) has been reported but its contribution to symptoms of rhinitis, sinusitis, or asthma is not known.

## 16. ACTIVATION OF FACTOR XII BY AGGREGATED PROTEINS

This is a relatively new area of investigation that requires further study. Aggregated proteins leading to amyloid deposition have been shown to activate factor XII (Maas et al., 2008) with subsequent conversion of PK to kallikrein, but not factor XII to factor XIIa. The molecular replantation for this predominance of PK activation is not known. Formation of factor XIIf rather than factor XIIa could explain it, but seems unlikely. If these initiators were to bind factor XII and activate it but not bind to HK, then factor XIIa could still convert PK to kallikrein in the fluid phase which is also relatively unaffected by binding of PK to HK. However, factor XI activation may require binding of HK factor XI to the surface.

Some of the aggregates studied include amyloid fibrils of A$\beta$, transthyretin, Bence-Jones proteins, and glycated bovine serum albumin. We have shown that A$\beta$ 1–42 and 1–40, are aggregated in the presence of zinc ion, can cause factor XII-dependent activation of PK and release of

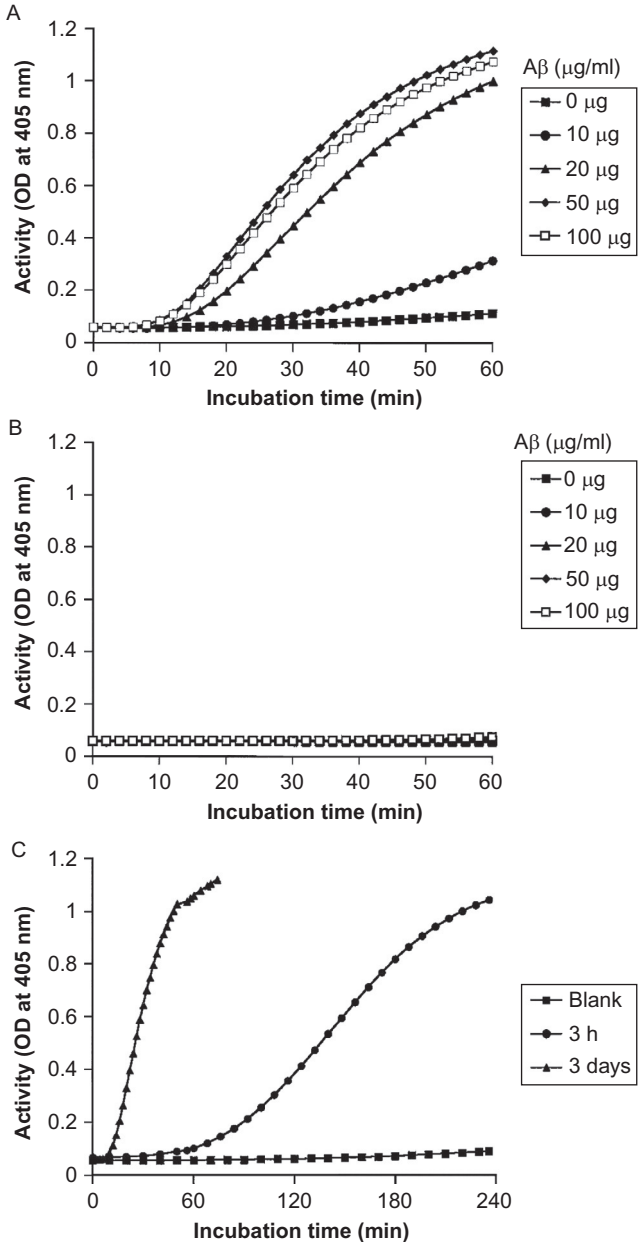


**Figure 2.14** A: Zinc-dependent formation of kallikrein upon incubation of increasing amounts of Aβ (1–38) aggregated for 3 days with purified factor XII, prekallikrein, and HK. B: Same experiment as above in the absence of zinc. C: Demonstration that formation of kallikrein depends on the extent of aggregation of Aβ(1–38). Unaggregated material (blank) is contrasted with Aβ(1–38) incubated for either 3 h or 3 days.

bradykinin (Shibayama et al., 1999; Fig. 2.14). This protein is responsible for plaque deposition in the brain of patients with Alzheimer's disease and plaques have been shown to stain for deposition of factor XII. Activation of this cascade has been documented in the spinal fluid of such patients (Bergamaschini et al., 1998) which is evidence that the process is occurring *in vivo*.

Bradykinin-dependent angioedema includes types I and II HAE, ACE-inhibitor induced angioedema, acquired C1 INH deficiency with antibody to C1 INH, are very likely, type 3 HAE and a subpopulation of antihistamine-resistant patients with angioedema of unknown origin (idiopathic). In this review we have also highlighted numerous diseases apart from angioedema where activation of this cascade may occur, and like HAE, are not associated with thrombosis. Among them are anaphylaxis, endotoxic shock/sepsis, recalcitrant chronic urticaria, cryoglobulinemia, Alzheimer's disease, and crystalline-induced arthropathies. These are interesting and potentially important areas for further research.

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CHAPTER THREE

# The Role of Short-Chain Fatty Acids in Health and Disease

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## Contents



## Abstract

There is now an abundance of evidence to show that short-chain fatty acids (SCFAs) play an important role in the maintenance of health and the development of disease. SCFAs are a subset of fatty acids that are produced by the gut microbiota during the fermentation of partially and nondigestible polysaccharides. The highest levels of SCFAs are found in the proximal colon, where they are used locally by enterocytes or transported across the gut epithelium into the bloodstream. Two major SCFA signaling mechanisms have been identified, inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs). Since HDACs regulate gene expression, inhibition of HDACs has a vast array of downstream consequences. Our understanding of SCFA-mediated inhibition of HDACs is still in its infancy. GPCRs, particularly GPR43, GPR41, and GPR109A, have been identified as receptors for SCFAs. Studies have implicated a major role for these GPCRs in the regulation of metabolism, inflammation, and disease. SCFAs have been shown to alter chemotaxis and phagocytosis; induce reactive oxygen species (ROS); change cell proliferation and function; have anti-inflammatory, antitumorigenic, and antimicrobial effects; and alter gut integrity. These findings highlight the role of

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SCFAs as a major player in maintenance of gut and immune homeostasis. Given the vast effects of SCFAs, and that their levels are regulated by diet, they provide a new basis to explain the increased prevalence of inflammatory disease in Westernized countries, as highlighted in this chapter.

## 1. INTRODUCTION

There is increasing evidence implicating the gut microbiota as critical contributors to host health and gut/immune homeostasis. This may be achieved, at least in part, through the release of short-chain fatty acids (SCFAs), which are the main bacterial metabolites produced following the fermentation of dietary fiber and resistant starches by specific colonic anaerobic bacteria. SCFAs are a subset of saturated fatty acids containing six or less carbon molecules that include acetate, propionate, butyrate, pentanoic (valeric) acid, and hexanoic (caproic) acid. Recent advances in the study of SCFAs, especially acetate, propionate, and butyrate, have highlighted their effects on various systems both at cellular and molecular levels. Indeed SCFAs or their deficiency may affect the pathogenesis of a diverse range of diseases, from allergies and asthma to cancers, autoimmune diseases, metabolic diseases, and neurological diseases.

### 1.1. The production of SCFAs

SCFAs are carboxylic acids defined by the presence of an aliphatic tail of two to six carbons. Although SCFAs can be produced naturally through host metabolic pathways particularly in the liver, the major site of production is the colon which requires the presence of specific colonic bacteria explaining their absence in germ-free mice (Hoverstad & Midtvedt, 1986). Acetate (C2), propionate (C3), and butyrate (C4), being the major SCFA released through fermentation of fiber and resistant starches, are mostly released in the proximal colon in very high concentrations (70–140 mM) while their concentrations are lower in the distal colon (20–70 mM) and in the distal ileum (20–40 mM) (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). The molar ratio of acetate, propionate, and butyrate production in the colon is 60:25:15, respectively (Tazoe et al., 2008), although proportions can vary depending on factors such as diet, microbiota composition, site of fermentation, and host genotype (Hamer et al., 2008). Butyrate is mostly utilized by colonocytes while acetate and propionate reach the liver through the portal vein. Propionate is subsequently metabolized by hepatocytes while acetate either remains in the liver or is released systemically

to the peripheral venous system (Pomare, Branch, & Cummings, 1985). Thus, only acetate is usually detectable in peripheral blood. Extensive research has highlighted the beneficial effects of SCFAs on health, detailed below in this chapter. Health authorities have thus established a recommended daily intake of fiber, which according to the World Health Organization is 20 g per 1000 kcal consumed (in adults) and this quantity is reached through the daily consumption of grains as well as 400 g per day of fresh fruits and vegetables (www.who.int). Notably, the typical consumption of fiber in most Western countries is much less than this (King, Mainous, & Lambourne, 2012) and consumption of fiber is inversely related to premature death from all causes of disease (Park, Subar, Hollenbeck, & Schatzkin, 2011)

### *1.1.1 Substrates for SCFA production*

Indigestible saccharides are the major substrates leading to SCFA production. Polysaccharides are subdivided into three categories: starch, starch-like, and nonstarch polysaccharides (NSPs). Starch, such as amylose, and starch-like polysaccharides, such as glycogen, consist of polymers of glucose linked by alpha 1–4 and alpha 1–6 glycosidic bonds. These bonds are broken down by salivary, pancreatic, and intestinal brush barrier enzymes and are thus digestible by mammals. Under healthy conditions, starch and starch-like polysaccharides are fully digested in the small intestine yielding glucose. Polysaccharides that are undigested or partially digested in the small intestine are able to undergo a process of fermentation by specific colonic anaerobic bacteria leading to the release of SCFAs in addition to gases and heat. These polysaccharides are called fermentable polysaccharides and are subclassified as NSPs, or dietary fibers, and resistant starch (RS). Depending on their degree of solubility, fibers are subclassified into insoluble or soluble fibers and in both cases are found in plant cell walls. Cellulose and lignin are examples of insoluble fibers while pectin substances or gums forming a gel in water are classified as soluble fibers. Insoluble fibers are highly fermentable and hence generate greater quantities of SCFA in the colon while soluble fibers have a rather low fermentability but increase fecal bulking and decrease colonic transit time. RS can be subdivided into four types: physically trapped starch (in coarse grains), RS granules naturally rich in amylose (i.e., raw potato flour), retrograded starch (i.e., cooked and cooled potato), and chemically modified starch (i.e., processed foods) (Englyst, Kingman, & Cummings, 1992). RS is considered as the most powerful butyrogenic substrate where fermentation of RS *in vitro* as well as *in vivo* generally results in a significant higher level of butyrate production compared to NSP

(Englyst et al., 1992). Oligosaccharides, defined by a short chain of monosaccharide units, such as galactooligosaccharides, fructooligosaccharides, mannanoligosaccharides, and chitooligosaccharides are also substrates for SCFAs (Pan, Chen, Wu, Tang, & Zhao, 2009). Finally, to a lesser extent, some SCFAs such as isobutyrate and isovalerate are produced during the catabolism of branched chain amino acids valine, leucine, and isoleucine and intermediate of fermentation in the microbiota such as lactate or ethanol can also be metabolized into SCFA (Macfarlane & Macfarlane, 2003).

### 1.1.2 Mechanism of SCFA production

The process involved in the production of SCFAs from fiber involves complex enzymatic pathways that are active in an extensive number of bacterial species. The most general pathway of SCFA production in bacteria is via the glycolytic pathway, although certain groups of bacteria such as the Bifidobacteria can utilize the pentose phosphate pathway to produce the same metabolites (Cronin, Ventura, Fitzgerald, & van Sinderen, 2011; Macfarlane & Macfarlane, 2003). Other pathways utilizing a variety of substrates are also able to produce SCFAs. Radioisotope analysis by Miller and Wolin (1996) demonstrated that a major pathway of acetate production by bacteria was via the oxygen-sensitive Wood–Ljungdahl pathway and is regarded as the most efficient pathway of acetate production (Fast & Papoutsakis, 2012). Using similar methods they show that propionate was generally generated by a carbon dioxide fixation pathway while butyrate was most commonly formed by conventional acetyl-*S* coenzyme A condensation (Miller & Wolin, 1996). Other pathways, such as the Bifidobacterium pathway (fructose-6-phosphate phosphoketolase pathway) found in the Bifidobacterium genus are able to utilize monosaccharides in a unique manner to ultimately generate SCFAs (Pokusaeva, Fitzgerald, & van Sinderen, 2011). These results suggest that different species possessing specific enzymes are involved in the production of the various SCFAs. Indeed, the Wood–Ljungdahl pathway is typically found in acetate-producing bacteria (known as acetogens) where the majority are of the Firmicutes phylum (Ragsdale & Pierce, 2008). On the other hand, the major groups involved in the production of butyrate are of the Cytophaga and Flavobacterium group belonging to the Bacteroidetes phylum (Guilloteau et al., 2010). Specific species of bacteria characterized by their high levels of butyrate production include *Clostridium leptum*, *Roseburia* species, *Faecalibacterium prausnitzii*, and *Coprococcus* species belonging to both the Firmicutes and Bacteroidetes phyla (Guilloteau et al., 2010).

The production of SCFAs is a highly complex and dynamic process. For example, butyrate and propionate may be degraded into the smaller two carbon chain acetate by sulfate- or nitrate-reducing acetogenic bacteria such as *Acetobacterium*, *Acetogenium*, *Eubacterium*, and *Clostridium* species (Westermann, Ahring, & Mah, 1989). However, increased proportion of butyrate-producing or -consuming species such as *F. prausnitzii* and *Roseburia* species can reverse this process (Duncan et al., 2004). Such interactions can involve the mutualistic production of SCFAs as demonstrated by the cocolonization of *Bacteroides thetaiotaomicron* and *Eubacterium rectale* where acetate produced by *B. thetaiotaomicron* acted as a substrate for butyrate generation by *E. rectale* (Mahowald et al., 2009).

In addition to enzymatic requirements, expression of protein transporters is also imperative for SCFA production. For example, the presence of ATP-binding cassette (ABC) transporters in *Bifidobacterium longum* is crucial for the uptake and transport of substrates, such as fructose, required for acetate production (Davidson & Chen, 2004; Fukuda et al., 2011). Another transporter, the PEP translocation group or the phosphotransferase system (PTS) is able to transport carbohydrates which can be subsequently metabolized to produce SCFAs (Postma, Lengeler, & Jacobson, 1993; Zoetendal et al., 2012). Genomic analysis revealed that Bacteroidetes possesses more polysaccharide-degrading enzymes but less ABC transporters and fewer PTS than the Firmicutes (Mahowald et al., 2009) suggesting that despite having the machinery to produce SCFAs they might not efficiently uptake the substrate necessary for their production. However, Firmicutes may be excellent scavenger of acetate through their ABC transporters and can uptake acetate to produce butyrate and propionate as fermentative by-products. It has therefore been hypothesized that the two predominant phyla could exist in a balance whereby acetate from Bacteroidetes is used to produce butyrate and propionate by Firmicutes (Mahowald et al., 2009). Therefore, the complex and delicate interaction within the microbiota may also control the proportion and levels of SCFAs in the gut lumen. Accordingly, prebiotics (agents favoring the growth of beneficial bacteria) or probiotic (introduction of beneficial bacteria) agents altering such balance may modulate the production of SCFAs.

### 1.1.3 Manipulation of SCFA production via modulation of microbiota

Dietary changes can alter the composition of the gut microbiota in as little as a day (Wanders, Graff, & Judd, 2012) and even minute alteration of dietary factors such as fiber content could shape microbial communities (Donohoe

et al., 2011). The biggest issue presented by a Western diet typically high in fat and digestible saccharides is that nutrients are mostly absorbed in the duodenum leaving very few substrates for the colonic bacteria. Consequently, this results in dysbiosis, the impairment of microbiota composition and increased susceptibility to inflammatory diseases such as inflammatory bowel diseases (IBDs) or colon cancer. On the other hand, in rural areas where the diet is closer to the Paleolithic diet comprising of fruit and vegetables enriched in fibers and RS, the prevalence of these inflammatory diseases is low while SCFA and presence of SCFA-producing bacteria are significantly more elevated (De Filippo et al., 2010). These data aligns with a "diet hypothesis" which suggests that adequate intake of fiber promotes a healthy microbiota that significantly reduces the prevalence of inflammatory diseases, notably through the release of SCFA (Macia et al., 2012; Maslowski & Mackay, 2011). Despite intense public health efforts to promote the beneficial effects of a healthy diet in Western countries, the incidence of obesity and inflammatory diseases are still increasing suggesting that other approaches must be explored. One alternative could be to provide food supplements such as the prebiotic inulin-type fructans, which have been shown to promote Bifidobacteria at the expense of *Roseburia* species and of Clostridium cluster XIVa in mice (Dewulf et al., 2011). The other alternative would be to directly introduce a cocktail of beneficial bacteria including the SCFA producer Bifidobacteria into solution, such as yogurt, similar to how some currently available probiotics products are consumed. One study has shown that gavage of mice with *B. longum* increased the production of acetate (Xiong et al., 2004) and reduced their susceptibility to infection. Another study showed that mice inoculated with VSL#3 (commercial formula containing eight naturally occurring probiotic strains of bacteria) showed protection against acute DSS-induced colitis (Mennigen et al., 2009). This suggests that even if all the mechanisms behind the use of probiotics are not fully understood, such as their rate of survival or site of action, they remain to be a very promising therapeutic strategy.

## 1.2. Transport of SCFAs

As discussed, while the majority of SCFAs are generated and utilized within the vicinity of the gut, a small proportion of propionate and acetate reaches the liver where they can be used as substrates for the energy-producing tricarboxylic acid cycle and efficiently metabolized to produce glucose. A small percent of SCFAs in the gut exists as unionized forms and can directly cross

the epithelial barrier. However, most exists in an ionized state and requires specialized transporters for their uptake. Therefore, the passage of the majority of SCFAs across the mucosa involves active transport mediated by two main receptors: the monocarboxylate transporter 1 (MCT-1) and the sodium-coupled monocarboxylate transporter 1 (SMCT-1) receptors. Both MCT-1 and SMCT-1 are highly expressed on colonocytes and also along the entire gastrointestinal tract including the small intestine and the cecum (Iwanaga, Takebe, Kato, Karaki, & Kuwahara, 2006). Additionally, MCT-1 is also highly expressed on lymphocytes suggesting the importance of intracellular SCFA uptake by these cells (Halestrap & Wilson, 2012). Additionally, SMCT-1 is expressed on the kidney and thyroid gland. SMCT-1 binds SCFAs in order of affinity butyrate > propionate > acetate (Ganapathy, Gopal, Miyauchi, & Prasad, 2005). Unabsorbed SCFAs are excreted.

## 2. SCFA SENSING AND SIGNAL TRANSDUCTION

The ability of SCFAs to modulate biological responses of the host depends on two major mechanisms. The first involves the direct inhibition of histone deacetylases HDACs to directly regulate gene expression. Intrinsic HDAC inhibitor (HDACi) activity is particularly characteristic of the SCFAs butyrate and propionate. The second mechanism for SCFA effects is signaling through G-protein-coupled receptors (GPCRs). The major GPCRs activated by SCFAs are GPR41, GPR43, and GPR109A.

### 2.1. HDAC inhibitors

Acetylation of lysine residues within histones induces gene activation by facilitating the access of transcription factors to promoter regions (MacDonald & Howe, 2009). HDACs remove acetyl groups from histones (Kim & Bae, 2011); as such, inhibition of HDAC activity or expression can increase gene transcription by increasing histone acetylation. SCFAs inhibit HDAC activity, and may therefore alter gene expression in a wide variety of cells.

Of all the SCFAs, butyrate is considered to be the most potent inhibitor of HDAC activity. Indeed, butyrate exhibits a stronger HDAC inhibitory activity than propionate as demonstrated in both HeLa (Boffa, Vidali, Mann, & Allfrey, 1978) and colon cancer cell lines whereas acetate appeared to have very little or no or effect (Hinnebusch, Meng, Wu, Archer, & Hodin, 2002; Kiefer, Beyer-Sehlmeyer, & Pool-Zobel, 2006; Waldecker, Kautenburger, Daumann, Busch, & Schrenk, 2008). However, this lack of effect on HDACs by acetate may be tissue dependent, since others have shown that acetate can inhibit

HDACs. In one study, treatment of hepatoma tissue (Sealy & Chalkley, 1978) with acetate, propionate, or butyrate leads to a global increase in histone acetylation. In the same vein, orally administered acetate has been shown to inhibit both HDAC2 activity and protein expression in the rodent brain (Soliman & Rosenberger, 2011). Thus, HDAC inhibition by SCFAs depends not only on the type of SCFA but also on which tissue or cell type they are acting.

### 2.1.1 Mechanism behind SCFA-mediated HDAC inhibition

While the exact mechanism behind SCFA inhibition of HDACs is not known, SCFAs might either act directly on HDACs by entering into the cells via transporters or indirectly through the activation of GPCRs (see below). Transporters such as SMCT-1 could be good candidates. Indeed, expression of SMCT-1 was required for butyrate- and propionate-induced blockade of murine dendritic cell development, which correlated with a global increase in HDAC inhibition and DNA acetylation (Singh et al., 2010). Thus, the transport of SCFAs into cells via SMCT-1 may account for the observed global inhibition of HDACs by propionate and butyrate and the subsequent blockade of enzymatic activity. Direct inhibitory activity of SCFAs on HDACs has been highlighted by the fact that while one butyrate molecule is a noncompetitive inhibitor that does not interfere with the binding of HDACs to their substrates, two molecules of butyrate may competitively occupy the hydrophobic cleft of the active site of HDACs (Cousens, Gallwitz, & Alberts, 1979). This is similar to the action of the well-characterized HDACi trichostatin A (TSA) (Davie, 2003).

Apart from a direct effect of SCFAs on HDACs, another interesting hypothesis is that they may have an indirect effect through GPCRs. Indeed, activation of GPR41 in Chinese hamster ovary cell lines suppressed histone acetylation possibly through the inhibition of HDACs (Wu, Zhou, Hu, & Dong, 2012). Thus, GPR41 but also GPR43 or GPR109 might contribute to HDAC inhibition mediated by SCFAs. Whether the SCFAs directly or indirectly block HDAC activation remains elusive and extensive research will be necessary to clarify these points.

### 2.1.2 Immunological relevance of SCFA-mediated HDAC inhibition

When SCFA-mediated HDAC inhibition can be established or associated, the overwhelming result is an anti-inflammatory immune phenotype (Table 3.1). Indeed, treatment of human macrophages with 1 mM of acetate *in vitro* significantly reduced their global HDAC activity and increased global histone acetylation correlating with decreased production of inflammatory cytokines IL-6,

IL-8, and TNFα (Kendrick et al., 2010). Similarly, butyrate and propionate decreased LPS-induced TNFα production *in vitro* from human peripheral blood mononuclear cells (PMBCs) in a similar manner to TSA (Usami et al., 2008). These results suggest an active control of the release of proinflammatory cytokines by SCFAs through HDAC inhibition in both rodents and humans. Activation of NF-κB is one of the major pathways involved in the release of inflammatory cytokines (Hayden, West, & Ghosh, 2006). Butyrate and propionate were shown to reduce NF-κB activity in PBMCs in a similar manner to TSA (Usami et al., 2008) suggesting that the anti-inflammatory effect of SCFAs might be mediated through the modulation of NF-κB via HDAC inhibition. However, a direct effect of these SCFAs on histone acetylation in PMBCs has not been shown. Finally, global inhibition of HDAC activity was also observed in rodent neutrophils after addition of acetate, propionate, or butyrate *in vitro* with increasing strength, respectively (Vinolo et al., 2011). In monocytes, butyrate and propionate, but not acetate, decreased LPS-induced TNFα expression and NOS expression in rodent neutrophils (Vinolo et al., 2011). This suggests that acetate might not mediate its anti-inflammatory effects through HDAC inhibition but rather through GPCR activation, as we have reported (Maslowski et al., 2009). Finally, HDAC inhibition by SCFAs is not restricted to cells of the innate immune system. Lymphocytes, in particular regulatory T cells (Tregs), may also be affected by HDAC inhibition. Indeed, HDAC inhibition, particularly HDAC9, increased expression of the forkhead box P3 (Foxp3) transcription factor in mice, which subsequently increased proliferative and functional capabilities of Tregs (Lucas et al., 2009; Tao et al., 2007). *In vitro*, addition of butyrate on human Treg was shown to moderately diminish their proliferation while increased their inhibitory capacities on T cell proliferation through a CTLA-4-mediated mechanism (Akimova et al., 2010). Furthermore, effector $CD4^+$ T cells could be anergized via the HDACi activities of butyrate, which occurred independently of Treg (Fontenelle & Gilbert, 2012). Although global HDAC activity is often associated with SCFA-mediated immunomodulation, specific HDAC inhibition or expression is rarely investigated and provides an avenue for further research.

## 2.2. G-protein-coupled receptors

### *2.2.1 GPR43*

GPR43, also known as free fatty acid receptor 2 (FFA2/FFAR2), is the primary receptor for the SCFA acetate. GPR43 recognizes an extensive range of SCFAs including propionate, butyrate, caproate, and valerate and while propionate was reported to be the most potent activator of GPR43, acetate is the

**Table 3.1** HDAC specific immunomodulation of the immune system

| HDAC No. | Immunological function | References |
|---|---|---|
| HDAC1 | • Reduces TNF-induced NF-κB-dependent reporter gene expression via direct interaction with corepressor p65 and p50<br>• Repression of IL-12 expression<br>• Increases expression of NF-κB-independent genes | Ashburner, Westerheide, and Baldwin (2001), Zhong, May, Jimi, and Ghosh (2002), Viatour et al. (2003), and Lu et al. (2005) |
| HDAC2 | • Reduces TNF-induced NF-κB-dependent reporter gene expression independent of interaction with p65<br>• Repression of major histocompatibility class II transactivator (CIITA) activity and subsequent repression of activation in macrophages | Ashburner et al. (2001) and Kong, Fang, Li, Fang, and Xu (2009) |
| HDAC3 | • Repression of NF-κB signaling by sequestration of NF-κB to the cytoplasm<br>• Increases expression of NF-κB-independent genes<br>• Required for inflammatory gene expression in macrophages<br>• Increased HDAC3 is associated with reduced apoptotic T lymphocytes from a reduction in p53 expression (tumor suppressor) | Chen, Fischle, Verdin, and Greene (2001), Viatour et al. (2003), and Zhang, Shi, Wang, and Sriram (2011) |
| HDAC7 | • Transcriptionally represses macrophage genes during B cell development<br>• Enhances Foxp3 function<br>• Histone deacetylation of the Foxp3 promoter | Bruna Barneda-Zahonero et al. (2013), Li et al. (2007), and Lal and Bromberg (2009) |
| HDAC8 | • Induces apoptosis of T cell lymphoma dependent on phospholipase C-γ1 signaling | Balasubramanian et al. (2008) |
| HDAC9 | • Inhibits proliferation and suppressive function of Tregs and is downregulated during TCR stimulation of Tregs<br>• HDAC9 knock-out mice have increased numbers of Tregs compared to WT. | Tao et al. (2007) |
| HDAC11 | • Regulates IL-10 expression from APCs<br>• Increasing HDAC11 caused an increase in IL-10 and promoted the restoration of responsiveness in tolerant $CD4^+$ T cells<br>• Reducing HDAC11 increased IL-10 expression in APCs and impaired antigen-specific T cell responses | Villagra et al. (2009) |

most selective (Le Poul et al., 2003). GPR43 expression has been identified along the entire gastrointestinal tract, including cells of both the immune and nervous system. In the intestinal tract, GPR43 is highly expressed on intestinal peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Tolhurst et al., 2012) producing endocrine L-cells of the ileum and colon (Vangaveti, Shashidhar, Jarrod, Baune, & Kennedy, 2010) as well as on colonocytes and enterocytes of the small and large intestine. Direct infusion of SCFAs in the colon of rats and rabbits induced the release of PYY, possibly through their binding on GPR43, that exerted anorexigenic effects (Roelofsen, Priebe, & Vonk, 2010) and GPR43 knock-out (*Gpr43*$^{-/-}$) mice have decreased SCFA-induced release of GLP-1, a key hormone controlling insulin release (Tolhurst et al., 2012). While SCFAs might modulate body weight via central effects by reducing food intake through secretion of PYY and GLP-1, they can also directly act in periphery on the adipose tissue. Indeed, high fat diet has been shown to upregulate GPR43 expression in subcutaneous adipose tissue in parallel with increased fat storage in adipocytes. On the other hand, supplementation of the diet with inulin-type fructans, fermentable carbohydrates, blunted the weight gain and the overexpression of GPR43 due to high fat feeding, suggesting that SCFAs might modulate adiposity (Dewulf et al., 2011). Moreover, inhibition of GPR43 expression in the adipocyte cell line 3T3-L1 using small interfering RNA inhibited their differentiation suggesting a possible role of GPR43 in adipocyte development (Dewulf et al., 2013). While RS consumption in rats leads to activation of the hypothalamic anorexigenic pathway shown by the increased expression of proopiomelanocortin in the arcuate nucleus, GPR43 does not seem to be expressed in the arcuate nucleus or other region of the hypothalamus (Sleeth, Thompson, Ford, Zac-Varghese, & Frost, 2010). More broadly, to our knowledge, there is no report of GPR43 expression in the central or peripheral nervous system.

In the immune system, GPR43 is expressed on eosinophils, basophils (Le Poul et al., 2003), neutrophils, monocytes, dendritic cells (Cox et al., 2009; Le Poul et al., 2003), and mucosal mast cells (Karaki et al., 2008) suggesting a broad role of SCFAs in immune responses. It is highly expressed in murine hemopoietic tissues such as the bone marrow and spleen suggesting the potential role for GPR43 in modulating the development or differentiation of immune cells (Maslowski et al., 2009; Senga et al., 2003).

Finally, a recent study has shown the expression of GPR43 in myometrium and fetal membranes after the onset of labor and a significant upregulation of GPR43 in preterm fetal membranes with evidence of

infection. This study also suggests an anti-inflammatory role of SCFAs through GPR43 that may reduce the risk of preterm labor induced by pathogens (Voltolini et al., 2012). This anti-inflammatory role of GPR43 is in accordance with our findings on the exacerbated inflammatory phenotypes of *Gpr43*$^{-/-}$ mice in colitis and arthritis models (Maslowski et al., 2009).

### 2.2.2 GPR41

Identified at the same time as GPR43, GPR41, also known as free fatty acid receptor 3 (FFA3/FFAR3), is a receptor for acetate and propionate and to a lesser degree butyrate. Like GPR43, it also recognizes other SCFAs including caproate and valerate, but to a lesser degree. GPR41 is expressed in the colonic mucosa in PYY but not GPR43-expressing cells. GPR41 is also expressed in the colonic smooth muscle and SCFAs induce phasic contraction of these muscles in a GPR41-dependent manner with the following order of potency: propionate $\geq$ butyrate $>$ acetate (Tazoe et al., 2009).

SCFAs stimulate sympathetic activation through GPR41 activation by acting on the sympathetic ganglion. This effect is abolished under fasted conditions by ketone bodies (Kimura et al., 2011). Based on these results, GPR41 agonists could be used as potential antiobesity therapeutics.

Moreover, the expression of GPR41 in adipose tissue and its potency to induce the release of the anorexigenic hormone leptin when activated by SCFAs confirms its beneficial effects on body weight (Xiong et al., 2004). The former findings are still controversial as Hong and colleagues did not find GPR41 expression on adipocytes and suggest that this effect on leptin release is mediated through GPR43. Langerhans cells in the pancreas also express GPR41 but its functional role in these cells is unknown. Finally, GPR41 is expressed in spleen and in PBMC but its role on immune cells remains uninvestigated.

### 2.2.3 GPR109A

GPR109a, also known as Niacin receptor 1, is a high affinity niacin (Vitamin B3) receptor and related to its low affinity analog GPR109B, which is only expressed in humans. Although niacin is the primary ligand of GPR109A, physiological concentrations of niacin do not reach a threshold required to activate the receptor (Wanders et al., 2012). However, butyrate is a suitable candidate ligand with the ability to bind GPR109A with low affinity in millimolar concentration (Thangaraju et al., 2009). GPR109A transcript is highly expressed in adipocytes but declines with age (Thangaraju et al., 2009). To a lesser extent, GPR109A is also expressed on immune cells such as dermal dendritic cells, monocytes, macrophages, and neutrophils (Wanders et al., 2012).

Activation of GPR109A in adipocytes has been shown to suppress lipolysis and lowering of plasma-free fatty acid levels (Kang, Kim, & Youn, 2011). The role of GPR109A in immune responses, and gut homeostasis, is yet to be reported. A summary of the major SCFA receptors, associated ligand, and their functions is presented in Table 3.2.

## 3. VARIED FUNCTIONS OF SCFAs

SCFAs, particularly butyrate, are key promoters of colonic heath and integrity. Butyrate is the major and preferred metabolic substrate for colonocytes providing at least 60–70% of their energy requirements necessary for their proliferation and differentiation (Suzuki et al., 2008). As such, colonocytes of germ-free mice, deficient in SCFAs, are highly energy deprived, as indicated by decreased expression of key enzymes involved in fatty acid metabolism in mitochondria (Tazoe et al., 2008). Consequently, these cells have a marked deficit of mitochondrial respiration, as shown by a decreased $NADH/NAD^+$ ratio, in ATP production as well as of oxidative phosphorylation, which can lead to autophagy. Addition of butyrate to colonocytes isolated from germ-free mice normalized this deficit (Donohoe et al., 2011). Apart from being a major energy source for colonocytes, SCFAs in the gut perform various physiological functions including dictating colonic mobility, colonic blood flow, and gastrointestinal pH, which can influence uptake and absorption of electrolytes and nutrients (Tazoe et al., 2008). These effects could be mediated through the activation of GPCRs as discussed earlier.

Finally, the physiological roles of SCFAs are broader than a local effect on the gut on enterocytes and on digestive function; they indeed play major immunological roles both systemically and locally in the gut that will be further expanded in the following sections.

### 3.1. Anti-inflammatory and antitumorigenic roles

SCFAs are well known for their anti-inflammatory functions by modulating immune cell chemotaxis, reactive oxygen species (ROS) release as well as cytokine release. Butyrate elicits anti-inflammatory effects via inhibition of IL-12 and upregulation of IL-10 production in human monocytes (Saemann et al., 2000), repressing production of proinflammatory molecules TNFα, IL-1β, nitric oxide (Ni et al., 2010), and reduction of NF-κB activity (Ni et al., 2010; Segain et al., 2000). The active suppression of NF-κB activity was shown by all three major SCFAs in order of potency being butyrate > propionate > acetate in Colo320DM cells (Tedelind, Westberg,

**Table 3.2** Summary of the major short-chain fatty acids-activated GPCR including its ligand, expression, and function

| GPCR | Ligands | Expression | Roles | Reference(s) |
|---|---|---|---|---|
| GPR41 | SCFA (C2–C7) Formate, acetate, propionate, butyrate, and pentanoate | Adipocytes, various immune cells, and enteroendocrine L cells | Leptin production, sympathetic activation | Kimura et al. (2011) and Xiong et al. (2004) |
| GPR43 | SCFA (C2–C7) Formate, acetate, propionate, butyrate, and pentanoate | Adipocytes, various Immune cells, enteroendocrine L cells, gut epithelium, fetal membrane | Anorexigenic effects via secretion of PYY and GLP-1, anti-inflammatory, and antitumorigenic | Cherbut et al. (1998), Maslowski et al. (2009), Tang, Chen, Jiang, Robbins, and Nie (2011), Suzuki, Yoshida, and Hara (2008), Tazoe et al. (2008), Le Poul et al. (2003), Cox et al. (2009), and Voltolini et al. (2012) |
| GPR109a | SCFAs (C4–C8), particularly butyrate Nicotinate | Adipocytes, various immune cells, intestinal epithelial cells, upregulated in hepatocytes during inflammation, epidermis in squamous carcinoma | High-density lipoprotein metabolism, cAMP reduction in adipocytes, DC trafficking, anti-inflammatory, and antitumorigenic | Li, Hatch, et al. 2010, Li, Millar, Brownell, Briand, and Rader (2010), Bermudez et al. (2011), Ingersoll et al. (2012), Thangaraju et al. (2009), and Wanders et al. (2012) |

Kjerrulf, & Vidal, 2007). Suppression of NF-κB activity and also TNFα production by SCFAs is also commonly observed in LPS-activated PMBCs such as neutrophils (Aoyama, Kotani, & Usami, 2010). This is consistent with the findings that butyrate could inhibit high mobility group box-1 (Aoyama et al., 2010), a nuclear transcription factor downstream of NF-κB signaling involved in eliciting inflammatory roles and promoting cellular proliferation that could promote cancer (Tang, Kang, Zeh Iii, & Lotze, 2010). Furthermore, butyrate (and also propionate) could induce apoptosis of neutrophils in nonactivated and LPS- or TNFα-activated neutrophil apoptosis by caspase-8 and caspase-9 pathways (Aoyama et al., 2010).

Under inflammatory conditions, addition of acetate has been shown to inhibit human neutrophil migration toward C5a or fMLP in a GPR43-dependent manner as phenylacetamide, a human GPR43 agonist mimicked these effects (Vinolo et al., 2011). *In vivo*, migration of neutrophils toward the peritoneum was exacerbated in *Gpr43*$^{-/-}$ mice when mice were challenged with C5a or fMLP, confirming the critical role of GPR43 as regulator of cell chemotaxis. It is, however, puzzling that under noninflammatory conditions, SCFAs attract both mouse and human neutrophils through a mechanism involving GPR43 activation (Le Poul et al., 2003; Maslowski et al., 2009; Vinolo et al., 2009). This illustrates the dual effects of SCFAs on chemotaxis and the phenomenon that SCFAs might attract inflammatory cells under basal conditions requires further investigation. SCFAs can enforce the epithelial barrier by affecting the mucus layer, epithelial cell survival, as well as tight junction proteins, and will be discussed in a later section of this chapter. SCFAs might enforce this epithelial barrier by increasing the infiltration of immune cells in the lamina propria. The most common immune mechanism known to induce content leakage from the gut is through the release of neutralizing IgA; however, the increase in phagocytes in the lamina propria might also be an important unexplored mechanism. Other than suppressing neutrophil functions, butyrate (and to a degree acetate and propionate) can inhibit IL-2 production and lymphocyte proliferation in culture (Cavaglieri et al., 2003).

SCFAs not only modulate cell migration but also their activity. As discussed earlier SCFAs are potent anti-inflammatory mediators, by inhibiting the release of proinflammatory cytokines from macrophages and neutrophils. Acetate was shown to promote the release of ROS when added on mouse neutrophils by activating GPR43 (Maslowski et al., 2009). ROS are efficient bactericidal factors involved in the clearance of pathogens. Thus, SCFAs might be key regulators of inflammatory diseases by tightly controlling the migration of immune cells toward inflammatory sites as well

as modulating their activation state, enabling accelerated pathogen clearance through ROS activation. As discussed earlier, all these processes would decrease host injury, which would not only allow for the survival of the host but also for survival of the SCFA-producing bacteria.

Butyrate has been associated with anticancer activity on a variety of human cancer cell lines. Treatment of human hepatoma cells *in vitro* increased expression of cell cycle inhibitory genes and appeared to reverse malignant phenotype, which has been associated with a reduction in telomerase activity via HDAC inhibition (Nakamura et al., 2001; Wakabayashi et al., 2005). Telomerase activity can maintain cancer cell proliferation, thereby providing a possible target for butyrate-induced antitumor effects. Furthermore, activation of GPR109a on human colon cancer cells by butyrate has been associated with increased apoptosis independent of HDAC inhibition and increased expression of the butyrate transporter MCT-1 (Borthakur et al., 2012; Thangaraju et al., 2009). Butyrate-induced GPR109a activation may directly inhibit colon cancer growth by inducing apoptosis or may act indirectly via increased MCT-1 expression and subsequent increase of butyrate transport into the cell. Expression of the butyrate transporter SMCT-1 on colon cancer cells is essential for its antitumorigenic function and correlates with global increases to histone acetylation (Gupta, Martin, Prasad, & Ganapathy, 2006). In addition, SMCT-1 is downregulated in human colon cancer cells, further accentuating the role of SMCT-1 in colon cancer (Miyauchi, Gopal, Fei, & Ganapathy, 2004). SMCT-1 may therefore transport butyrate into colonic cells and prevent development of a cancerous phenotype, though the involvement of HDAC inhibition remains largely unknown. Even if the mechanisms behind the beneficial role of SCFAs on cancer are not fully understood, it is widely accepted that intake of fiber lowers risk of cancer, especially colorectal cancer. The analysis of 25 studies demonstrated that cereals and whole grain intake was associated with reduced risk of colorectal cancer supporting the potential beneficial role of SCFAs in cancer (Aune et al., 2011).

## 3.2. SCFAs and antimicrobial activities

Free fatty acids (such as medium- and short-chain fatty acids) exhibit intrinsic broad-spectrum antimicrobial activity and are used as such in the agriculture industry. For example, propionate is routinely used as an antimicrobial additive in food (Arora, Sharma, & Frost, 2011) while *in vivo* administration of butyrate is used to control Salmonella infections (Fernandez-Rubio et al., 2009). Several key mechanisms were attributed to the antimicrobial

activities of free fatty acids including disruption of osmotic and pH balance, nutrient uptake, and energy generation and their working concentrations were well below the toxicity threshold to host cells (Dewulf et al., 2011). This was shown in a study by Hong et al. (2005) demonstrating that formic acid, acetate, propionate, butyrate, and hexanoic acid exerted various biocidal (lethal) or biostatic (growth inhibitory) effects on oral microorganisms at concentrations as low as micromolar. Propionate and hexanoic acid can also exert antimicrobial activities by promoting host antimicrobial peptide expression (Alva-Murillo, Ochoa-Zarzosa, & Lopez-Meza, 2012). Similarly, host defense peptides of the innate immune system were potently induced by oral treatment of butyrate and were responsible for the clearance of *Salmonella enteritidis* without triggering a proinflammatory response indicated by a lack of IL-1β production (Sunkara, Jiang, & Zhang, 2012). In humans, the activity of cathelicidin, an antimicrobial agent released by polymorphonuclear leukocytes was induced by butyrate, possibly via its HDAC inhibitory activities (Kida, Shimizu, & Kuwano, 2006). A recent study has shown that the antimicrobial activities of individual SCFAs were relatively inert toward species of bacteria that produced them but were otherwise potent toward other microorganisms (Alva-Murillo et al., 2012). Therefore, the production of SCFAs themselves may play a significant role in the shaping of the gut microbial ecology; however, the precise effects of SCFAs on bacterial selection require further investigation.

### 3.3. SCFAs and gut integrity

Gut integrity is an essential factor in maintaining mucosal homeostasis. It is ensured by an efficient separation between the gut luminal contents and the host, which is partly due to an effective epithelial barrier. Disruption of gut integrity has been attributed to various intestinal diseases such as inflammatory bowel disease, celiac diseases, irritable bowel syndrome (Voltolini et al., 2012), and colorectal cancer (Tolhurst et al., 2012). It is interesting to note that alteration of gut integrity seems to have much broader health implications than locally in the gut. Indeed, a phenomenon called "leaky gut," characterized by increased gut permeability, is associated with diseases such as asthma or type 1 diabetes (T1D) showing that an effective physical separation of host tissues from the gut microbiota is critical for general health.

A layer of mucus forms a physical barrier that separates the epithelium from the luminal environment, and this contributes to gut integrity by limiting physical access of bacteria to the epithelium, thus limiting prospects for

breach and inflammation (Tolhurst et al., 2012). Mucus is comprised of secretory (MUC2, MUC5A/B, MUC6) and epithelial membrane-bound (MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, and MUC17) mucin glycoproteins (Cherbut et al., 1998; Tolhurst et al., 2012). Deficiencies in mucins exacerbate various intestinal diseases such as mucositis but can be remediated via oral supplementation of butyrate, which modulates gut permeability (Ferreira et al., 2012). Consistent with this, supplementation of either butyrate or propionate could induce both MUC2 mRNA expression and MUC2 secretion in human goblet-like cell line LS174T (Burger-van Paassen et al., 2009) suggesting that SCFAs might be critical bacterial products promoting gut integrity. However, whether the mechanisms behind these effects are through HDAC inhibition or via the stimulation of GPR41, GPR43, or GPR109 has not been elucidated.

Functional tight junction proteins, such as ZO-1 and occludin between epithelial cells, are also required for maintaining gut integrity by limiting gut permeability (Balda & Matter, 2008). As mentioned earlier, increased gut permeability is a common feature in diseases such as food allergy and asthma (Hijazi et al., 2004; Perrier & Corthesy, 2011), however, whether it is the cause or the consequence of these diseases remains largely unresolved. *In vitro*, butyrate supplementation to Caco-2 cell monolayers enhances the transepithelial resistance (TER), which is a marker of gut integrity, by accelerating the assembly of tight junction proteins ZO-1 and occludin dependent on AMPK activation without altering their expression levels (Tolhurst et al., 2012). *In vivo*, mice treated with *B. longum*, a probiotic strain of bacteria that releases large amounts of acetate, decreased the translocation of Shiga toxin from enterohemorrhagic *Escherichia coli* O157:H7 toward the bloodstream and thus increased survival (Xiong et al., 2004). *In vitro*, this study shows that while acetate *per se* did not affect the TER of Caco-2 cells, it did increase their survival when they were coinfected with this pathogen resulting in increased gut integrity.

Finally, it has been shown in numerous studies that obesity or inflammatory bowel disease, that dysbiosis is associated with increased gut permeability. These conditions are probably associated with much lower concentrations of SCFAs in both the GI tract and the blood. Apart from acting on the epithelial layer, SCFAs might promote gut integrity by maintaining symbiosis. Indeed, by lowering the luminal pH, SCFAs can directly promote the growth of symbionts, and on the other hand inhibit growth of pathobionts (Roy, Kien, Bouthillier, & Levy, 2006). However, some opportunistic pathobionts have evolved to take advantage of the presence of SCFAs. Indeed it has been

shown that butyrate promotes virulence gene factor expression in pathogenic *E. coli* and thus, colonize the colon where levels of butyrate are the highest (Nakanishi et al., 2009). Furthermore, SCFAs (particularly butyrate) could also induce the production of flagella and regulate its motility function in enterohemorrhagic *E. coli* (Herold, Paton, Srimanote, & Paton, 2009; Tobe, Nakanishi, & Sugimoto, 2011).

From an evolutionary point of view, it is not surprising that beneficial bacteria protect the host, notably by maintaining gut homeostasis to ensure their own survival. Our view is that vertebrates have evolved systems that allow bacterial metabolites such as SCFAs to regulate immunity and gut physiology. Expression of GPR43 on innate/inflammatory immune cells and the gut epithelium is an excellent example of this relationship. In Western countries where consumption of dietary fiber is low, boosting the levels of SCFAs appears as a promising new approach to promote gut integrity and homeostasis. SCFAs or HDAC/GPR43 agonists might find uses to treat or prevent a broad range of diseases from cancers to allergies and autoimmune diseases.

## 4. INTEGRATIVE VIEW OF THE GUT MICROBIOTA, SCFAs, AND DISEASE

The incidence of both inflammatory and autoimmune diseases has increased dramatically in Westernized countries over the past several decades. While both genetic and environmental factors influence the induction of such diseases, the contribution of diet and the relevance of SCFAs have only been appreciated recently. The effect of SCFAs on various inflammatory and autoimmune diseases will be discussed below.

IBDs such as Crohn's disease (CD) and ulcerative colitis (UC) are characterized by inflammation of the gastrointestinal tract and colonic mucosa. The induction of IBDs is multifactorial with genetic, environmental, and microbial components. The increased incidence of IBD in developed countries over the last 20 years is too rapid to be explained by genetic changes. However, what has dramatically changed over the last 20 years is the lifestyle, particularly the introduction of Western style diets, which are generally low in fiber, and rich in fat and digestible sugars. Thus, "Western" diets could be driving this increase of IBD in Western countries (Shapira, Agmon-Levin, & Shoenfeld, 2010).

As mentioned previously, changes in diet can lead to rapid changes in the composition of gut microbiota, which in turn could influence the relative

amounts of the different SCFAs produced. Observations in both mice and humans support the link between diet, SCFA production via the gut microbiota, and IBDs. Indeed, metagenomic analyses of fecal bacteria have shown significant dysbiosis in patients suffering from CD or UC, where there is a lower representation of Bacteroidetes and Firmicutes, typical commensal bacterial species, especially Clostridial clusters IV (*C. leptum* subgroup) and XIVa (*Clostridium coccoides* subgroup) compared to healthy individuals (Frank et al., 2007). Whether this dysbiosis is causative or a consequence of IBD is unknown, however, targeting the microbiota through antibiotic treatments has shown promising results by decreasing bacterial infiltration to tissues. Combined treatment with probiotics and prebiotics also appears beneficial in IBD, however, the use of anti-, pro-, and prebiotics as treatments for IBD is yet to be fully established (Sartor, 2004). An emerging and promising therapeutic approach is fecal transplantation, which has been highly successful in some *Clostridium difficile*-infected patients (Borody, Brandt, Paramsothy, & Agrawal, 2013; Brandt, 2012), as well as some UC patients (Damman, Miller, Surawicz, & Zisman, 2012).

In mouse models, the role of the microbiota in the development of DSS-induced colitis, a mouse model of ulcerative colitis, has been demonstrated. While under SPF conditions, IL-10-deficient mice developed exacerbated colitis, whereas they were protected under germ-free conditions (Sellon et al., 1998). These results suggest that IL-10-deficient mice have a colitogenic microbiota. Although it has not been shown in humans, we can speculate that patients with IBD may also house a colitogenic microbiota that if transmitted from mother to child at birth may confer susceptibility to CD (Akolkar et al., 1997).

Interestingly, in parallel with the dysbiosis, two studies have shown that IBD was correlated with lower levels of SCFAs in feces by nuclear magnetic resonance spectroscopy (Marchesi et al., 2007) and by HPLC with acetate (162.0 μM/g), propionate (65.6 μM/g), and butyrate (86.9 μM/g) in the feces of IBD patients compared to healthy individuals (209.7, 93.3, and 176.0 μM/g, respectively) (Huda-Faujan et al., 2010). Given these differences, SCFAs may play an important role in the pathogenesis of IBD. However, the stage of these diseases at which SCFAs are lowered, before the first signs of inflammation, early signs, or once the diseases are clearly established, remains unknown. Sabatino et al. (2005) explored the therapeutic effect of administering butyrate orally to patients with CD. Administration of 4 g of butyrate per day for 8 weeks via an enteric-coated tablet induced clinical improvement and remission in 53% of patients where butyrate successfully

downregulated mucosal levels of NF-κB and IL-1β. Mouse studies have also shown that SCFAs were beneficial in colitis as mice treated with butyrate had reduced inflammation in their colonic mucosa with reduced neutrophil infiltration (Vieira et al., 2012) and treatment with acetate had similar beneficial effects (Maslowski et al., 2009). Moreover, lack of SCFA signaling through GPR43 in *Gpr43*$^{-/-}$ mice exacerbated the development of colitis (Maslowski et al., 2009). Thus, normalizing levels of SCFAs as well as remediating dysbiosis may have synergistic and beneficial effects in the treatment of IBD.

The beneficial anti-inflammatory effects of SCFAs extend beyond the gut. Indeed, Brown et al. (2011) completed a metagenomic analysis of the gut microbiome of T1D matched case–control subjects. 16S rRNA sequencing revealed a larger proportion of bacterial species producing butyrate in controls compared to individuals suffering from T1D. This confirms the notion that in healthy individuals, the presence of butyrate-producing bacteria might maintain gut integrity, while in T1D patients, nonbutyrate-producing bacteria impede the synthesis of mucin, which could lead to increased gut permeability. In rats, oral treatment with butyrate during the preweaning period tended to delay the development of diabetes (Li, Hatch, et al., 2010) suggesting that butyrate might play a role. In this study, only one dose of butyrate was investigated, thus alternative dosing strategies and perhaps in combination with other SCFAs such as acetate would be necessary to draw firmer conclusions about the effects of SCFAs on diabetes development. Moreover, analysis of fecal microbiota revealed that *Myd*88$^{-/-}$ NOD mice, which are protected from diabetes development under SPF conditions, had an increase in Bacteroidetes species when housed under SPF conditions (Wen et al., 2008). Bacteroidetes produce large amounts of SCFAs, thus protection from T1D in these *Myd*88$^{-/-}$ NOD mice under SPF conditions could be via the anti-inflammatory effects provided by SCFAs. Similarly, fecal microbiota of patients suffering from rheumatoid arthritis (RA), another autoimmune disease, revealed that RA patients had significantly less Bifidobacteria and Bacteroides species compared to patients suffering from fibromyalgia, a noninflammatory musculoskeletal disease (Vaahtovuo, Munukka, Korkeamaki, Luukkainen, & Toivanen, 2008). Thus, low levels of SCFAs might contribute or result from the development of RA; however, prospective studies that assess the production of SCFAs in RA patients as well as other inflammatory diseases would be of great interest to determine if a defect in SCFA levels contributes to disease onset.

Finally, Böttcher et al. (2000) compared the production of SCFAs in allergic and nonallergic children and found that allergic infants had lower levels of propionate, acetate, and butyrate in their feces compared to nonallergic individuals. This may account for the observation that *Gpr43*$^{-/-}$ mice exhibit exacerbated development of allergic airway inflammation (Maslowski et al., 2009). These results suggest that SCFAs might play a protective role in allergic disease. This would support a diet/fiber deficiency model (Maslowski et al., 2009) for the increase in inflammatory diseases in Western countries.

## 5. PERSPECTIVE

The incidence of autoimmunity, IBD, and allergy has increased dramatically in Western and Westernized countries. This increase parallels a decrease in the consumption of fiber and indigestible starches. Carefully designed studies are now required to evaluate the effect of diet, independent of other possible contributing factors (i.e., hygiene, infection, sunlight, etc.). These studies will be critical for determining the role of diet, particularly fiber and SCFAs, in the development of Western diseases. If indeed diet and the resulting changes to the gut microbiota underlie certain Western lifestyle diseases, then there is enormous potential for prevention or correction through diet, probiotics, or new drugs targeting metabolite sensing mechanisms such as HDACs or GPCRs.

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CHAPTER FOUR

# Combined Immunodeficiencies with Nonfunctional T Lymphocytes

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## Contents



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## Abstract

Immunodeficiencies with nonfunctional T cells comprise a heterogeneous group of conditions characterized by altered function of T lymphocytes in spite of largely preserved T cell development. Some of these forms are due to hypomorphic mutations in genes causing severe combined immunodeficiency. More recently, advances in human genome sequencing have facilitated the identification of novel genetic defects that do not affect T cell development, but alter T cell function and homeostasis. Along with increased susceptibility to infections, these conditions are characterized by autoimmunity and higher risk of malignancies. The study of these diseases, and of corresponding animal models, has provided fundamental insights on the mechanisms that govern immune homeostasis.

## 1. INTRODUCTION

Human primary immunodeficiencies (PIDs) comprise over 170 different genetic disorders, most of which are due to single gene defects (Al-Herz et al., 2011). Classification of PID is based on the nature of the immune defect, and this has important consequences on clinical manifestations of the disease. In particular, genetic abnormalities that compromise the development of T lymphocytes are responsible for severe combined immunodeficiency (SCID), a heterogeneous group of conditions characterized by susceptibility to severe, early-onset infections and failure to thrive (van der Burg & Gennery, 2011). Lymphopenia, with lack of circulating T lymphocytes and absence of T cell function, is the immunological hallmark of SCID and accounts for increased susceptibility to life-threatening viral and opportunistic infections. Moreover, specific antibody production is also severely impaired, either because of concurrent B cell defects or because of lack of helper T cells. Consequently, patients with SCID are also highly susceptible to bacterial infections. Because of the extreme susceptibility to infections, SCID is rapidly fatal, unless treated by hematopoietic cell transplantation (HCT) (Gennery et al., 2010) or, in selected cases, by enzyme replacement therapy (Gaspar, 2010) or gene therapy (Cavazzana-Calvo, Fischer, Hacein-Bey-Abina, & Aiuti, 2012).

More recently, a growing number of conditions have been identified, in which circulating T lymphocytes are detected, but their function is compromised. In addition to increased susceptibility to infections, patients affected with these conditions present a broader spectrum of clinical manifestations, including autoimmunity, inflammatory disease, lymphoproliferation,

and increased risk of malignancies (Felgentreff et al., 2011). The study of the genetic basis of these disorders has revealed that in some case, the disease reflects mutations of genes that play an important, but not critical, role in T cell development and/or function. In other cases, hypomorphic mutations in SCID-causing genes have been identified. The molecular, immunological, and clinical features of these disorders are the focus of this review.

## 2. IMMUNODEFICIENCY PHENOTYPES DUE TO HYPOMORPHIC MUTATIONS IN SCID-ASSOCIATED GENES

In spite of genetic heterogeneity, SCID is characterized by extreme reduction in the number of circulating T cells and absence of their function, reflecting the essential role played by SCID-associated genes in T cell development. However, in the last 15 years, several studies have shown that hypomorphic mutations in the same genes, allowing residual expression and function of the encoded protein, are often partially permissive for T cell development (Felgentreff et al., 2011; van der Burg & Gennery, 2011). Accordingly, patients with such mutations may have a detectable number of circulating T lymphocytes. Moreover, their function is defective, but not completely abrogated. These abnormalities are responsible for impaired immune homeostasis and the frequent occurrence of clinical manifestations of immune dysregulation.

### 2.1. Omenn syndrome: Hypomorphic mutations affecting thymic lymphostromal cross-talk and impairing immune tolerance

Omenn syndrome (OS) is the prototype of phenotypic manifestations of immune dysregulation due to hypomorphic mutations in SCID-causing genes. Patients with OS present in the first weeks of life with diffuse erythroderma, lymphadenopathy, hepatosplenomegaly, eosinophilia, and elevated serum IgE (Villa, Notarangelo, & Roifman, 2008). Circulating T cells are present in variable number; importantly, they are oligoclonal, have an activated phenotype, and infiltrate peripheral tissues causing extensive tissue damage (de Saint-Basile et al., 1991; Signorini et al., 1999). T cells in patients with OS are of autologous origin, allowing distinction from Omenn-like disease which represents a manifestation of graft-versus-host disease due to maternal T cell engraftment in SCID infants (Denianke, Frieden, Cowan, Williams, & McCalmont, 2001; Palmer et al., 2007).

Studies in patients and in suitable animal models have demonstrated that the immune dysregulation of OS reflects severe impairment of the mechanisms of immune tolerance (Marrella, Maina, & Villa, 2011). In addition to permitting generation of a diversified repertoire of T lymphocytes, the thymus serves an essential function also in controlling development and function of self-reactive T cells, which are generated because of the stochastic nature of V(D)J rearrangement. Extensive cross-talk between thymocytes and thymic stromal cells plays a critical role in this process (Akiyama et al., 2008; Hikosaka et al., 2008; Hollander et al., 1995; Irla et al., 2008; van Ewijk, Hollander, Terhorst, & Wang, 2000). In particular, a subset of mature medullary thymic epithelial cells (mTECs) express autoimmune regulator (AIRE), a transcription factor that induces transcription of a number of tissue-restricted genes (Anderson et al., 2002). As a result of this process, tissue-restricted antigens are expressed in association with major histocompatibility class II (MHC-II) molecules on the surface of mTECs and are presented to nascent mature thymocytes that traffic through the medulla. Moreover, tissue-restricted peptides can be also cross-presented by thymic dendritic cells (DCs) (Hubert et al., 2011). Recognition of these peptides by antigen-specific T cells induces clonal deletion of newly generated self-reactive T lymphocytes (Fig. 4.1). Although initial expression of AIRE in the thymus is not controlled by TCR$\alpha\beta^+$ T lymphocytes (White et al., 2008), there is substantial evidence that at later stages during development it is maintained by NF-κB signaling in response to T cell-mediated activation (Desanti et al., 2012; Roberts et al., 2012). Analysis of thymic biopsies from patients with hypomorphic mutations in SCID-associated genes that reduce (without abrogating) T cell development has shown poor maturation of TECs and markedly impaired expression of AIRE (Poliani et al., 2009). Similarly, reduced expression of AIRE and of tissue-restricted antigens has been described in knock-in animal models of OS with homozygous missense mutations in *Rag1* (Rucci et al., 2011) and *Rag2* (Marrella et al., 2007). Overall, these data suggest that deletion of self-reactive T cells is impaired in patients with OS and may account for immune dysregulation.

Moreover, in the normal thymus, mTECs and thymic DCs participate also at the conversion of self-reactive T lymphocytes into natural regulatory T (nTreg) cells (Watanabe et al., 2005; Fig. 4.1), which are exported to the periphery and contribute to immune tolerance by suppressing autoimmune responses. Impaired generation of T lymphocytes in patients with OS is associated with profound abnormalities of thymic architecture, defective

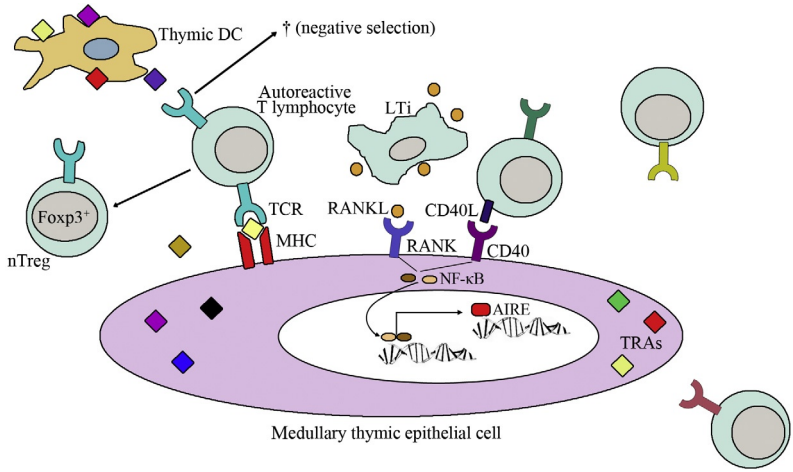


**Figure 4.1** Mechanisms of central T cell tolerance. Schematic representation of the mechanisms controlling central tolerance in the thymus. RANK ligand (secreted by lymphoid tissue induced [LTi] cells at early stages during thymic development) and CD40 ligand (CD40L, expressed by nascent thymocytes) induce activation of NF-κB in medullary thymic epithelial cells and allow expression of the transcription factor autoimmune regulator (AIRE). AIRE promotes expression of tissue-restricted antigens (TRAs), which are presented in association with major histocompatibility complex class II (MHC-II) molecules on the surface of medullary thymic epithelial cells or of thymic dendritic cells (DC). Recognition of TRAs leads to clonal deletion of self-reactive T cells or to their conversion into natural regulatory T cells (nTreg) expressing the Foxp3 transcription factor.

maturation of TECs, reduced number of thymic DCs, and severe impairment of nTreg cell development (Poliani et al., 2009). The hypothesis that immune dysregulation in OS is secondary to defective T cell generation, with inefficient provision of instructive signals to thymic stromal cells to support mechanisms of central tolerance, has been confirmed in animal models. In particular, administration of anti-CD3ε monoclonal antibody (mAb) to *Rag2*$^{R229Q/R229Q}$ mice (a model of OS) of neonatal age, mimicking pre-T cell receptor (pre-TCR) and TCR signaling, induced significant amelioration of thymic architecture, with increase of the medulla/cortex ratio, and induction of expression of *Ulex europaeus* agglutinin-1, a marker of mature mTECs (Marrella et al., 2012). These changes of thymic morphology and maturation were associated with reduced activation of peripheral T cells and tissue infiltration, hence with improvement of the disease phenotype. Similarly, treatment of *Rag2*$^{-/-}$ mice with anti-CD3ε mAb, followed by transplantation of *Rag2*$^{R229Q/R229Q}$ fetal liver cells, resulted in appearance

of a sizeable number of naïve T lymphocytes and lack of skin abnormalities (skin rash, alopecia). By contrast, transplantation of *Rag2*$^{R229Q/R229Q}$ fetal liver cells into untreated *Rag2*$^{-/-}$ mice was associated with typical signs of OS and severe tissue infiltration by activated T cells (Marrella et al., 2012). These data may have important therapeutic implications, because they suggest that induction of thymic stromal cell maturation may prevent T cell-mediated infiltration and damage of target tissue, even in the presence of a cell autonomous *Rag* gene defect.

Knowledge that typical manifestations of immune dysregulation in OS reflect defective lymphostromal cross-talk in the thymus and impaired development of mechanisms of central tolerance has also important implications for the characterization of the genetic basis of this disease. Described for the first time in 1965 (Omenn, 1965), OS remained with undefined pathogenesis for several decades. In 1991, de Saint Basile et al. reported the simultaneous occurrence of OS and of SCID with absence of both T and B lymphocytes ($T^-$ $B^-$ SCID) in two siblings (de Saint-Basile et al., 1991), and hypothesized that OS could be allelic to $T^-$ $B^-$ SCID. This description had shortly followed cloning of the *RAG1* (Schatz, Oettinger, & Baltimore, 1989) and *RAG2* (Oettinger, Schatz, Gorka, & Baltimore, 1990) genes and their role in initiating V(D)J recombination. Soon after the description of *RAG* mutations in patients with $T^-$ $B^-$ SCID (Schwarz et al., 1996), Villa et al. established that OS is more often due to hypomorphic mutations in the *RAG* genes that severely impair, but do not completely abrogate V(D)J recombination activity. Similar findings were obtained by others, thus confirming the role of *RAG* mutations in OS. However, it was soon appreciated that not all cases of OS are due to *RAG* mutations. Moreover, while *RAG* gene defects typically abrogate B cell development, some patients with OS have a normal number of circulating B lymphocytes. Recognition that inflammatory and immune dysregulation manifestation of OS are secondary to impaired lymphostromal cross-talk in the thymus with defective central tolerance has led to speculate that OS may be due to a variety of genetic defects that impair (but do not abolish) early stages in T cell development and affect maturation of thymic stromal cells. This hypothesis has been confirmed with the demonstration that OS may also be caused by hypomorphic mutations in interleukin-2 receptor gamma chain (*IL2RG*) (Shibata et al., 2007), *LIG4* (Grunebaum, Bates, & Roifman, 2008), *IL7R* (Giliani et al., 2006), *ADA* (Roifman, Zhang, Atkinson, Grunebaum, & Mandel, 2008), *DCLRE1C* (Ege et al., 2005), *AK2* (Henderson et al., 2013), *RMRP* (Roifman, Gu, & Cohen, 2006), *ZAP70* (Turul et al., 2009), and *CHD7* (Gennery et al., 2008) genes.

## 2.2. Other "leaky" phenotypes due to hypomorphic mutations in SCID-associated genes

OS is only one of the possible phenotypic manifestations of hypomorphic mutations of SCID-associated genes, and *RAG* defects in particular. In the last few years, hypomorphic *RAG* mutations have been identified in patients with delayed-onset disease characterized by granulomatous and/or autoimmune manifestations (Avila et al., 2010; De Ravin et al., 2010; Schuetz et al., 2008; Sharapova et al., 2013). Interestingly, circulating B cells and autoantibodies could be demonstrated in several of these cases. Similarly, autoantibodies were also detected in mice carrying hypomorphic *Rag1* (Walter et al., 2010) or *Rag2* (Cassani et al., 2010) mutations. Disturbance of B cell tolerance in these models was shown to reflect impaired receptor editing and increased peripheral levels of B cell activating factor (BAFF), which can rescue self-reactive B cells (Cassani et al., 2010; Walter et al., 2010). Finally, the spectrum of phenotypes associated with hypomorphic *RAG* mutations in humans also includes idiopathic CD4 lymphopenia (Kuijpers et al., 2011) and dysgammaglobulinemia with hyper-IgM phenotype (Chou et al., 2012).

X-linked SCID is the most common form of SCID in Western countries, and is due to mutations of the *IL2RG* gene, that encodes for the common gamma chain (γc), also shared by receptors for IL-4, IL-7, IL-9, IL-15, and IL-21 (Noguchi et al., 1993; Rochman, Spolski, & Leonard, 2009). Typically, patients with X-linked SCID present lack of circulating T and NK lymphocytes, reflecting an essential role played by IL-7 and IL-15 in thymocyte and NK cell development, respectively (Kovanen & Leonard, 2004). The thymus of patients with X-linked SCID is hypoplastic, with lack of corticomedullary demarcation, severe lymphopenia, and absence of Hassall corpuscles (Poliani et al., 2009). However, an atypical immunological phenotype has been reported in several infants carrying missense mutations in the *IL2RG* gene, resulting in a single amino acid substitution (R222C) in the extracellular domain of γc (Mella et al., 2000; Sharfe, Shahar, & Roifman, 1997; Somech & Roifman, 2005). In these patients, normal thymic architecture was demonstrated (Poliani et al., 2009; Sharfe et al., 1997); moreover, circulating T cells were present in normal numbers, had a polyclonal repertoire and, in most cases proliferated normally in response to mitogens and antigens (Sharfe et al., 1997; Somech & Roifman, 2005). However, the mutant γc bound IL-2 with lower affinity, and no increase in lymphocyte proliferation to anti-CD3 was observed upon addition of exogenous IL-2 (Mella et al., 2000; Sharfe et al., 1997;

Somech & Roifman, 2005), possibly accounting for the increased susceptibility to opportunistic infections despite normal T cell number.

A normal number of circulating T, B, and NK cells associated with defective antigen-specific immune responses was reported in a patient with a splice-site mutation of the *IL2RG* gene that allowed generation of two transcripts, one of which contained a single amino acid substitution (D17N) that caused reduced expression of the high-affinity IL-2R at the cell surface (DiSanto, Rieux-Laucat, Dautry-Varsat, Fischer, & de Saint Basile, 1994). Reduced, but not abolished, γc-dependent JAK3 binding intracellular signaling have been implied to account for the progressive development of T cells with an activated phenotype in patients with L271Q γc mutation (Russell et al., 1994; Schmalstieg et al., 1995). Finally, progressive increase in the number of circulating T cells has been reported in some patients with X-linked SCID (Mella et al., 2000; Notarangelo et al., 2000). This is reminiscent of the appearance of $CD4^+$ T cells in γc-deficient mice (Cao et al., 1995; DiSanto, Muller, Guy-Grand, Fischer, & Rajewsky, 1995). In one case, development of autologous T lymphocytes with partially preserved function was demonstrated several months after haploidentical HCT (Morelon et al., 1996). In this case, a premature termination in the intracytoplasmic tail of the γc was demonstrated that impaired cytokine-mediated JAK3 phosphorylation. In addition to typical manifestations of SCID, patients with *IL2RG* mutations and detectable circulating T cells are also prone to manifestations of immune dysregulation (skin rash, autoimmunity) and lymphoproliferation.

JAK3 is an intracytoplasmic tyrosine kinase that is physically and functionally coupled to γc, allowing cytokine-dependent signal transduction. Autosomal recessive JAK3 deficiency recapitulates the phenotype observed in patients with γc deficiency, that is, $T^-$ $B^+$ $NK^-$ SCID (Macchi et al., 1995). However, residual JAK3 protein expression and function has been associated with development of autologous, activated, oligoclonal, and poorly functioning T lymphocytes (Brugnoni et al., 1998; Li et al., 2010). An unusually variable phenotype has been reported in multiple affected family members of a single family, who were compound heterozygotes for an initiation start site mutation and a splice-site mutation (Frucht et al., 2001). Clinical features in affected family members ranged from early-onset and fatal opportunistic infections to severe lymphoproliferative disease to survival into adulthood with warts. Circulating T cells showed an oligoclonal repertoire and failed to upregulate Fas ligand (FasL) upon *in vitro* activation. This may have contributed to the increased risk of lymphoproliferative

disease in affected patients. Finally, long-term coexistence of maternally engrafted T lymphocytes and autologous T cells was demonstrated in a patient with compound heterozygosity for a missense and a splice-site *JAK3* mutation, who was reported to be in excellent clinical conditions at 4 years of age without HCT (Cattaneo et al., 2013). Interestingly, the autologous T cells initially had a polyclonal repertoire that became restricted over time and retained some proliferation capacity.

Development of TCR$\gamma\delta^+$ T cells, but not of TCR$\alpha\beta^+$ lymphocytes, has been reported in patients with mutations of the TCR$\alpha$ constant region (*TRAC*) gene (Morgan et al., 2011) or of the *CD3D* gene, encoding for CD3$\delta$ (Gil et al., 2011).

Mutations of the invariant CD3$\delta$, CD3$\varepsilon$, and CD3$\zeta$ chains of the TCR are typically associated with a $T^-$ $B^+$ $NK^+$ SCID phenotype (Dadi, Simon, & Roifman, 2003; de Saint Basile et al., 2004; Rieux-Laucat et al., 2006; Roberts et al., 2007). However, homozygosity for the same *CD3D* splice-site mutation in two unrelated patients was associated with selective lack of TCR$\alpha\beta^+$ T cells, preserved generation of TCR$\gamma\delta^+$ T cells, and impaired *in vitro* proliferation to mitogens (Gil et al., 2011). Molecular analysis revealed residual levels of wild-type *CD3D* transcript and of CD3$\delta$ protein expression. The few TCR$\alpha\beta^+$ T cells were oligoclonal and showed an activated phenotype.

Similarly, Morgan et al. reported two apparently unrelated patients who shared a homozygous single nucleotide substitution in the *TRAC* gene, immediately after the translation termination codon at the end of exon 3 (Morgan et al., 2011). This resulted in skipping of exon 3, with production of an aberrant transcript joining exon 2 to the normally untranslated exon 4. As a result of this aberrant splicing, the transmembrane and intracytoplasmic domain of the TCR$\alpha$ chain were lost. The immunological phenotype was characterized by a normal number of circulating T cells, with increased proportion of TCR$\gamma\delta^+$ cells that showed a polyclonal repertoire. *In vitro* proliferation to mitogens and antigens was variably reduced. In addition to increased susceptibility to infections, patients had evidence of immune dysregulation, with eczema, autoimmune cytopenias, and lymphoproliferation.

## 2.3. Gene reversion events and environmental factors may modify the disease phenotype of SCID infants

Phenotypic heterogeneity in patients with mutations of SCID-associated genes may also reflect somatic mosaicism, due to second-site mutations or true somatic reversions that restore, at least in part, protein expression

and function. This scenario was initially reported in adenosine deaminase deficiency (Hirschhorn, Yang, Israni, Huie, & Ownby, 1994), but has since been described also in X-linked SCID (Bousso et al., 2000; Kawai, Saito, et al., 2012; Speckmann et al., 2008; Stephan et al., 1996; Wada et al., 2008), RAG1 deficiency (Wada et al., 2005), and CD3ζ deficiency (Rieux-Laucat et al., 2006). Moreover, several other cases of second-site mutations in the ADA genes have been described (Ariga et al., 2001; Arredondo-Vega et al., 2002; Hirschhorn et al., 1996; Moncada-Velez et al., 2011). In some of these patients, multiple reversion events have been identified (Kawai, Saito, et al., 2012; Rieux-Laucat et al., 2006). The clinical and immunological consequences of somatic reversion cannot be easily predicted. Among patients with SCID, somatic reversion has been detected especially within the T cell lineage, indicating selective differentiation and/or proliferation advantage for the revertant T cell progenitors (Bousso et al., 2000). Generation of a diversified T cell repertoire, improved *in vitro* proliferation to mitogens and antigens, and clinical amelioration have been reported in some patients (Bousso et al., 2000; Hirschhorn et al., 1996; Kawai, Saito, et al., 2012), providing a strong basis to the development of gene therapy. However, no benefits could be demonstrated in other cases, as indicated by persistent T cell lymphopenia and poor proliferative responses (Rieux-Laucat et al., 2006; Speckmann et al., 2008). Occasionally, emergence of the revertant clone coincided with worsening of the disease manifestations and autoimmunity (Wada et al., 2005, 2008).

Finally, development of poorly functioning T lymphocytes in patients with mutations of SCID-associated genes may be also precipitated by infections. In particular, expansion of TCRγδ T cells has been reported following cytomegalovirus infection (de Villartay et al., 2005; Ehl et al., 2005; Gil et al., 2011), whereas sudden development of typical features of OS (generalized erythroderma, lymphadenopathy, and accumulation of activated and oligoclonal T cells) has been demonstrated in an infant with $T^-B^-$ SCID due to *RAG2* mutations after parainfluenza 3 virus infection (Dalal et al., 2005).

## 3. OTHER COMBINED IMMUNODEFICIENCIES WITH DYSFUNCTIONAL T CELLS

The study of patients with SCID has been essential to identify genes that regulate cell survival, cytokine-induced proliferation of T cell progenitors, expression of the pre-TCR, and pre-TCR-mediated signaling.

Consistent with the notion that these are key processes during early stages of T cell development, null mutations in these genes are associated with lack of circulating T cells and therefore result in a SCID phenotype. By contrast, residual development of T cells, most often associated with impaired T cell function, has been observed in patients with genetic defects that affect later stages in T cell development. Some of these defects, such as purine nucleoside phosphorylase (PNP) deficiency and zeta-associated protein of 70 kDa (ZAP-70) deficiency are known since several years and will be briefly discussed here. Importantly, in recent years advances in genome analysis, in particular whole exome/genome sequencing (WES/WGS) have permitted identification of several disorders characterized by defective T cell function. The immunological, molecular, and clinical basis of these novel forms of combined immunodeficiency will be discussed in greater detail.

## 3.1. PNP deficiency

PNP catalyzes conversion of guanosine and deoxyguanosine (dGuo) to inosine and deoxyinosine, respectively. In the absence of PNP, accumulation of phosphorylated metabolites of dGuo, and dGuo triphosphate in particular, has toxic effects on proliferating cells, by inhibiting ribonucleotide reductase and preventing synthesis of deoxynucleotides. These effects are especially pronounced in the thymus (Fairbanks, Taddeo, Duley, & Simmonds, 1990). It has recently been shown that double-positive (DP) of dGuo, causing dissipation of mitochondrial membrane potential and release of cytochrome C, followed by apoptosis (Papinazath et al., 2011). Interestingly, *in vivo* pulse experiments with 5-bromo-2-deoxyuridine have shown that proliferation of thymocytes is not affected by PNP deficiency (Papinazath et al., 2011), although peripheral T cell proliferation is often impaired (Arpaia et al., 2000; Roifman et al., 2012). At variance with ADA deficiency, that in its most severe form presents in the first months of life with infections and severe lymphopenia affecting T, B, and NK lymphocytes, PNP deficiency is characterized by recurrent infections with onset in the first year of life, associated with progressive neurological deterioration and autoimmunity (especially cytopenias). Thymopoiesis is not as dramatically affected in PNP-deficient mice and humans as it is in ADA deficiency (Papinazath et al., 2011). Consistent with this, a variable number of circulating T cells are detected at birth. However, this is followed by progressive T cell lymphopenia. B lymphocytes may also be affected, but less severely

than in ADA deficiency. Repeated injections of PNP coupled to HIV-TAT protein transduction domain allowing intracellular delivery of the enzyme have resulted in significant improvement of immune function and extended survival in PNP-deficient mice (Toro & Grunebaum, 2006).

## 3.2. Lck deficiency

The lymphocyte-specific protein tyrosine kinase (Lck) is constitutively associated with the cytoplasmic tail of CD4 and CD8, and initiates the TCR signaling process by phosphorylating the immunoreceptor tyrosine activation motifs (ITAMs) located in the intracytoplasmic domains of CD3 subunits and in the ZAP-70 (Salmond, Filby, Qureshi, Caserta, & Zamoyska, 2009; van Oers, Killeen, & Weiss, 1996). These events are important in triggering phosphorylation of downstream signaling molecules, such as the Linker of Activation in T cells (LAT) and the Src homology 2 (SH2)-domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) protein (Fig. 4.2). During T cell development, Lck plays a critical role in relaying pre-TCR- and TCR-mediated signaling. Consistent with this, Lck-deficient mice show a pronounced thymic atrophy, a block at DP stage, with lack of single-positive (SP) thymocytes, and few peripheral T cells expressing low levels of CD4 and CD8 molecules at the cell surface (Molina et al., 1992). Moreover, transition from double-negative 3 (DN3) to DN4 stage, which requires pre-TCR signaling, is impaired in $Lck^{-/-}$ mice (Wallace et al., 1995), and development of TCR$\alpha\beta^+$ CD8$\alpha\alpha^+$ intraepithelial lymphocytes is also defective (Page, van Oers, Perlmutter, Weiss, & Pullen, 1997). Moreover, in combination with Fyn, Lck provides TCR-mediated survival signals to peripheral naïve T lymphocytes (Seddon & Zamoyska, 2002), but is also required for activation-induced cell death (AICD) in response to TCR engagement (Yu, Levin, Madrenas, & Anasetti, 2004). The number of $CD8^+$ lymphocytes and *in vitro* proliferative responses are reduced in transgenic mice that express Lck in the thymus but not in peripheral T cells (Trobridge & Levin, 2001). Finally, Lck is required for $CD8^+$ T cell primary responses during acute viral infection (Tewari, Walent, Svaren, Zamoyska, & Suresh, 2006). Altogether, these data indicate that Lck plays an important role in T cell development and function.

Defective expression of Lck has been reported in patients with various forms of immunodeficiency, including one infant with clinical features of SCID and severe $CD4^+$ cell deficiency, (Goldman et al., 1998), one adult woman with idiopathic CD4 lymphopenia (Hubert et al., 2011), and in

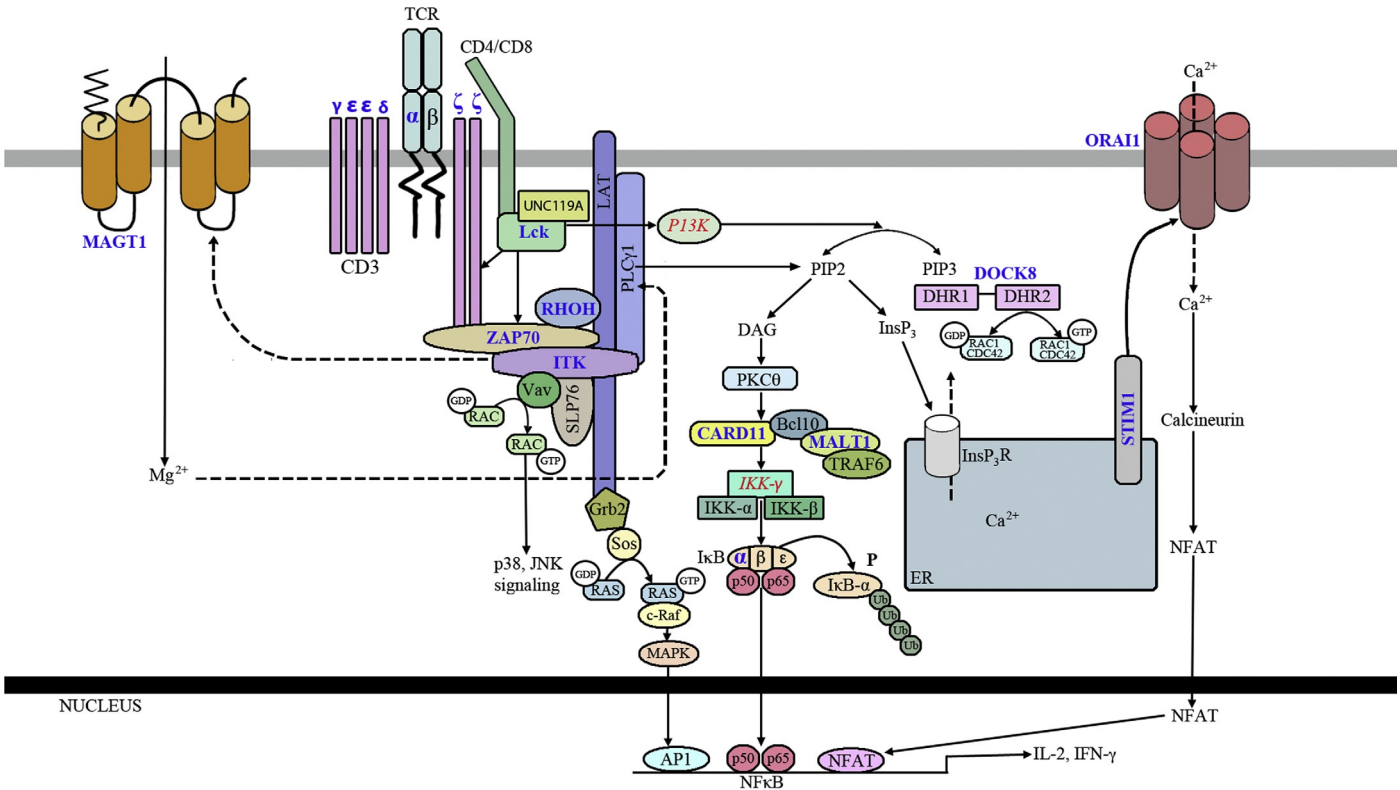


**Figure 4.2** TCR-mediated signaling. Schematic representation of the signaling pathways elicited upon engagement of the T cell receptor (TCR)/CD3 complex. Molecules whose mutations in humans are associated with defects of T cell development and/or function are highlighted in bold. Molecules whole mutations have been associated in humans with immunodeficiencies that do not have prominent signs of T cell dysfunction are indicated in Italic.

another patient with common variable immunodeficiency and CD4 lymphopenia. In the latter, an aberrantly spliced *LCK* transcript lacking exon 7 was demonstrated; however, in none of the three patients could *LCK* gene mutations be identified. More recently, Hauck et al. have described a child with recurrent infections, failure to thrive, and immune dysregulation (nodular skin lesions, arthritis, retinal vasculitis, and autoimmune thrombocytopenia) (Hauck et al., 2012). This patient was homozygous for a missense mutation (L341P) that affected Lck protein expression and function, and that reflected to uniparental isodisomy of the maternally inherited chromosome 1 carrying the same mutation (Hauck et al., 2012). Severe $CD4^+$ T cell lymphopenia with oligoclonal $TCR\alpha\beta^+$ T cells and an increase in proportion of central memory ($CD45R0^+$ $CCR7^+$) $CD4^+$ cells and of $CD45RA^+$ $CCD27^-$ $CD62L^-$ "exhausted" T effector memory ($T_{EMRA}$) $CD8^+$ cells were demonstrated. Moreover, circulating $CD3^+$ T cells showed markedly reduced expression of CD4 and CD8 molecules. *In vitro* lymphocyte proliferation in response to anti-CD3, phytohemagglutinin (PHA), and immunization antigens was abolished, but proliferation to phorbol-myristate acetate (PMA) was preserved, consistent with a proximal defect in T cell activation. Indeed, in response to CD3 stimulation, tyrosine phosphorylation of CD3ζ, ZAP-70, LAT, SLP-76, and phospholipase C-γ1 (PLC-γ1) was markedly decreased, and $Ca^{2+}$ mobilization was abrogated. Finally, clinical manifestations of immune dysregulation were associated with elevated serum levels of IgM, presence of antinuclear and antidouble stranded DNA antibodies and rheumatoid factor, and a low number of regulatory T (Treg) cells. Moreover, consistent with a role for Lck in AICD, T cell blasts from the patient showed reduced AICD than control blasts, and this may also have contributed to immune dysregulation.

### 3.3. UNC119 deficiency

Uncoordinated 119 (UNC119) is a chaperone molecule involved in transport and targeting of myristoylated proteins to specific subcellular membrane compartments. The human *UNC119A* and *UNC119B* genes have a similar structure with 5 exons and encode for highly homologous proteins of 240 and 251 amino acids, respectively (Higashide & Inana, 1999). Crystal structure analysis has shown that UNC119A adopts an immunoglobulin-like β-sandwich fold that forms a hydrophobic cavity in which side lipid chains may be inserted (Zhang et al., 2011). Yeast two-hybrid screening has revealed that UNC119A interacts with a large number of proteins, including

acylated Gα-protein subunits, src tyrosine kinases, nonreceptor tyrosine kinases, the GTPase dynamin, and small ARF-like GTPases (ARL proteins) (Constantine, Zhang, Gerstner, Frederick, & Baehr, 2012). UNC119B interacts with nephrocystin-3, a myristoylated protein whose mutations in humans are associated with a rare form of nephronophthisis (Wright et al., 2011).

In T cells, UNC119A interacts with Lck and Fyn, two src tyrosine kinases associated with TCR (Gorska, Stafford, Cen, Sur, & Alam, 2004). In resting conditions, src kinases are maintained in a close, inactive conformation through intramolecular interactions between the SH2/SH3 domains and the kinase domains. Upon binding to src kinases, UNC119A induces src autophosphorylation and kinase activity (Gorska et al., 2004). Moreover, UNC119A regulates Lck intracellular trafficking to the cell membrane, and this activity is dependent on Rab11 (Gorska, Liang, Karim, & Alam, 2009). Finally, UNC119A regulates recruitment of myosin 5B, and the organization of multiprotein complex, on endosomes (Gorska et al., 2009).

A heterozygous dominant-negative missense mutation of *UNC119A*, affecting the N-terminus of the molecule, has been identified in an adult patient with recurrent respiratory tract infections, multiple episodes of shingles, chronic fungal infections, and idiopathic CD4 lymphopenia (Gorska & Alam, 2012). Consistent with the role of UNC119A in promoting Lck activity, *in vitro* proliferative response to mitogens and antigens was markedly decreased. Lck was sequestered in the endosomal compartment, with little expression at the cell membrane. Expression of mutant UNC119A in primary $CD4^+$ lymphocytes and in Jurkat cells blocked activation of Lck, confirming the dominant-negative effect of the mutant (Gorska & Alam, 2012).

Besides the immune system, UNC119A plays an important role also in rod photoreceptor signaling and interacts with transducing α subunits (Zhang et al., 2011). A single patient with cone dystrophy and a heterozygous nonsense mutation of *UNC119A* has been reported (Kobayashi et al., 2000).

## 3.4. RhoH deficiency

RhoH is a hematopoietic-specific atypical small GTPase that preferentially binds GTP and lacks GTPase activity (Gu et al., 2006; Li et al., 2002; Tybulewicz & Henderson, 2009). Its C-terminus CAAX box is a lipid modification site domain that allows localization of the molecule to the cell membrane. There is substantial evidence that RhoH plays an important role in T cell and mast cell activation. In particular, following TCR-mediated

activation, RhoH undergoes phosphorylation at two tyrosine residues within a noncanonical ITAM. This phosphorylation event allows RhoH to bind to the ZAP-70 SH2 domain (Gu et al., 2006). RhoH also interacts with Lck and Csk (Wang, Zeng, Fan, & Lim, 2011). Therefore, following TCR engagement, RhoH plays an important role in recruiting ZAP-70 and Lck to the LAT signalosome at the immunological synapse in the detergent-insoluble membrane fraction that contains lipid rafts (Chae, Siefring, Hildeman, Gu, & Williams, 2010; Dorn et al., 2007; Fig. 4.2). In the absence of RhoH, these events are abrogated (Chae et al., 2010). TCR stimulation induces downregulation of RhoH transcription, thereby providing a feedback regulatory mechanism (Li et al., 2002). Moreover, RhoH participates also at FcεR-mediated signaling during mast cell activation (Oda et al., 2009). Finally, RhoH is involved in regulation of cell survival and proliferation. Overexpression of RhoH in hematopoietic progenitor cells induces apoptosis and inhibits cell proliferation, by suppressing activation of NF-κB and p38 MAP kinase by other Rho/Rac GTPases (Gu et al., 2006; Li et al., 2002).

The hypothesis that RhoH plays an important role in T cell development and function was initially supported by studies in mice. *Rhoh*$^{-/-}$ mice show a severe, but incomplete block at two distinct stages in T cell development, corresponding to DN3 and at DP cells, respectively (Gu et al., 2006; Wang et al., 2011). This block is associated with impaired activation of CD3ζ, LAT, and p44/42 MAP kinase upon TCR stimulation in thymocytes (Gu et al., 2006; Wang et al., 2011). Expression of a myristoylated form of ZAP-70 that allows translocation of ZAP-70 to the cell membrane can partially rescue the developmental defect of *Rhoh*$^{-/-}$ mice (Wang et al., 2011). Altogether, these data demonstrate that RhoH permits recruitment of ZAP-70 and Lck to the LAT signalosome, and that RhoH deficiency causes T cell deficiency by affecting both pre-TCR-mediated signaling and positive selection, similar to what reported in *Zap70*$^{-/-}$ mice (Palacios & Weiss, 2007). Recent data suggest that RhoH is also involved in negative selection. In particular, male mice with a transgenic TCR specific for the male-associated HY antigen (HY TCR-tg mice) have a small thymus and lack DP cells due to negative selection in the thymus. By contrast, the thymus of male *Rhoh*$^{-/-}$ HY TCR-tg mice is larger, with an increased proportion of DP cells (Oda et al., 2009). Furthermore, *Rhoh*$^{-/-}$ mice have a reduced number of nTreg cells and of NKT lymphocytes, indicating that RhoH plays an important role also in the development of nonconventional T cells (Oda et al., 2009). The majority of peripheral T cells from *Rhoh*$^{-/-}$

mice have an activated phenotype, possibly reflecting homeostatic expansion. Proliferation in response to anti-CD3 stimulation and to alloantigens is impaired, and cell-mediated cytotoxicity and cytokine release by activated T cells are defective (Dorn et al., 2007; Porubsky et al., 2011).

T cell immunodeficiency with increased susceptibility to persistent cutaneous human papillomavirus (HPV) infection, Burkitt's lymphoma, psoriatic-like skin rash, and lung granulomatous disease has been recently reported in two young adult siblings with RhoH deficiency (Crequer et al., 2012). Thymopoiesis is impaired in RhoH-deficient patients, as indicated by markedly reduced number of naïve $CD4^+$ cells and lack of recent thymic emigrants. Moreover, restricted TCR Vβ repertoire, and reduced *in vitro* proliferative response to anti-CD3 stimulation, have been observed (Crequer et al., 2012). Both $CD4^+$ and $CD8^+$ cells from RhoH-deficient patients express markers indicative of chronic activation and exhaustion, such as CD244, CXCR1, CD57, granzyme B, and perforin. By contrast, expression of CD127 and CD27 is reduced (Crequer et al., 2012). It is not clear whether accumulation of memory T cells with an exhausted phenotype is a direct consequence of the mutation, or whether it is secondary to chronic viral infections.

The immunodeficiency of RhoH-deficient patients may also reflect impaired trafficking of T cells and defective interaction with antigen-presenting cells. Studies in mice have shown that RhoH plays opposing roles on LFA-1 activation during T cell migration and activation. In particular, it inhibits chemokine-induced activation of Rap1 and of LFA-1, whereas it potentiates Rap1-mediated LFA-1 activation in response to TCR ligation. These contrasting effects are paralleled by differential subcellular localization in response to various stimuli. Upon activation with chemokines, LFA-1 and RhoH localize at the opposing edges of the cell (leading edge and uropod, respectively), whereas TCR-mediated signaling induces clustering of both RhoH and LFA-1 at the immunological synapse (Baker et al., 2012). Importantly, RhoH deficiency impairs the directional motility of T lymphocytes and affects the ability of T lymphocytes to form stable conjugates with antigen-presenting cells (Baker et al., 2012). Altogether, these abnormalities may induce RhoH-deficient T cells to enter and depart from lymph nodes too quickly, causing inability to mount efficient immune responses.

Finally, it is likely that development of chronic warts due to HPV infection in patients with RhoH deficiency reflects defective homing of antigen-specific T cells to the skin. In support of this hypothesis, the number of

circulating T cells expressing skin-homing receptors, such as CCR4, CCR6, CCR10, cutaneous lymphocyte antigen (CLA), and β7 integrin is reduced in patients with RhoH deficiency (Crequer et al., 2012). Defective expression of β7 integrin has been also observed in T lymphocytes from *Rhoh*$^{-/-}$ mice, suggesting that this may be a direct consequence of the gene defect, and not a consequence of HPV infection.

## 3.5. ZAP-70 deficiency

Following TCR-induced activation, Lck mediates phosphorylation of the ITAMs of the CD3ζ chain, enabling recruitment of ZAP-70 to the TCR/CD3 signaling complex through its SH2 domains binding to CD3ζ phosphorylated ITAMs (Jin et al., 1990; Wange, Kong, & Samelson, 1992; Fig. 4.2). During this process, conformational changes of ZAP-70 occur, so that the molecule no longer remains in autoinhibited conformation (Deindl et al., 2007). Moreover, Lck phosphorylates ZAP-70 at three tyrosine residues (Y292, Y315, and Y319) located in the interdomain B that separates the C-terminal SH2 domain from the kinase domain. Phosphorylation of these tyrosine residues has important implications on ZAP-70 function and T cell signaling. In particular, phosphorylation of Y292 allows recruitment of c-Cbl and regulates internalization of the TCR complex (Davanture et al., 2005; Wang et al., 2001), whereas phosphorylation of Y315 enables interaction of ZAP-70 with CrkII and promotes LFA-1 activation and T cell adhesion (Gelkop, Gish, Babichev, Pawson, & Isakov, 2005). Finally, phosphorylation of Y319 allows docking of Lck (Pelosi et al., 1999) and of PLC-γ (Williams et al., 1999), thereby promoting calcium flux and T cell signaling. The ZAP-70 kinase domain mediates phosphorylation of the signal transducing molecules LAT (Zhang, Sloan-Lancaster, Kitchen, Trible, & Samelson, 1998) and SLP-76 (Bubeck Wardenburg et al., 1996), ultimately inducing activation of the MAP kinases ERK1/2 (Jordan, Singer, & Koretzky, 2003; Fig. 4.2).

Human ZAP-70 deficiency was described for the first time in 1994 (Arpaia, Shahar, Dadi, Cohen, & Roifman, 1994; Chan et al., 1994; Elder et al., 1994). ZAP-70-deficient patients lack circulating CD8$^+$ lymphocytes; CD4$^+$ T cells are present, but they fail to proliferate in response to CD3 cross-linking. This phenotype contrasts with that of *Zap70*$^{-/-}$ mice, that show a complete block in T cell development at the DP stage, with lack of SP CD4$^+$ and of SP CD8$^+$ cells (Negishi et al., 1995). However, generation of TCRγδ$^+$ T cells is unaffected in *Zap70*$^{-/-}$ mice (Kadlecek et al., 1998).

Significant differences in phenotypic expression of the disease have been reported in patients and mice carrying the same mutation. In particular, complete lack of mature TCRαβ T cells is observed in the inbred strange strain that carries a missense mutation in the conserved DLAARN domain required for Zap-70 catalytic activity (Wiest et al., 1997). By contrast, homozygosity for the same mutations in humans is associated with lack of $CD8^+$ cells, but normal number of nonfunctional $CD4^+$ lymphocytes (Elder et al., 2001). This species-specific discrepancy of the ZAP-70 deficiency phenotype may reflect different patterns of expression of ZAP-70 and of its homologue tyrosine kinase Syk during T cell development in humans and mice. In particular, expression of the Syk kinase is rapidly downregulated during transition from DN to DP thymocytes in mice, whereas its expression is maintained in DP human thymocytes (Chu et al., 1999). Moreover, studies in mice have shown that expression of Zap-70 is upregulated beyond the DN stage, and that highest levels of expression are observed in SP cells (Palacios & Weiss, 2007). Importantly, higher amounts of Zap-70 are required for the generation of SP $CD8^+$ thymocytes than for SP $CD4^+$ cells (Saini et al., 2010). Altogether, these data suggest that Syk may play a compensatory role for the generation of $CD4^+$ T cells in ZAP-70-deficient patients. However, circulating $CD4^+$ cells from these patients lack expression of both kinases, and hence they fail to proliferate in response to CD3 stimulation.

ZAP-70 may play an important role also in response to pre-TCR engagement. In particular, circulating T cells from ZAP-70-deficient patients have reduced levels of the δRAC-ψJα rearrangement product, which is generated as a result of a *TCRD*-deleting rearrangement that initiates at early stages during T cell development and peaks at transition from DP to SP cells (Roifman, Dadi, Somech, Nahum, & Sharfe, 2010). Defective signaling in response to pre-TCR and TCR stimulation in ZAP-70 deficient thymocytes is associated with lower levels of T cell receptor excision circles (TRECs) in peripheral blood (Roifman et al., 2010). TRECs are generated as a by-product of V(D)J recombination during development of $TCR\alpha\beta^+$ T cells and are a robust indicator of thymopoiesis (Douek et al., 1998).

Circulating T cells from ZAP-70 deficient patients show a broadly polyclonal repertoire, however their cytokine gene expression profile is abnormal, with lower levels of *TGFB*, *IL4*, and *IL10* genes (Roifman et al., 2010). Finally, impaired formation of stable focal adhesion, rapid turnover of pseudopods, and defective chemotactic motility have been reported after

stimulation of ZAP-70-deficient $CD4^+$ cells with chemokines (Lin, Cheng, Huang, Lin, & Yang, 2010). These abnormalities reflect the role played by ZAP-70 in chemokine-mediated signaling, whereby phosphorylated ZAP70 interacts with talin, a cytoskeletal protein involved in regulation of actin polymerization and focal adhesion (Lin et al., 2010). Moreover, ICAM-1-induced activation of LFA-1 promotes ZAP70 phosphorylation and outside-in signaling (Evans, Lellouch, Svensson, McDowall, & Hogg, 2011). The GM-CSF/IL-3/IL-5 receptor common beta-chain-associated protein (CBAP) has been recently shown to bind to ZAP-70 and to function as an adaptor that regulates chemokine-induced T cell trafficking (Chiang et al., 2013). Overall, these data indicate that ZAP-70 deficiency, while permissive for $CD4^+$ T cell development, caused profound functional abnormalities of peripheral blood lymphocytes.

Lack of circulating $TCR\alpha\beta^+$ $CD8^+$ T cells and severe functional impairment of $CD4^+$ lymphocytes account for severe, early-onset infections that characterize the clinical phenotype of most patients with ZAP-70 deficiency. However, some phenotypic heterogeneity has been reported in patients with hypomorphic *ZAP70* mutations. In particular, infiltrative erythematous skin lesions due to infiltration with activated $CD4^+$ $CD25^+$ $CD45R0^+$ $DR^+$ cells and eosinophils have been described in one case (Katamura et al., 1999). Two patients with homozygous missense mutations in the kinase domain have been described to suffer mainly from immune dysregulation, with wheezing and severe erythroderma resembling OS (Turul et al., 2009). Another patient with a homozygous R514C missense mutation in the kinase domain has been recently reported, whose phenotype was characterized by superficial perivascular dermatitis, icthyosis, elevated serum IgE, eosinophilia, and pneumonia (Karaca et al., 2013). Finally, homozygosity for an intronic mutation that introduced a cryptic splice site was associated with residual protein expression and a milder clinical phenotype, with delayed onset of symptoms and preserved generation of $CD4^+$ T follicular helper ($T_{FH}$) cells, $T_H1$, $T_H17$, and Treg cells (Picard, Dogniaux, et al., 2009).

Autoimmunity has been reported also in hypomorphic mouse models of Zap-70 deficiency. In particular, autoimmune arthritis is present in a spontaneous mouse model ($Zap70^{SKG}$ mice), due to a homozygous missense mutation in the SH2 domain of the protein, that diminishes TCR signaling (Sakaguchi et al., 2003). Moreover, mouse models carrying *Zap70* mutations that cause a stepwise decrease in TCR signaling develop progressively worsening autoimmunity and a hyper-IgE phenotype (Siggs et al., 2007).

These data indicate that intact ZAP-70 function is required not only to allow efficient generation and function of T cells, but also to maintain immune homeostasis and prevent immune dysregulation.

The mechanisms by which *ZAP70* mutations may cause immune dysregulation may include abnormalities in intrathymic negative selection of self-reactive clones and/or impaired function of Treg cells. The study of thymic biopsies from patients with ZAP-70 deficiency has shown preservation of corticomedullary demarcation. However, the number of $AIRE^{+}$ mTECs is reduced (Poliani, Fontana, Roifman, & Notarangelo, 2013), and similar observations have been made in $Zap70^{-/-}$ mice (White et al., 2010). Moreover, there is a lack of Hassall's corpuscle mTECs expressing involucrin (Poliani et al., 2013; White et al., 2010). These are terminally differentiated mTECs that express a distinct set of self-antigens (Derbinski et al., 2005). Finally, the number of nTreg cells is also reduced in the thymus of ZAP-70-deficient patients (Poliani et al., 2013), and it is possible that this may also contribute to immune dysregulation in patients with hypomorphic mutations.

## 3.6. Interleukin-2-inducible T cell kinase deficiency

The interleukin-2-inducible T cell kinase (ITK) is a member of the Tec family of nonreceptor tyrosine kinases that is expressed in T cells and is activated in response to TCR stimulation (Berg, Finkelstein, Lucas, & Schwartzberg, 2005; Fig. 4.2). ITK has a pleckstrin homology domain that contains three aromatic amino acid residues (FYF) that are important for ITK interaction with phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$), recruitment to the cell membrane, and intracellular signaling (Hirve et al., 2012). Upon TCR stimulation, ITK promotes optimal PLC-γ1 activation and $Ca^{2+}$ influx. Although ITK is not strictly required for TCR-mediated signaling (Schaeffer et al., 2000, 1999), its deficiency is associated with impaired activation of key signaling molecules, including PLC-γ1, and defective activation of the ERK/mitogen-activated protein kinase (Qi, Kannan, & August, 2011).

Insights into the biological role of ITK have initially been provided by the observation that positive selection of T lymphocytes is impaired in $Itk^{-/-}$ mice due to reduced strength of TCR signaling (Hu, Qi, & August, 2010; Liao & Littman, 1995; Schaeffer et al., 2000). Moreover, the majority of peripheral $Itk^{-/-}$ T cells have an unusual phenotype ($CD44^{hi}$ $CD122^{hi}$ $CD62L^{-}$), depend on IL-15, and rapidly produce IFN-γ upon stimulation (Atherly, Lucas, et al., 2006; Broussard et al., 2006;

Horai et al., 2007; Hu et al., 2010). This phenotype corresponds to "innate memory" T cells. Defective PLC-γ1 activation and reduced $Ca^{2+}$ influx in response to TCR stimulation cause impaired nuclear translocation of NFAT and are responsible for defective *In vitro* proliferation and cytokine production in $Itk^{-/-}$ T lymphocytes (Berg et al., 2005; Fowell et al., 1999; Gomez-Rodriguez et al., 2009; Liao & Littman, 1995; Liu, Bunnell, Gurniak, & Berg, 1998; Schwartzberg, Finkelstein, & Readinger, 2005). In particular, production of IL-2, IL-4, IL-5, IL-13, IL-17A, and IL-17F is defective (Blomberg et al., 2009; Gomez-Rodriguez et al., 2009; Kannan, Sahu, Mohanan, Mohinta, & August, 2013; Mueller & August, 2003). By contrast, Itk-deficient $CD8^+$ T cell shows increased expression of eomesodermin (Blomberg et al., 2009), a transcription factor that promotes expression of CD122, a marker shared by innate- and memory-like T cells. $CD8^+$ cells cytotoxic activity and IFN-γ production in response to viral infections are impaired in $Itk^{-/-}$ mice, and there is a defect of primary and memory T cell responses (Atherly, Brehm, Welsh and Berg, 2006; Bachmann, Littman, & Liao, 1997). Development of invariant NKT (iNKT) cells and their survival in the periphery are significantly impaired in $Itk^{-/-}$ mice (Felices & Berg, 2008; Qi et al., 2012). However, these mice show an expansion of $V\gamma1.1^+$ $V\delta6.3^+$ γδ T cells, also known as γδ NKT lymphocytes (Yin et al., 2013). These cells express the PLZF transcription factor and secrete high levels of Th2 cytokines. Consistent with this, elevated serum IgE levels have been reported in $Itk^{-/-}$ mice (Mueller & August, 2003; Schaeffer et al., 2001). Altogether, these data indicate that ITK serves an important role in development, maturation, and function of both T and NKT lymphocytes.

Similar developmental and functional abnormalities have been reported in ITK-deficient patients. This condition was described for the first time in 2009, when Huck et al. reported two sisters with a homozygous missense mutation in the SH2 domain of ITK that affected stability of the protein. The clinical phenotype of the patients was characterized by EBV-driven lymphoproliferative disease and cytopenias (Huck et al., 2009). The elder sibling developed EBV-associated Hodgkin lymphoma. A higher risk of opportunistic infections (candidiasis, BK polyomavirus infection, and *Pneumocystis jiroveci* pneumonia) was also recognized. Since then, additional patients with ITK deficiency have been described (Linka et al., 2012; Mansouri et al., 2012; Stepensky et al., 2011). A very high risk of EBV-driven lymphoproliferative disease has been confirmed, often manifesting with pulmonary nodules and mediastinal lymphadenopathy. ITK-deficient patients are also prone to other *herpesvirus* infections, in particular

disseminated CMV infection and severe varicella. Progressive T cell lymphopenia (especially pronounced for naïve $CD4^+$ cells), with an increased proportion of activated/memory T cells, defective *in vitro* proliferation in response to CD3 stimulation, and worsening hypogammaglobulinemia are present (Huck et al., 2009; Linka et al., 2012; Mansouri et al., 2012; Stepensky et al., 2011). Similar to what observed in $Itk^{-/-}$ mice, T lymphocytes from ITK-deficient patients express very high levels of eomesodermin, and NKT cells are usually absent (Huck et al., 2009). The association of NKT cell deficiency and unusual susceptibility to severe EBV infection has been reported also in X-linked lymphoproliferative disease (Nichols et al., 2005; Pasquier et al., 2005). Overall these data indicate that ITK plays an important role in T and NK cell development and peripheral homeostasis.

## 3.7. DOCK8 deficiency

The dedicator of cytokinesis 8 (DOCK8) protein is a member of the DOCK180 superfamily of atypical guanine nucleotide exchange factors that activate GTPases of the Rho/Rac/Cdc42 family (Cote & Vuori, 2002; Ruusala & Aspenstrom, 2004). Its gene is organized in 48 exons, spanning about 250 kb of genomic DNA on chromosome 9p24 (Griggs, Ladd, Saul, DuPont, & Srivastava, 2008). This subtelomeric region contains multiple Alu-repeats sequences and other short repetitive sequences that facilitate intrachromosomal recombination events. Similar to other DOCK family members, DOCK8 has DOCK-homology region 1 (DHR1) and DHR2 domains. The DHR1 domain binds to $PIP_3$, allowing recruitment of DOCK8 to the cell membrane (Cote, Motoyama, Bush, & Vuori, 2005; Premkumar et al., 2010). The DHR2 domain interacts with GTPases and mediates the GTP-GDP exchange reaction (Cote & Vuori, 2006; Fig. 4.2).

Although *DOCK8* mRNA transcript can be detected in multiple tissues, higher levels of expression have been demonstrated in B and T lymphocytes (Zhang et al., 2009). Moreover, multiple transcript isoforms have been described, and expression of a transcript lacking exon 1 has been reported in primary T cells (Zhang, Davis, Dove, & Su, 2010).

Studies in mice and in humans have demonstrated that DOCK8 plays an important role in immune system development and function. In 2009, two groups identified biallelic mutations of the *DOCK8* gene in patients with an autosomal recessive form of combined immunodeficiency with hyper-IgE (Engelhardt et al., 2009; Zhang et al., 2009). In many cases, large deletions

have been identified that are due to Alu-mediated recombination (Engelhardt et al., 2009; Zhang et al., 2009). These deletions may also extend to neighboring genes. By contrast, missense mutations are rare and are often associated with protein instability (Sanal et al., 2012). Overall, lack of DOCK8 protein expression has been observed in most patients.

The clinical features of the disease have been well characterized (Al-Herz et al., 2012; Chu et al., 2012; Engelhardt et al., 2009; Sanal et al., 2012; Zhang et al., 2009). Eczema is observed in >90% of the patients and is often associated with severe manifestations of food, environmental, or drug-related allergy. Upper and lower respiratory tract infections, skin abscesses, and mucocutaneous candidiasis are very common. Patients are at very high risk for cutaneous and mucosal viral infections, including recurrent Herpes Simplex infection, molluscum contagiosum, and warts. However, systemic viral infections have been also described, such as severe varicella, recurrent herpes zoster, and JC virus-related progressive multifocal leukoencephalopathy. Cryptosporidium infection complicated by sclerosing cholangitis, vascular thrombosis in the central nervous system, aortic aneurysms, autoimmune cytopenias, and eosinophilic esophagitis have been reported in a minority of patients. Facial dysmorphisms and retention of primary teeth may also be seen. Finally, patients are at increased risk for malignancies, including squamous cell carcinoma (often associated with HPV infection), T and B cell lymphoma (often with extranodal distribution), and leiomyosarcoma (Al Mutairi et al., 2013; Alsum et al., 2013; Chu et al., 2012; Engelhardt et al., 2009; Sanal et al., 2012; Zhang et al., 2009). The disease has a severe prognosis, and many patients die in childhood or young adulthood from infections or malignancies. Allogeneic HCT may be curative (Bittner et al., 2010).

A variety of immunological abnormalities have been described in patients with DOCK8 deficiency and may account for the pleomorphic clinical manifestations of the disease. T cell lymphopenia may affect both $CD4^+$ and $CD8^+$ cells (Zhang et al., 2009) or predominantly the $CD4^+$ subset (Engelhardt et al., 2009; Sanal et al., 2012). Among $CD8^+$ lymphocytes, there is marked reduction of naïve $CD45RA^+$ $CCR7^+$ cells, and an increased proportion of cells with an "exhausted" $CD45RA^+$ $CCR7^-$ phenotype, typical of effector memory $CD45RA^+$ ($T_{EMRA}$) cells (Randall et al., 2011). Furthermore, both naïve and memory $CD8^+$ cells fail to proliferate in response to CD3/CD28 stimulation, and this defect is not corrected by exogenous IL-2 (Engelhardt et al., 2009; Randall et al., 2011; Zhang et al., 2009). T cell lymphopenia in DOCK8 deficiency may reflect both

reduced thymic output (Dasouki et al., 2011) and impaired survival of peripheral T lymphocytes (Al Khatib et al., 2009). Defective differentiation of $T_H17$ cells may contribute to the increased frequency of chronic mucocutaneous candidiasis (CMC) (Al Khatib et al., 2009; Su, Jing, & Zhang, 2011).

DOCK8 deficiency is also characterized by defects of B cell immunity. The number of circulating B lymphocytes may be reduced (Zhang et al., 2009) or normal (Engelhardt et al., 2009). Laboratory markers of immune dysregulation include markedly elevated serum IgE levels and eosinophilia. By contrast, IgM levels are often low (Engelhardt et al., 2009; Sanal et al., 2012; Zhang et al., 2009). Serum IgG levels can be normal or elevated. Upon immunization with T-dependent antigens, levels of specific antibodies may initially be protective, but often tend to decline with time (Al-Herz et al., 2012; Jabara et al., 2012; Zhang et al., 2009).

Generation of Dock8-deficient mice has further helped define the role played by this protein in T and B cell development, function, and homeostasis. Two models of Dock8 deficiency have been generated by ENU-induced mutagenesis (Randall et al., 2009). In particular, the "*captain morgan*" (*cpm*) mutant carries a homozygous donor splice-site mutation in intron 20 of the *Dock8* gene, causing abnormal splicing, frameshift, and premature termination that eliminates the DHR-2 domain (Randall et al., 2009). The "*primurus*" (*pri*) mutant is homozygous for a missense mutation in exon 43, causing a serine to proline substitution in the DHR-2 domain (Randall et al., 2009). Finally, a third model of Dock8 deficiency has been generated by gene targeting (Harada et al., 2012).

Analysis of the immunological phenotype and function in Dock8-deficient mice has revealed many similarities with the human phenotype. Thymocyte development is largely unaffected (Harada et al., 2012; Randall et al., 2011). Impaired thymic egress (Lambe et al., 2011) in Dock8-deficient mice parallels reduced TREC levels observed in patients (Dasouki et al., 2011). In spite of normal homing of Dock8-deficient T cells (Harada et al., 2012), the number of T lymphocytes in peripheral lymphoid organs is markedly reduced, especially of naïve $CD4^+$ and $CD8^+$ T cells (Harada et al., 2012; Lambe et al., 2011; Randall et al., 2011). However, under pathogen-free conditions, there is no accumulation of $CD8^+$ cells with an exhausted memory phenotype, at variance with what observed in patients (Randall et al., 2011). The T cell lymphopenia of Dock8-deficient mice is cell autonomous and reflects poor T cell survival (Lambe et al., 2011; Randall et al., 2011). Reduced expression of CD127 (IL-7R$\alpha$) on the surface of naïve $CD4^+$ and $CD8^+$ T cells from

*Dock8*$^{pri/pri}$ mice might contribute to impaired IL-7-dependent survival signaling (Randall et al., 2011).

Dock8 binding to Cdc42 through the DHR-2 domain is important for localizing activation of Cdc42 at the leading edge of DCs in response to chemokine stimulation (Harada et al., 2012). In the absence of Dock8, DCs are impaired in their ability to move in the dermal interstitium and to transmigrate through the subcapsular sinus of lymph nodes (Harada et al., 2012). Consequently, they fail to accumulate in draining lymph nodes, causing impaired T cell priming and defective generation of memory $CD8^+$ T cell responses in response to viral infections (Lambe et al., 2011; Randall et al., 2011).

The increased occurrence of viral infections in DOCK8-deficient patients may also reflect impaired cell-mediated cytotoxicity. Although Dock8-deficient mouse cytotoxic T lymphocytes (CTLs) have no obvious killing defect (Randall et al., 2011), it has been recently shown that DOCK8 deficiency causes defective actin accumulation at the lytic immunological synapse and impairs human NK cell function (Mizesko et al., 2013). Importantly, this defect was not associated with a global deficiency of F-actin. Furthermore, defective NK cytolytic function could be restored by addition of exogenous IL-2 (Mizesko et al., 2013). Finally, depletion of DOCK8 in human NK cell lines caused a significant reduction in their cytolytic activity (Ham et al., 2013). Similar defects of F-actin cytoskeleton reorganization, NK cell-mediated cytotoxicity, and DC trafficking, have been reported also in patients with the Wiskott–Aldrich syndrome (WAS), that shares several features with DOCK8 deficiency, including increased susceptibility to viral infections and immune dysregulation (Thrasher & Burns, 2010). Interestingly, also the WAS protein (WASp) interacts with Cdc42 (Aspenstrom, Lindberg, & Hall, 1996), and recent data indicate that DOCK8 exists in a macromolecular complex with WASp and talin, a protein required for integrin-mediated adhesion (Ham et al., 2013).

Dock8 deficiency may also lead to global abnormalities of $T_H$ cell differentiation. In particular, stimulation of naïve $CD4^+$ lymphocytes under $T_H2$ polarizing conditions leads to a higher fraction of IL-4 expressing cells in Dock8-deficient mice than in wild-type animals (Lambe et al., 2011). This is reminiscent of the unbalanced and increased secretion of IL-4 (vs. IFN-$\gamma$ and IL-2) upon stimulation of peripheral blood lymphocytes from DOCK8-deficient patients with PHA (Al-Herz et al., 2012). Overall, this skewing to $T_H2$ responses may play a role in the hyper-IgE phenotype and immune dysregulation of the disease.

Finally, DOCK8 plays an important role also in B cell immunity. Lack of the marginal zone, severe reduction of peritoneal B-1 B cells, and atrophy of germinal centers have been reported in Dock8-deficient mice (Randall, Lambe, Goodnow, & Cornall, 2010; Randall et al., 2009). Although BCR-mediated signaling is apparently intact in these mice, persistence and survival of germinal center B lymphocytes and development of long-lasting high-affinity memory antibody responses are profoundly impaired, probably because Dock8-deficient B cells fail to form a proper immunological synapse with antigen-presenting follicular dendritic cells (FDCs) (Randall et al., 2010, 2009). Similarly, although immunization may initially lead to normal specific antibody production, impaired maturation and lack of sustained antibody responses have been observed also in patients and in mice with DOCK8 deficiency (Al-Herz et al., 2012; Jabara et al., 2012). Besides impaired interaction with FDC, this defect may reflect B cell-intrinsic abnormalities. In particular, it has been recently shown that DOCK8 functions as an adaptor that links TLR9-MyD88 signaling to B cell activation (Jabara et al., 2012). DOCK8 is constitutively associated with MyD88 in B lymphocytes. TLR9 stimulation leads to Pyk2-mediated tyrosine phosphorylation of DOCK8, allowing recruitment of Src and/or LYN, and phosphorylation of SYK and STAT3 (Jabara et al., 2012). Inability to trigger this signaling pathway causes defects of B lymphocyte proliferation and *in vitro* production of IgM and IgG in response to *in vitro* stimulation with CpG-induced in DOCK8-deficient patients (Jabara et al., 2012).

## 3.8. CARD11 deficiency

Caspase recruitment domain 11 (CARD11), also known as caspase recruitment domain membrane-associated guanylate kinase protein 1 (CARMA1) is a member of a family of membrane-associated guanylate kinases, and acts as a scaffold protein that facilitates formation of macromolecular complexes that integrate TCR- and BCR-mediated signaling and promote activation of the canonical NF-κB signaling pathway (Bertin et al., 2001). It is predominantly expressed in hematopoietic and lymphoid tissues, such as spleen, thymus, and peripheral leukocytes (Bertin et al., 2001). Following antigen-receptor engagement and activation of PLC-γ, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) is converted into inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). The latter activates protein kinase C (PKC)-β in B lymphocytes and PKC-θ in T lymphocytes. PKC

phosphorylates the linker region of CARD11 (Matsumoto et al., 2005; Sommer et al., 2005), inducing conformational changes that enable CARD11 to associate with B cell lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (McCully & Pomerantz, 2008). The CARD11–BCL10–MALT1 (CBM) complex recruits the tumor necrosis factor receptor-associated factor 6 (TRAF6) protein, which activates the IκB kinase (IKK) complex that phosphorylates the inhibitor IκBα, resulting in its phosphorylation and ubiquitination. This releases subunits of NF-κB from IκBα. NF-κB dimers can thus translocate to the nucleus and bind to consensus sequences of target genes thereby mediating their transcription (Fig. 4.2). In particular, CARD11 binds to the regulatory subunit IKK-γ (also known as NF-κB essential modulator, NEMO) (Stilo et al., 2004) and modulates its polyubiquitination following antigen-receptor stimulation (Shambharkar et al., 2007). This modification is essential for IKK kinase activity (Shambharkar et al., 2007).

A role for the CBM complex in immune function and homeostasis was further demonstrated when somatic gain-of-function mutations of CARD11, and chromosomal translocations involving BCL10 and MALT1 were identified in patients with diffuse large B cell lymphoma and MALT lymphoma (Shaffer, Young, & Staudt, 2012). Interestingly, introduction of CARD11 point mutations found in lymphoma into antigen-activated mature B lymphocytes in mice determined a switch from self-antigen-induced B cell death to T cell-independent proliferation, indicating that CARD11 acts also as a modulator of B cell tolerance (Jeelall et al., 2012).

More recently, biallelic loss-of-function mutations of *CARD11* have been identified in patients with combined immunodeficiency. Stepensky et al. have described a 13-month-old female infant, born to consanguineous parents, who presented with recurrent infections, including *P. jiroveci* pneumonia, and progressive panhypogammaglobulinemia (Stepensky et al., 2013). Similarly, Greil et al. reported the case of a 6-month-old female born to consanguineous parents, who presented with *P. jiroveci* pneumonia, and agammaglobulinemia (Greil et al., 2013). Both patients had a normal number of T and B lymphocytes in the periphery, with a polyclonal TCR repertoire and preserved levels of TRECs and of kappa receptor excision circles, indicating that generation of T and B lymphocytes was not affected. However, B cells were immature, with an increased proportion of transitional B lymphocytes (Greil et al., 2013; Stepensky et al., 2013). Furthermore, both naïve and transitional B lymphocytes expressed lower amounts of

B cell activating factor receptor (BAFF-R) (Stepensky et al., 2013). In both cases, significant abnormalities were identified in the T cell compartment. In particular, both infants had markedly decreased number of circulating Treg cells. *In vitro* proliferation of T lymphocytes to soluble anti-CD3 was abolished. Stimulation of T and B lymphocytes with PMA and ionomycin resulted in defective IκBα degradation, reduced p65 phosphorylation and nuclear translocation, and impaired cytokine production as compared to what observed in healthy controls. Moreover, T lymphocytes activated *in vitro* with PMA and ionomycin, or with soluble anti-CD3/CD28, produced reduced amounts of IL-2, and failed to upregulate expression of ICOS, CD25, and OX40. Similarly, stimulation of B lymphocytes with anti-IgM led to reduced expression of CD25 and ICAM-1 as compared to controls (Greil et al., 2013; Stepensky et al., 2013). These data were strongly indicative of defective NF-κB activation. WES revealed biallelic *CARD11* mutations in both patients, in particular a nonsense mutation (Q945*) (Greil et al., 2013), and a deletion of exon 21 (Stepensky et al., 2013). Introduction of the Q945* mutation into CARD11-deficient JPM50.6 Jurkat T cell line resulted in inability to activate NF-κB and markedly defective IL-2 production (Greil et al., 2013). *Card11*$^{-/-}$ mice and *unmodulated* mice (carrying a hypomorphic *Card11* mutation), show a similar phenotype, with selective defect in NF-κB activation, decreased proliferation and impaired cytokine production in response to TCR/BCR stimulation, hypogammaglobulinemia, and poor antibody responses to T-dependent and T-independent antigens (Hara et al., 2003; Jun et al., 2003). Overall, these observations identify germline loss-of-function mutations of *CARD11* as a novel cause of combined immunodeficiency with dysfunctional T and B lymphocytes.

Interestingly, germline heterozygous gain-of-function mutations in *CARD11* have been also shown to cause T and B cell abnormalities. In particular, Snow et al. reported four patients from two families, who presented with splenomegaly and B cell lymphocytosis. Immunological investigations revealed increased number of polyclonal late transitional B cells in the periphery, enhanced B cell proliferation *in vitro* in response to IgM stimulation, and increased nuclear translocation of p65 protein in circulating B and T lymphocytes (Snow et al., 2012). The accumulation of late transitional B cells in the periphery did not reflect increased proliferation or enhanced survival, and was likely due to elevated output from the bone marrow. Histological examination of lymphoid tissues showed prominent primary follicles, with atrophic germinal centers. The patients had a reduced

number of circulating memory B cells and failed to mount antibody responses to polysaccharide antigens. Moreover, differentiation of the patients' B lymphocytes into plasmablasts *in vitro* was impaired.

Use of massively parallel mRNA sequencing allowed identification of a heterozygous E127G mutation of *CARD11* in affected patients from the first family. Introduction of this mutant into CARD11-deficient JPM50.6 T cells resulted in constitutive activation of NF-κB. The affected patient from the second family was found to carry a heterozygous G116S CARD11 mutation, which had been previously reported to be a gain-of-function somatic mutant in a DLBCL tumor (Lenz et al., 2008). In spite of the activating nature of the CARD11 mutations, the patients' T lymphocytes showed defective proliferation, reduced expression of CD25 and CD69, and impaired production of IL-2 in response to stimulation with soluble anti-CD3/CD28. Introduction of the E127G mutant into normal T cells led to increased CD69 expression and IL-2 production; however, when the cells were stimulated via CD3/CD28, they produced significantly less IL-2 and showed reduced proliferation as compared to control transfectants (Snow et al., 2012). These data indicate that germline heterozygous *CARD11* gain-of-function mutations cause constitutive NF-κB signaling that promotes increased release of transitional B cells from the bone marrow, selective expansion of transitional and naïve peripheral B cells, associated with inability to maintain a memory B cell pool and induction of T cell anergy. This condition has been also referred to as "B cell expansion with NF-κB and T cell anergy" syndrome (Snow et al., 2012). Altogether, these data demonstrate that both loss-of-function and gain-of-function mutations of CARD11 interfere with T cell function.

## 3.9. MALT1 deficiency

MALT1 is a caspase-like cysteine protease that is recruited in a CARD/BCL10/MALT1 (CBM) complex that contains BCL10 and scaffolding proteins that contain a CARD and a coiled-coil domain, such as CARD9, CARD10, or CARD11 (Thome, 2008). The CBM complex recruits and activates TRAF6, thereby permitting NF-κB signaling in response to activation of the TCR (Fig. 4.2), BCR, some G-protein-coupled receptors, and other receptors containing ITAM motifs.

The role of MALT1 in NF-κB signaling has been further illustrated by observations in tumors. In particular, a t(11;18)(q21;q21) translocation in extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (Levine et al., 1989) results in formation of a chimeric molecule containing

the N-terminus of the cellular inhibitor of apoptosis 2 (API2) and the C-terminus of MALT1. This API2-MALT1 fusion protein undergoes spontaneous oligomerization, thereby causing constitutive activation of NF-κB (Garrison, Samuel, & Reed, 2009).

Recently, a homozygous loss-of-function MALT1 mutation has been identified in patients with clinical features of combined immunodeficiency belonging to a consanguineous family (Jabara et al., 2013). The clinical features of the disease included early-onset infections due to bacteria, viruses, and *Candida* spp. The patients had a normal number of T and B lymphocytes and normal distribution of naïve and memory T cells. However, *in vitro* proliferation to anti-CD3 and antigens was markedly reduced. Immunoglobulin serum levels were normal, but the patients failed to mount protective antibody responses to tetanus toxoid and *S. pneumoniae* after immunization. Moreover, isohemagglutinin levels were also low. WGS identified a homozygous S89I mutation in the N-terminus CARD domain of MALT1 that disrupted protein stability leading to lack of protein expression. Stimulation of the patient's PHA blast with PMA and ionomycin failed to induce IκBα degradation and to promote IL-2 expression, consistent with a defect of NF-κB signaling. Reconstitution experiments based on retrovirus-mediated gene transfer into primary T cells from *Malt1*$^{-/-}$ mice showed that wild-type MALT1, but not the S89I mutant, was able to rescue IκBα degradation and IL-2 expression (Jabara et al., 2013).

These data share many similarities with previous observations in *Malt1*$^{-/-}$ mice, including impaired NF-κB induction and defective *in vitro* proliferation upon stimulation of T cells via the TCR/CD3 complex or with PMA and ionomycin (Ruland, Duncan, Wakeham, & Mak, 2003). BAFF regulates MZ B cell survival and is required for optimal antibody responses to T-dependent and T-independent antigens. While BAFF-R signaling was not directly investigated in MALT1-deficient patients, it is impaired in *Malt1*$^{-/-}$ mice, and this defect is associated with a severe reduction of MZ B cells and peritoneal B1 cells, which produce antibodies to type II T-independent antigens, and natural antibodies respectively, and with impaired antibody response to T-dependent antigens (Tusche et al., 2009). Overall, these data indicate that MALT1 is required for TCR-mediated NF-κB signaling, cell proliferation, and IL-2 production. Moreover, MALT-1 is dispensable for BCR-induced B cell activation, but plays an important role in BAFF-R-mediated signaling.

In addition to T and B lymphocytes, other hematopoietic cells are likely affected by *MALT1* mutations, and these defects may also contribute to the

clinical features of the disease, such as susceptibility to infections with *C. albicans*. In particular, MALT1 associates with CARD9 and BCL10, linking the β-glucan receptor dectin-1 and the α-mannan receptor dectin-2 to NF-κB activation in DCs and macrophages (Geijtenbeek & Gringhuis, 2009). *CARD9* mutations cause CMC in humans (Glocker et al., 2009). Furthermore, MALT1 promotes also dectin-1- and dectin-2-dependent production of the $T_H17$-polarizing cytokines IL-1β and IL23 by DCs stimulated with *C. albicans* (Gringhuis et al., 2011). $T_H17$ lymphocytes play also an important role in immune defense against candida, as shown by patients with CMC and mutations in IL-17 or IL-17Rα (Puel et al., 2011). Overall, the clinical phenotype of MALT1-deficient patients shares many similarities with that of CARD11 deficiency, but the broader spectrum of infections suggests that in addition to TCR- and BCR-induced signaling, other NF-κB-dependent pathways are also affected in MALT1 deficient patients.

## 3.10. IκBα mutations causing defective protein degradation and impaired NF-κB signaling

The transcription factor NF-κB plays a major role in regulating T cell activation and effector functions in response to TCR engagement (Ruland & Mak, 2003). NF-κB is composed of multiple proteins, including p50, p65 (also known as RelA), RelB, c-Rel, and p52 (and its precursor p100). These proteins form homo- and heterodimers; upon activation, they translocate to the nucleus where they bind to NF-κB consensus sequences of gene, inducing their transcription. Under resting conditions, NF-kB is restrained by sequestration of NF-κB molecules in the cytoplasm by inhibitory of NF-κB (IκB) proteins, which include IκB-α, IκB-β, and IκB-ε (Bonizzi & Karin, 2004). TCR-mediated signaling results in activation of the CBM complex, recruitment of TRAF6, and activation of the IKK complex, which includes the catalytic IKKα and IKKβ proteins, and the regulatory subunit IKKγ (Fig. 4.2). Activation of IKK proteins induces phosphorylation and ubiquitin-mediated degradation of the IκB proteins, thereby releasing NF-κB molecules and allowing their nuclear translocation. Besides TCR and BCR engagement, the IKK complex can be activated also by many other extracellular signals that include toll-like receptor, IL-1βR and IL-18R (TIR), and members of the tumor necrosis factor-α receptor (TNFR) superfamily, among which the ectodysplasin A receptor and the receptor activator of NF-κB (RANK). Finally, NF-κB is activated also in response to vascular endothelial growth factor receptor. The IKK-dependent

activation of NF-κB is also known as the canonical pathway of NF-κB signaling. An alternative, noncanonical pathway, exists that is triggered by differentiation and developmental stimuli, such as lymphotoxin-α, BAFF, and RANK ligand. These signals induce activation of the NF-κB-inducing kinase which leads to phosphorylation and processing of the NF-κB precursor subunit p100, ultimately generating p52 that can dimerize with RelB and translocate to the nucleus (Bonizzi & Karin, 2004).

Mutations of the inhibitor of NF-κB kinase subunit gamma (*IKBKG*) gene, also known as *NEMO* and encoding for IKK-γ, cause a variety of clinical syndromes. In particular, heterozygosity for null mutations of this gene causes incontinentia pigmenti in females and is embryonically lethal in males. By contrast, hypomorphic mutations of the *IKBKG* gene in males cause a complex phenotype, which includes X-linked recessive anhidrotic ectodermal dystrophy and immunodeficiency (XR-EDA-ID), with increased susceptibility to bacterial and mycobacterial infections, defective production to polysaccharide antigens, and a variable degree of hypogammaglobulinemia (Doffinger et al., 2001; Zonana et al., 2000). In a minority of these patients, osteopetrosis and/or lymphatic vessel abnormalities leading to lymphedema (XR-EDA-ID-OL) may also be present (Doffinger et al., 2001; Dupuis-Girod et al., 2002). Finally, some patients may present with immunodeficiency without or with modest signs only of ectodermal dystrophy (Mooster et al., 2010; Niehues et al., 2004; Puel et al., 2006). This heterogeneity of clinical manifestations reflects, at least in part, a different effect of *IKBKG* mutations on NF-κB activation (Hanson et al., 2008). Although several immune pathways are altered in patients with XR-EDA-ID, the number and function of T lymphocytes are usually preserved. In a series of patients reported into a database, mitogen- and antigen-induced proliferative responses were normal in 91% and 76% of the cases, respectively (Hanson et al., 2008).

By contrast, a profound T cell immunodeficiency has been observed in several patients with heterozygous, gain-of-function mutations of the *IKBA* gene, encoding for IκB-α (Courtois et al., 2003; Janssen et al., 2004; Lopez-Granados et al., 2008; McDonald et al., 2007; Ohnishi et al., 2012; Schimke et al., 2013). Under normal conditions, stimuli that activated the canonical pathway of NF-κB signaling induce IKK-mediated phosphorylation of the IκB-α protein at two critical residues, serine 32 and serine 36 (DiDonato et al., 1996). This triggers IκB-α polyubiquitination of residues Lys21 and Lys22 and subsequent protein degradation (Karin & Ben-Neriah, 2000), thus allowing nuclear translocation of NF-κB dimers.

The *IKBA* mutations identified in patients with EDA-ID interfere with IκB-α serine phosphorylation. In particular, Courtois et al. have described a patient with a heterozygous S32I mutation that caused dramatic reduction of Ser32 phosphorylation and impeded IκB-α degradation in TNF-stimulated patient-derived fibroblasts (Courtois et al., 2003). The same mutation was also reported by Janssen et al. in a father and in one of his children (Janssen et al., 2004). Two additional patients were shown to carry heterozygous nonsense mutations (W11X and E14X) that result in usage of downstream translation initiation site at codon M37, thereby deleting the N-terminus 36 amino acid residues that contain the two serine residues whose phosphorylation is essential for IκB-α polyubiquitination and degradation (McDonald et al., 2007; Ohnishi et al., 2012). Another patient was demonstrated to be heterozygous for an even more 5′ premature termination (Q9X) (Lopez-Granados et al., 2008). This mutant allele was associated with production of two distinct forms of N-terminus-deleted *IKBA* transcript, one lacking the first 12 codons, and the other one lacking the first 36 codons. In all of these six patients, defective serine phosphorylation of the mutant IκB-α serine phosphorylation was associated with resistance to degradation, impaired activation and nuclear binding of NF-κB, and defective transcription of NF-κB-dependent genes. More recently, Schimke et al. have reported a patient with heterozygous M37K mutation that several bioinformatics tools predicted to be deleterious (Schimke et al., 2013). Although the authors did not analyze the effect of this mutation on activation-induced IκB-α serine phosphorylation, they demonstrated defective IκB-α degradation, NF-κB nuclear translocation, and transcriptional activation in the patient's cells upon appropriate stimulation *in vitro*.

The clinical features of patients with heterozygous, gain-of-function *IKBA* mutations share similarities with the EDA-ID phenotype observed in patients with *IKBKG* defects, but also important differences. Patients with *IKBA* mutations present typical features of ectodermal dystrophy and show severe defect of NF-κB activation in response to stimulation via TIR or TNFR. However, their clinical history is characterized by severe, early-onset infections, including *P. jiroveci* pneumonia, CMC, hepatitis due to *Cytomegalovirus*, often associated with chronic diarrhea and failure to thrive (Courtois et al., 2003; Janssen et al., 2004; Lopez-Granados et al., 2008; Ohnishi et al., 2012; Schimke et al., 2013). These features are not typically observed in patients with XR-EDA-ID and are highly suggestive of a significant T cell defect. Although the total number of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes is preserved (and in some cases is even increased),

accumulation of cells with a $CD45RA^+$ phenotype, and marked reduction of $CD45R0^+$ memory T lymphocytes, has been reported in the majority of the patients (Courtois et al., 2003; Janssen et al., 2004; Ohnishi et al., 2012; Schimke et al., 2013). Decreased number of $T_H17$ cells has been also observed and may contribute to impaired resistance to pathogens (Schimke et al., 2013). In most cases, *in vitro* lymphocyte proliferation to PHA is normal, however proliferative response to anti-CD3 and to recall antigens is impaired (Courtois et al., 2003; Schimke et al., 2013), and production of IFN-γ in response to anti-CD3 is markedly reduced (Lopez-Granados et al., 2008). However, variability of T cell function has been observed, with some patients showing defective proliferation also to mitogens (Janssen et al., 2004; Ohnishi et al., 2012), and others normal T cell proliferation to all stimuli tested (Lopez-Granados et al., 2008; McDonald et al., 2007). Phenotypic variability has been observed also within the same family. In the case of the father and child carrying the S32I mutation, partially preserved response to anti-CD3, and of NF-κB nuclear translocation in response to activating signals in the father's cells, were associated with a milder clinical course of the disease (Janssen et al., 2004). Interestingly, somatic reversion restoring the wild-type *IKBA* sequence was demonstrated in the father's peripheral blood mononuclear cells and was especially frequent within T and B lymphocytes. In particular, while a mutant allele was identified in the naïve T cell population, only the wild-type allele was detected in memory T cells (Janssen et al., 2004). Somatic mosaicism has been also described in patients with *IKBKG* mutations and in carrier females of XR-EDA-ID (Kawai, Nishikomori, et al., 2012). Also in these cases, a higher fraction of cells with somatic gene reversion has been identified within the memory T cell compartment, suggesting that NF-κB signaling confers a selective advantage for memory T cell development and/or survival.

## 3.11. Defects of store-operated $Ca^{2+}$ entry: STIM1 and ORAI1 deficiencies

$Ca^{2+}$ signaling plays a critical role in activation and homeostasis of multiple cell types, including nonexcitable cells of the immune system, such as T, B, and NK lymphocytes, DCs, and mast cells (Vig & Kinet, 2009). In T lymphocytes, under resting conditions, higher $Ca^{2+}$ concentrations (0.4–1 mM) are present in the endoplasmic reticulum (ER) than in cytoplasm, where $[Ca^{2+}]$ is ~50–100 nM. A much higher $Ca^{2+}$ concentration (~2 mM) is present in the extracellular milieu, thereby determining a

progressive $Ca^{2+}$ gradient between the extracellular milieu, the ER, and the cytoplasm. TCR stimulation activates Lck and ZAP-70, allowing formation of the LAT signalosome and activation of PLC-γ1. Similarly, in B lymphocytes, stimulation of the BCR leads to activation of Syk, formation of a signalosome complex that includes the B cell linked adaptor protein and the Bruton tyrosine kinase, and activation of PLC-γ2. In both T and B lymphocytes, activation of PLC-γ mediates conversion of $PIP_2$ into DAG and $InsP_3$. The latter binds to and opens $InsP_3$ receptor channels in the membrane of the ER causing efflux of $Ca^{2+}$ from the ER stores and a reduction of $Ca^{2+}$ concentration in the ER. STIM1 and STIM2 are $Ca^{2+}$-binding ER transmembrane proteins that sense depletion of $Ca^{2+}$ stores contained in the ER. In resting cells, STIM proteins are diffusely distributed throughout the ER membrane (Zhang et al., 2005). Following depletion of ER $Ca^{2+}$ stores, STIM proteins oligomerize. This process is mediated by coiled-coil domains at the cytoplasmic C-terminus of the molecule (Cahalan, 2009). STIM oligomers translocate to the plasma membrane-proximal ER and form protein clusters known as STIM puncta (Wu, Buchanan, Luik, & Lewis, 2006), allowing binding to N- and C-termini of the ORAI1 molecule, opening of the $Ca^{2+}$-release-activated-$Ca^{2+}$ (CRAC) channels, and entry of $Ca^{2+}$ from the extracellular milieu (Fig. 4.2). (McNally, Somasundaram, Jairaman, Yamashita, & Prakriya, 2013). Both STIM1 and STIM2 may participate at activation of the CRAC channels, however these proteins have a different pattern of expression and display a different kinetics of oligomerization in response to depletion of $Ca^{2+}$ ER stores. In particular, STIM1 is constitutively expressed in naïve T cells, whereas STIM2 is induced upon differentiation into T helper cells (152). Importantly, STIM1 oligomerization follows a rapid kinetics in response to antigen receptor-mediated signals that induce a significant depletion of $Ca^{2+}$ ER concentrations ($<0.4$ μM). By contrast, STIM2 oligomerization occurs more slowly and is triggered by smaller variations of ER $Ca^{2+}$ concentration ($<0.8$ μM) (Oh-Hora et al., 2008). These data suggest that while STIM1 is activated in response to signals that require rapid $Ca^{2+}$ signaling, STIM2 activation is meant to sustain prolonged store-operated $Ca^{2+}$ entry (SOCE) function by facilitating filling of $Ca^{2+}$ stores in the ER.

The ORAI1 protein (ORAI1, -2, and -3) forms the pore of CRAC channels on the cell membrane. They are tetraspanning membrane proteins, with N- and C-terminus intracellular domains. ORAI1 is more abundantly expressed in T lymphocytes, but is also expressed in B and NK lymphocytes,

DCs, neutrophils, mast cells, platelets, skeletal muscle cells, and in various secretory epithelial cells of exocrine tissues (McCarl et al., 2009). In contrast, ORAI2 and ORAI3 have a more restricted pattern of expression, and there is limited functional data available to demonstrate a role for ORAI2 and ORAI3 in immune cells (Feske, Skolnik, & Prakriya, 2012; McCarl et al., 2009). Crystal structure of Orai from *Drosophila melanogaster* has shown that the calcium channel is composed of a hexameric assembly of Orai subunits arranged around a central ion pore that crosses the membrane and extends into the cytosol (Hou, Pedi, Diver, & Long, 2012). A ring of glutamate residues on the extracellular side of the Orai proteins forms the selectivity filter, whereas a basic region near the intracellular side can bind anions that may stabilize the closed state of the channel. The architecture of the CRAC channel presents marked differences in comparison to other ion channels, justifying selective permeability to $Ca^{2+}$ (Hou et al., 2012). The first transmembrane domain of ORAI1 contains a negatively charged amino acid residue (E106) which functions as a $Ca^{2+}$-binding site in the CRAC channel pore (Prakriya et al., 2006). STIM-induced opening of the CRAC channels determines $Ca^{2+}$ influx from the extracellular milieu. The resulting increase in $Ca^{2+}$ intracellular concentration triggers activation of downstream signaling pathways, such as PKC, ERKs, NFAT, cAMP response element binding, activating transcription factor 2, and myeloid elf1-like factor 2. Eventually, this cascade controls a variety of cellular processes in lymphocytes, including cell proliferation, differentiation, cytotoxicity, and cytokine production (Shaw & Feske, 2012).

Within the immune system, activation of SOCE plays an important role not only in T and B lymphocytes, but also in other cell types. In particular, it induces the production of leukotriene C4 and the release of histamine in response to FcεRI engagement in mast cells (Baba et al., 2008; Vig et al., 2008); it facilitates maturation of DCs (Felix et al., 2013); it induces production of reactive oxygen species, cell adhesion, and motility in neutrophils (Brechard, Plancon, Melchior, & Tschirhart, 2009; Schaff et al., 2010); and it promotes FcγR-mediated oxidase activity (Steinckwich, Schenten, Melchior, Brechard, & Tschirhart, 2011) and phagocytosis of opsonized particles by macrophages (Braun et al., 2009).

Genetic studies in patients with mutations in *ORAI1* or *STIM1* and in mice in which genes encoding for various components of the SOCE system have been targeted, have established nonredundant roles for CRAC channels in the immune system (Feske et al., 2012). In particular, biallelic loss-of-function mutations of the *ORAI1* and *STIM1* genes in humans are inherited

as autosomal recessive traits and are characterized by clinical features of immunodeficiency, autoimmunity, nonprogressive myopathy, and other extra-immune manifestations (Feske et al., 2006; Picard, McCarl, et al., 2009). Overall, there is substantial clinical overlap between ORAI1 and STIM1 deficiencies. Early-onset infections due to bacterial, fungal, and viral pathogens (especially herpes viruses) are almost invariably present. One patient with STIM1 deficiency has been reported, who developed Kaposi sarcoma associated with HHV8 infection (Byun et al., 2010). Manifestations of immune dysregulation include autoimmune cytopenias and lymphoid proliferation. These features are especially prominent in patients with STIM1 deficiency (Fuchs et al., 2012; Picard, McCarl, et al., 2009), but have been reported also in patients with ORAI1 deficiency (McCarl et al., 2009). Extra-immune manifestations of the disease include congenital myopathy, and ectodermal dysplasia, manifesting with anhidrosis, defects of dental enamel calcification, and partial iris hypoplasia (Feske, Picard, & Fischer, 2010; McCarl et al., 2009; Picard, McCarl, et al., 2009).

The spectrum of symptoms observed in patients with ORAI1 or STIM1 deficiency reflects abnormalities of $Ca^{2+}$ signaling in various cell types, and studies in gene-targeted mice have confirmed these defects. Neither STIM1 nor ORAI1 deficiency affects development of T, B, and NK lymphocytes. However, both in humans and in mice, deficiency of ORAI1 or STIM1 impairs NFAT activation (Feske, Giltnane, Dolmetsch, Staudt, & Rao, 2001; Oh-Hora et al., 2008), causing defective production of many cytokines by $CD4^+$ and $CD8^+$ T lymphocytes, including IL-2, IL-17, IFN-γ, and TNF-α (Feske et al., 2001, 1996; Feske, Prakriya, Rao, & Lewis, 2005; Fuchs et al., 2012; Ma, McCarl, Khalil, Luthy, & Feske, 2010; McCarl et al., 2010; Oh-Hora et al., 2008). Delayed-type hypersensitivity responses upon challenge with recall antigens are defective in patients (Feske et al., 1996). T cell proliferation in response to mitogens or antigens is markedly reduced in patients with STIM1 or ORAI1 deficiency (Feske et al., 1996; Le Deist et al., 1995; McCarl et al., 2009; Picard, McCarl, et al., 2009). By contrast, *Orai1*$^{-/-}$ and *Stim1*$^{-/-}$ naïve T cells proliferate normally in response to CD3 stimulation (Beyersdorf et al., 2009; Gwack et al., 2008; McCarl et al., 2010). However, T lymphocyte proliferative responses are markedly defective in *Stim1*$^{-/-}$ *Stim2*$^{-/-}$ mice (McCarl et al., 2010; Oh-Hora et al., 2008).

Susceptibility to viral infections in patients with defects of SOCE suggests impaired function of $CD8^+$ and/or NK lymphocytes. Generation of virus-specific $CD8^+$ T lymphocytes is not compromised by *ORAI1* or

*STIM1* mutations. However, release of cytolytic granules and production of inflammatory cytokines (IFN-γ, TNF-α) upon recognition of target cells are markedly reduced in patients with *ORAI1* or *STIM1* defects (Feske et al., 2005; Fuchs et al., 2012)(158, 176), and these abnormalities are associated with complete lack of SOCE activity (Feske et al., 2005; Picard, McCarl, et al., 2009). By contrast, residual $Ca^{2+}$ influx has been reported in naïve $CD8^+$ lymphocytes from *Orai1*$^{-/-}$ or *Stim1*$^{-/-}$ mice (Gwack et al., 2008; McCarl et al., 2010; Oh-Hora et al., 2008). However, SOCE function is abolished in $CD8^+$ lymphocytes from *Stim1*$^{-/-}$ *Stim2*$^{-/-}$ mice (Oh-Hora et al., 2008). Overall, these data indicate some redundancy of function between Stim1 and Stim2 in mice, but not in humans.

The function of NK cells is also probably affected by *ORAI1* or *STIM1* mutations, and this defect may contribute to susceptibility to viral infections. In particular, patients' NK lymphocytes produced reduced amounts of IFN-γ, TNF-α, and CCL2 and failed to degranulate and kill target cells (Fuchs et al., 2012; Maul-Pavicic et al., 2011). Finally, virtual absence of NKT lymphocytes has been demonstrated in a patient with STIM1 deficiency (Fuchs et al., 2012) (158). By analogy with what reported in patients with X-linked lymphoproliferative disease (Nichols et al., 2005; Pasquier et al., 2005), lack of NKT cells may contribute to the increased susceptibility to infections due to herpesviruses in patients with *ORAI1* or *STIM1* mutations.

Recent data indicate that CRAC channels are important also for peripheral T cell homing. In particular, T lymphocytes expressing a dominant-negative Orai1 E106A mutant fail to activate high-affinity LFA-1 integrin and to migrate across endothelial cells (Greenberg et al., 2013). Similar trafficking defects *in vivo* may compromise the ability of T lymphocytes to recognize and respond to pathogens.

Autoimmune manifestations in patients with *ORAI1* or *STIM1* deficiency have been attributed to a possible defect of Treg development and/or function. In particular, a reduced number of circulating $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells has been reported in some STIM1-deficient patients (Picard, McCarl, et al., 2009) and in *Stim1*$^{-/-}$ *Stim2*$^{-/-}$ mice (Oh-Hora et al., 2008). A normal number of Treg cells have been documented in other patients with STIM1 deficiency, but the majority of their $FOXP3^+$ cells had an activated ($CD45R0^+$ $CD39^-$) phenotype, and many of them did not express CD25 (Fuchs et al., 2012). Defects of CRAC channels cause impaired activation of NFAT and reduced IL-2 production, and this may affect Treg cell function. Indeed, *Stim1*$^{-/-}$ *Stim2*$^{-/-}$ Treg cells have reduced suppressive activity, and adoptive transfer of wild-type Treg

lymphocytes into *Stim1*$^{-/-}$ *Stim2*$^{-/-}$ mice rescues the autoimmune phenotype (Oh-Hora et al., 2008). However, development and function of Treg lymphocytes is minimally altered in *Orai1*$^{-/-}$ and *Stim1*$^{-/-}$ mice, and this is associated with residual $Ca^{2+}$ influx (Beyersdorf et al., 2009; McCarl et al., 2010). It is possible that Orai2, Orai3, and Stim2 may play a partially compensatory role in mice. Moreover, no defects of Treg suppressive activity have been reported in another patient with STIM1 deficiency (Fuchs et al., 2012). Finally, generation of a T cell-specific conditional knockout mouse model of Orai1 deficiency has permitted to demonstrate that CRAC channel inhibition prevents expression of Foxp3 in inducible Treg cells, but not in nTreg lymphocytes (Jin et al., 2013), suggesting a differential impact of ORAI1 and STIM1 deficiency in the generation and function of peripherally induced Treg cells.

Defects of SOCE function may compromise T cell homeostasis also by interfering with the role that TCR signaling plays in maintaining immune tolerance. Strong TCR-mediated signaling in the thymus promotes negative selection of autoreactive T cells, and AICD plays a critical role in peripheral T cell homeostasis (Krammer, Arnold, & Lavrik, 2007). *Orai1*$^{-/-}$ effector $CD4^+$ T cells are resistant to AICD, and this phenomenon is associated with reduced expression of FasL and of some proapoptotic genes (Noxa, Bok, Bax), whereas levels of the antiapoptotic molecule Mcl-1 are increased (Kim et al., 2011). Moreover, *Orai1*$^{-/-}$ mice have an increased number of SP lymphocytes in the thymus, and injection of anti-CD3 monoclonal antibody *in vivo* into these mice induces reduced depletion of T cells both in the thymus and in the periphery and in the thymus of *Orai1*$^{-/-}$ mice (Kim et al., 2011). Overall, these data suggest that both intrathymic negative selection and peripheral deletion of activated T cells are affected by SOCE defects.

$Ca^{2+}$ flux is also elicited in response to BCR activation, and aberrant B lymphocyte responses might also contribute to the immune dysregulation observed in patients with ORAI1 and STIM1 deficiency. Stim1 expression is expressed at higher levels in transitional B cells than in mature naïve B lymphocytes (Yarkoni & Cambier, 2011). Triggering of SOCE promotes apoptosis of self-reactive B cells through a Stim1-dependent mechanism (Limnander et al., 2011). B lymphocytes from mice with defects in Orai, Stim1, or Stim2 show reduced proliferative responses upon BCR stimulation (Gwack et al., 2008; Matsumoto et al., 2011). Moreover, intact SOCE function is required also for optimal production of IL-10 by $CD1d^{hi}$ $CD5^+$ regulatory B lymphocytes. B cell-specific conditional knock-out of Stim1

and Stim2 in mice is associated with reduced production of IL-10 and increased development of experimental autoimmune encephalomyelitis (Matsumoto et al., 2011). On the other hand, B cell number and immunoglobulin serum levels are normal in patients and mice with defects of SOCE (Fuchs et al., 2012; McCarl et al., 2009). Specific antibody responses to T-dependent and T-independent antigens are also unaffected in *Stim1*$^{-/-}$ mice (Beyersdorf et al., 2009) and in *Stim1*$^{flox/flox}$ *Stim2*$^{flox/flox}$ *Mb1-Cre* mice (Matsumoto et al., 2011). On the other hand, impaired antibody production has been observed in some patients (Feske et al., 2010).

Finally, ORAI1 and STIM1 defects affect also nonimmune cells. In particular, ORAI1 and STIM1 proteins in the skeletal muscle allow SOCE function and refilling of sarcoplasmic reticulum $Ca^{2+}$ stores (Stiber et al., 2008), thus explaining the myopathy observed in patients with SOCE defects. Defective integrin activation and degranulation have been observed in platelets from mice carrying the *Orai1* R93W mutation (Bergmeier et al., 2009); however, these abnormalities are associated with modest defects of platelet aggregation and adherence, and no significant bleeding problems have been observed in patients. Inactivation of the *Orai1* gene in zebrafish causes severe heart failure, associated with loss of myofiber integrity and ultrastructural abnormalities of the z-disc in the heart muscle (Volkers et al., 2012). While heart failure has not been reported in patients, longer follow-up of surviving patients may be needed.

## 3.12. Magnesium transporter protein 1 deficiency

$Mg^{2+}$ is the most abundant divalent cation in eukaryotic cells, with an intracellular $Mg^{2+}$ concentration of approximately 10–30 mM. The vast majority (~95%) of intracellular $Mg^{2+}$ is bound to ATP or other molecules, and only 1–5% (0.2–1 mM) is represented by cytosolic-free $Mg^{2+}$ ($[Mg^{2+}]_i$) (Li, Lenardo, & Chaigne-Delalande, 2011). $Mg^{2+}$ is a cofactor for ATP and regulates several enzymes (Romani, 2011). The magnesium transporter 1 (MAGT1) protein selectively conducts $Mg^{2+}$ across the plasma membrane (Zhou & Clapham, 2009). Moreover, it plays an important role also in intracellular signaling. It potentiates T lymphocyte activation in response to PHA, but not ionomycin (Abboud, Scully, Lichtman, Brennan, & Segel, 1985; Modiano, Kelepouris, Kern, & Nowell, 1988). These data suggest that it may play a role in regulating membrane-proximal signaling events.

The important role of $Mg^{2+}$ as a second messenger in the immune system has been elucidated with the identification of defective T cell signaling

in two siblings and a third unrelated patient who shared mutations in the X-linked *MAGT1* gene (Li, Chaigne-Delalande, et al., 2011; Li, Lenardo, et al., 2011). More recently, additional patients with the disease have been identified (Chaigne-Delalande et al., 2013).

*MAGT1* encodes for a membrane-associated transporter with high selectivity for $Mg^{2+}$ (189). Two MAGT1 isoforms have been described: a 335 amino acid form that contains 4 transmembrane domains (190), and a longer (367 amino acids) form with five transmembrane domains and an intracytoplasmic tail that may be involved in signaling (188).

MAGT1 deficiency is characterized by uncontrolled chronic EBV infection often leading to Hodgkin, non-Hodgkin, or Burkitt lymphoma. Polyclonal $EBV^+$ lymphoproliferative disease has been also reported. Moreover, there is increased susceptibility to infections sustained by other viruses or bacteria (Chaigne-Delalande et al., 2013). The disease is also known under the acronym XMEN, for X-linked immunodeficiency with $Mg^{2+}$ defect, EBV infection, and neoplasia (Li, Chaigne-Delalande, et al., 2011).

Immunological studies have shown that the patients with XMEN have CD4 lymphopenia, with a reduced number of $CD4^+$ $CD27^+$ $CD45R0^-$ $CD31^+$ recent thymic emigrant cells (Li, Chaigne-Delalande, et al., 2011). Interestingly, nonrandom X-chromosome inactivation has been demonstrated in T lymphocytes from female carriers (Li, Chaigne-Delalande, et al., 2011), suggesting that $Mg^{2+}$ signaling confers an important advantage for T cell development and/or survival. Upon stimulation via CD3, T lymphocytes show defective activation, with impaired upregulation of CD25, CD69, and CD95; defective nuclear translocation of NFAT and NF-κB; and reduced proliferation (Li, Chaigne-Delalande, et al., 2011). By contrast, T cell activation to PMA and ionomycin is unaffected, suggesting a proximal defect in intracellular signaling. Additional biochemical studies have elucidate the step at which intracellular signaling is altered. In particular, following TCR engagement, T lymphocytes from patients with XMEN show normal phosphorylation of CD3ζ, ZAP70, and LAT (Li, Chaigne-Delalande, et al., 2011). By contrast, activation of PLC-γ1 is impaired, causing decreased generation of $InsP_3$. Consistent with this, $Mg^{2+}$ influx in response to TCR stimulation is abrogated in T lymphocytes from patients with XMEN, and $Ca^{2+}$ flux is also severely decreased (Li, Chaigne-Delalande, et al., 2011).

By contrast, *MAGT1* mutations do not affect B cell activation in response to stimulation via BCR or TLR. Although B cell stimulation via BCR or CD40 is associated with defective $Mg^{2+}$ influx, $Ca^{2+}$ signaling

is not affected (Li, Chaigne-Delalande, et al., 2011). T and B lymphocytes express PLC-γ1 and PLC-γ2, respectively. It is possible that the different effect of MAGT1 deficiency on $Ca^{2+}$ signaling in T and B lymphocytes reflects a selective impact on PLC-γ1, but not PLC-γ2 activation. Consistent with largely preserved B cell activation, levels of immunoglobulins and of specific antibodies upon immunization are either normal or low in patients with XMEN (Li, Chaigne-Delalande, et al., 2011).

In spite of a significant defect in T cell activation, patients develop normal adaptive responses *in vivo*. In particular, following infection with EBV, they generate EBV-specific memory $CD8^+$ T cells (Chaigne-Delalande et al., 2013). However, cytotoxicity against autologous EBV-transformed lymphoblastoid cell lines is defective (Chaigne-Delalande et al., 2013). Moreover, impaired NK cell-mediated cytotoxicity against K562 target cells suggests a more global, non-EBV-restricted defect of cytotoxicity (Chaigne-Delalande et al., 2013).

Defective cell-mediated cytotoxicity in XMEN is associated with markedly reduced expression of NKG2D on the surface of NK and CTLs (Chaigne-Delalande et al., 2013). NKG2D mediates cytotoxic responses (Bryceson & Ljunggren, 2008; Guerra et al., 2008) and is associated with the adapter DAP10, whose expression is also decreased in patients' CTL and NK lymphocytes (Chaigne-Delalande et al., 2013). NKG2D recognizes multiple ligands, including the MHC class I-like proteins MICA and MICB, and the UL16-binding glycoproteins 1–6, whose expression is induced by infection, transformation, and cellular stress (Champsaur & Lanier, 2010). NK lymphocytes from patients with XMEN show defective cytotoxic responses in response to NKG2D engagement (Chaigne-Delalande et al., 2013). Low basal free $[Mg^{2+}]i$ has been observed in peripheral blood mononuclear cells from XMEN patients (Chaigne-Delalande et al., 2013). Culture with $Mg^{2+}$-supplemented medium increases free $[Mg^{2+}]_i$ and results in increased expression of NKG2D on the surface of NK cells and CTLs from XMEN patients. Moreover, the cytotoxicity defect is also partially restored (Chaigne-Delalande et al., 2013), suggesting that chronically low free $[Mg^{2+}]_i$ may cause defective cytotoxicity. By contrast, addition of exogenous $Mg^{2+}$ does not rescue T cell activation in response to TCR stimulation (Chaigne-Delalande et al., 2013). Following oral supplementation with magnesium gluconate, a small but significant increase of NKG2D has been observed on both CTLs and NK lymphocytes from XMEN patients, and this was associated with a decline of the proportion of EBV-infected B lymphocytes. However, upon suspension of treatment,

levels of NKG2D surface expression rapidly drop, and the proportion of EBV-positive B cells rises, indicating that the effect of $Mg^{2+}$ supplementation is reversible and that chronic treatment may be necessary (Chaigne-Delalande et al., 2013).

## 3.13. MST1 deficiency

The macrophage stimulating 1 (MST1), also known as serine threonine kinase 4 was originally described in *D. melanogaster* as part of the Hippo signaling pathway, that controls cell proliferation and apoptosis (Wu, Huang, Dong, & Pan, 2003). Studies in mice have shown that Mst1 plays an important role in thymocyte egress, T cell adhesion, migration, proliferation, and survival (Choi et al., 2009; Dong et al., 2009; Katagiri et al., 2009; Mou et al., 2012; Zhou et al., 2008). Mst1 is expressed at low levels in DP thymocytes, but is upregulated in SP cells (Mou et al., 2012), in which Mst1 is phosphorylated and is found in a 1:1 complex with RAPL, a Rap1-GTP-binding factor (Avruch et al., 2009; Praskova, Khoklatchev, Ortiz-Vega, & Avruch, 2004). Following TCR-mediated or chemokine-induced stimulation, the Mst1-RAPL complex is recruited to the leading edge of the cell, allowing clustering of LFA-1 molecules. SP thymocytes from *Mst1*$^{-/-}$ mice show defects of cytoskeleton rearrangement and of integrin activation, and have impaired *in vitro* chemotactic response to sphingosine-1-phosphate (S1P) and various chemokines, reduced Rac1 GTP charging in response to CCL19, impaired RhoA GTP charging upon activation with S1P, and markedly impaired phosphorylation of Mob1A/B, a direct target of Mst1 and a binding partner to DOCK8 (Dong et al., 2009; Mou et al., 2012). As a result of these abnormalities of TCR- and chemokine-induced activation, thymic egress of lymphocytes is defective in *Mst1*$^{-/-}$ mice. Moreover, there is inefficient migration and antigen recognition of $CD4^+$ thymocytes in the thymic medulla of *Mst1*$^{-/-}$ mice, and this abnormality is associated with impaired negative selection of self-reactive T cells (Ueda et al., 2012). Consistent with this, *Mst1*$^{-/-}$ mice show prominent inflammatory lymphocytic infiltrates in multiple organs and develop autoantibodies (Ueda et al., 2012). Moreover, *Mst1*$^{-/-}$ mice show also defective migration of peripheral lymphocytes, with impaired ability to adhere to high endothelial venules and to enter lymph nodes (Katagiri et al., 2009; Mou et al., 2012; Zhou et al., 2008). Impairment of cell trafficking is also at the basis of a reduced number of MZ B cells and DCs observed in the spleen of *Mst1*$^{-/-}$ mice (Katagiri et al., 2009).

In spite of increased *in vitro* proliferation, the number of naïve T cells is markedly reduced in *Mst1*$^{-/-}$ mice (Zhou et al., 2008). This reflects a critical role played by Mst1 in cell lymphocyte survival. In particular, Foxo1 and Foxo3a are two direct targets of Mst1, and their levels are reduced in peripheral T cells from *Mst1*$^{-/-}$ mice (Choi et al., 2009). Foxo1 controls IL-7Rα expression in naïve T cells (Ouyang, Beckett, Flavell, & Li, 2009), and hence also delivery of IL-7-mediated survival signals. Moreover, expression of a constitutively active form of Foxo3a protects T cells from oxidative stress and apoptosis, and restores the number of peripheral T cells in Mst1-deficient mice (Choi et al., 2009). Finally, Foxo1 controls also the expression of L-selectin and CCR7, and it is possible that this mechanism may also contribute to impaired trafficking of peripheral T cells in *Mst1*$^{-/-}$ mice.

Two groups have reported MST1 deficiency in three families in which affected individuals suffered from a novel form of autosomal recessive combined immunodeficiency (Abdollahpour et al., 2012; Nehme et al., 2012). The clinical features of the disease included recurrent and severe viral and bacterial infections, extensive warts, molluscum contagiosum, mucocutaneous candidiasis, and autoimmunity. An increased risk of EBV-driven lymphoproliferative disease was documented. Heart defects and intermittent neutropenia have been also described.

The immunological phenotype of MST1 deficiency is characterized by reduced T cell count, which is especially pronounced for naïve $CD4^+$ and $CD8^+$ T cells, an increased proportion of $CD8^+$ lymphocytes with a $CD45RA^+$ $CCR7^-$ $CD127^-$ phenotype ($T_{EMRA}$), and oligoclonal T cell repertoire. *In vitro* proliferation to mitogens and antigens is markedly reduced, due to increased apoptosis rate and abnormalities of cell cycle progression (Abdollahpour et al., 2012; Nehme et al., 2012). Defective expression of FOXO1 and FOXO3a has been observed in patients' T lymphocytes, similar to what detected in *Mst1*$^{-/-}$ mice. Moreover, T lymphocytes from MST1-deficient patients show reduced expression of CD127 (IL-7Rα) and of the antiapoptotic protein BCL2, as well as of the CD62L and CCR7 homing receptors (Nehme et al., 2012). Increased apoptosis of the patients' T cells is associated with loss of mitochondrial transmembrane potential ($\Delta\psi m$) upon *in vitro* activation or exposure to staurosporine (Abdollahpour et al., 2012; Nehme et al., 2012). A similar loss of $\Delta\psi m$ is observed when patients' neutrophils are exposed to valinomycin *in vitro*, implying apoptosis as the mechanism responsible for neutropenia *in vivo* (Abdollahpour et al., 2012).

Finally, MST1 deficiency is also characterized by reduced number of circulating B cells, with an increased proportion of transitional B lymphocytes

and a reduction of marginal zone-like ($IgD^+$ $IgM^+$ $CD27^+$) and switched memory ($IgD^-$ $CD27^+$) B cells (Abdollahpour et al., 2012; Nehme et al., 2012).

## 3.14. Coronin-1A deficiency

The coronin family of proteins includes several members that have F-actin and Arp2/3-binding property, and regulate cytoskeleton-dependent cellular processes (Rybakin & Clemen, 2005). Coronins have a similar structure, with five WD40 repeats, flanked by conserved N- and C-terminal extensions (Appleton, Wu, & Wiesmann, 2006). However, members of the coronin family have a distinct cell type-specific distribution. In particular, coronin-1A is expressed predominantly in hematopoietic cells. Its structure is organized into a seven-bladed β-propeller, with two potential F-actin binding sites on the top and bottom faces of the structure. A cluster of buried hydrophobic amino acids in blades 2 and 3 is supposed to play a structural role in the tight packaging of these blades (Moshous et al., 2013). The C-terminal extension of coronin-1A contains a leucine-zipper coiled-coil domain that mediates homotrimerization of the molecule (Gatfield, Albrecht, Zanolari, Steinmetz, & Pieters, 2005).

During T cell development, coronin-1A is expressed at low levels in DP thymocytes and at higher levels in SP cells (Mueller et al., 2008). Coronin-1A has been implicated in TCR signaling (Mueller, Liu, & Pieters, 2011; Mugnier et al., 2008) and T cell homeostasis (Foger, Rangell, Danilenko, & Chan, 2006; Mueller et al., 2008). In particular, by controlling TCR-mediated activation and calcium signaling, coronin-1A promotes survival of T lymphocytes (Mueller et al., 2011). Moreover, by binding to the Arp2/3 complex (Machesky et al., 1997), coronin-1A inhibits branched F-actin formation, and therefore modulates cytoskeleton dynamics, integrin outside-in signaling (Gatfield et al., 2005), chemokine-induced cell migration, and T cell homeostasis (Foger et al., 2006). Finally, coronin-1A also participates at regulation of neutrophil- and macrophage-mediated phagocytosis (Ferrari, Langen, Naito, & Pieters, 1999; Grogan et al., 1997).

The study of mice with genetic defects in the *coronin-1A* (*Coro1A*) gene has provided fundamental insights into the role of this molecule in T cell development, homeostasis, and function. $Coro1A^{-/-}$ mice show an increase of DP thymocytes, associated with a decreased proportion of mature ($CD24^{lo}$) SP thymocytes. The increased number of thymocytes in $Coro1A^{-/-}$ contrasts with a dramatic reduction of $TCR\alpha\beta^+$ peripheral

T cells (Foger et al., 2006; Mugnier et al., 2008). By contrast, TCR$\gamma\delta^+$ T cells are unaffected. The few peripheral TCR$\alpha\beta^+$ T cells show an activated phenotype ($CD44^{hi}$ $CD62L^{lo}$) and are highly susceptible to apoptosis (Foger et al., 2006; Mugnier et al., 2008). Upon stimulation via CD3/CD28 or with alloantigens, *Coro1A*$^{-/-}$ T lymphocytes demonstrate delayed cell division, reduced production of IL-2, and increased apoptosis (Mueller et al., 2008; Mugnier et al., 2008).

Enhanced apoptosis of *Coro1a*$^{-/-}$ T lymphocytes may reflect inefficient TCR-mediated signaling and/or accumulation of branched F-actin. Upon TCR stimulation, coronin-1A interacts with PLC-$\gamma$1 and promotes generation of $InsP_3$, release of $Ca^{2+}$ from intracellular stores, and cell proliferation. Consistent with this, *Coro1A*$^{-/-}$ T lymphocytes show defective PLC-$\gamma$1 activation, $InsP_3$ generation, and $Ca^{2+}$ flux in response to TCR stimulation (Mueller et al., 2008). However, the hypothesis that defective TCR signaling and impaired $Ca^{2+}$ flux in coronin-1A-deficient cells may affect T cell survival has been challenged following demonstration that T lymphocytes from the Coro1A mutant "Koyaanisqatsi" mouse strain show reduced survival in spite of marginal defects of $Ca^{2+}$ flux upon CD3 stimulation (Shiow et al., 2008). These data suggest that reduced viability of *Coro1A*$^{-/-}$ T cells is uncoupled from TCR-induced $Ca^{2+}$ flux defects. Alternatively, impaired T lymphocyte survival in Coro1A-deficient mice may reflect abnormal accumulation of branched F-actin, which may promote apoptosis (Foger et al., 2006). However, accumulation of F-actin alone is not sufficient for induction of apoptosis (Haraldsson et al., 2008). Therefore, the molecular mechanisms accounting for decreased T lymphocyte survival in coronin-1 deficiency remain unclear.

A role of coronin-1A in regulation of actin cytoskeleton has been also established through the study of the "peripheral T cell deficiency" (*Ptcd*) mouse strain, with a naturally occurring gain-of-function mutation (K26E) in the *Coro1A* gene (Shiow et al., 2008; Yagi et al., 1996). The *Ptcd* mouse shows accumulation of mature SP cells in the thymus associated with peripheral T cell lymphopenia. This phenotype reflects defective thymic egress because of impaired response of SP thymocytes to various chemoattractants, including S1P, CCL21, and CXCL12 (Shiow et al., 2008). This defect affects also peripheral trafficking of *Ptcd* T lymphocytes, with impaired migration into, and defective egress from, lymph nodes. These abnormalities are associated with intracellular mislocalization of the mutant coronin-1A molecule that fails to accumulate at the leading edge of chemokine-stimulated T lymphocytes (Shiow et al., 2008).

Coronin-1A deficiency has been reported in four patients from two families (Haraldsson et al., 2008; Shiow et al., 2009, 2008). In particular, Shiow et al. described a female patient with a sporadic presentation of the disease, characterized by recurrent respiratory tract infections, candidiasis, severe varicella following immunization with live attenuated viral vaccine, failure to thrive, and delayed language and motor development (Shiow et al., 2009, 2008). This patient carried a dinucleotide deletion in exon 3 of the *CORO1A* gene on one allele, and a large (600 kb) deletion on chromosome 16p11.2, encompassing *CORO1A* and 24 other genes on the other allele (Shiow et al., 2009, 2008). Moshous et al. reported three siblings from a consanguineous family who presented with aggressive, EBV-associated lymphoproliferative disease. One of these siblings also suffered from recurrent infections, and another one had important neurocognitive impairment (Moshous et al., 2013). Affected patients from this family shared a homozygous missense mutation (V134M) in blade 2 of the coronin-1A β-propeller that destabilized the protein structure (Moshous et al., 2013). Immunological investigations showed significant T cell lymphopenia, with striking reduction of naïve $CD4^+$ and $CD8^+$ T cells, oligoclonal T cell repertoire, and increased proportion of central memory and effector memory T lymphocytes (Moshous et al., 2013). In spite of virtual absence of recent thymic emigrants, a normal-sized thymus was detected by chest computed tomography, indicating active thymopoiesis (Moshous et al., 2013; Shiow et al., 2008). Proliferative responses to mitogens and antigens were variably affected (Moshous et al., 2013; Shiow et al., 2009, 2008), but activation of ERK1 and ERK2 in response to TCR activation was delayed (Moshous et al., 2013). Similarly, defective antibody responses to immunization antigens were detected in some patients, but normal responses were observed in others (Moshous et al., 2013; Shiow et al., 2009, 2008). Finally, invariant NKT lymphocytes and $V\alpha7.2^+$ $CD161^+$ mucosal-associated invariant T cells were present in markedly reduced numbers, suggesting that coronin-1A deficiency also affects development and/or survival of nonconventional T cells (Shiow et al., 2008). Altogether, these data are consistent with observations in coronin-1A-deficient mice and indicate a critical role for coronin-1A in T lymphocyte survival.

If untreated, coronin-1A deficiency has a severe clinical course. Two of the four patients have died of large BCL and of severe, posttransplant graft-versus-host disease, respectively (Moshous et al., 2013). One patient has attained immune reconstitution after unrelated cord blood transplantation (Shiow et al., 2009, 2008), and another patient was reported to be in

complete remission at 11.5 years of age after chemotherapy for diffuse large BCL (Moshous et al., 2013).

## 4. CONCLUSIONS

Careful description of clinical and immunological features of patients presenting with features suggestive of a significant T cell defect in spite of largely preserved T cell development, along with detailed biochemical and molecular studies and advances in human genome investigation, has been essential to identify a growing number of genetic conditions characterized by dysfunctional T lymphocytes. Although the molecular basis underlying these disorders is heterogeneous, defects in TCR cell signaling lead to common clinical and laboratory features that mark a clear distinction between these diseases and typical forms of SCID. In particular, the strength of the TCR signal regulates intrathymic differentiation, central and peripheral T cell homeostasis, AICD, and quality and extent of cytokine production (Salmond et al., 2009; Sprent & Surh, 2011). Not surprisingly, patients with dysfunctional T cells often suffer from autoimmune manifestations secondary to impairment of mechanisms of central and peripheral tolerance, irrespective of the nature of the underlying genetic defect. Moreover, reduced ability to generate and sustain antigen-specific memory T cell responses and to mount potent effector mechanisms of disease, especially against viruses, cause increased susceptibility to viral infections, in particular to herpesviruses, whose natural cycle of infection is characterized by frequent reactivations that require adequate immune response. Thus, susceptibility to EBV infection is very common in patients with genetic disorders characterized by dysfunctional T lymphocytes, and often leads to lymphoma. Furthermore, chronic viral infections may also trigger continuous stimulation of T cells and lead to exhaustion of $CD8^+$ cells. Indeed, accumulation of $CD45RA^+$ $CCR7^-$ exhausted memory $CD8^+$ lymphocytes is observed in patients with *LCK* (Hauck et al., 2012), *RHOH* (Crequer et al., 2012), *DOCK8* (Randall et al., 2011), and *MST1* (Nehme et al., 2012) defects. Although it is possible that accumulation of $T_{EMRA}$ cells is a direct consequence of the genetic defect affecting T cell homeostasis, it has not been reported in animal models kept under controlled, standard pathogen-free conditions, suggesting that environmental factors may have an important impact on the immunological phenotype.

Impaired survival of T lymphocytes has emerged as another mechanism involved in the pathophysiology of diseases with altered T cell function.

Altered induction of *FOXO* genes in patients with *MST1* mutations may affect expression of IL-7R and hence impair IL-7-mediated delivery of survival signals (Abdollahpour et al., 2012; Nehme et al., 2012). Increased apoptosis has also been shown to contribute to lymphopenia in patients with coronin-1A deficiency (Moshous et al., 2013).

Finally, it should be noted that identification of many forms of immunodeficiency with nonfunctional or dysfunctional T cells has been based on the study of few patients, and in some cases even single patients. Importantly, discovery of human diseases often preceded generation of corresponding animal models, emphasizing the importance of investigating PIDs as "Experiments of Nature" (Good, 1968). This task has been greatly facilitated by recent advances in human genome sequencing (WGS, WES) that may permit identification of novel gene defects even starting from single cases. Undoubtedly, the success registered in the last few years with identification of several novel immunodeficiency genes is just the "tip of the iceberg", and the discovery of many more genetic defects should be expected in the near future. Availability of novel functional platform that permit rapid analysis of the effects of the gene variants identified will be of fundamental importance, because this remains a major bottleneck in the validation of the disease-causing role of novel gene defects.

It is also becoming increasingly clear that different mutations in the same gene may lead to distinct phenotypes, as best exemplified by hypomorphic versus null mutations in SCID-causing genes. It is hard to predict the impact of this phenotypic variability on our ability to correctly anticipate the nature of genetic defects underlying clinical manifestations of disease in individual patients.

Finally, from a public health perspective, newborn screening based on quantification of TRECs at birth represents a formidable tool that has already permitted early and correct identification of babies with SCID, and prompt referral to HCT (Dvorak et al., 2013). Whether (and to what extent) a similar strategy may be applied to early diagnosis of immunodeficiencies with nonfunctional T cells remains to be seen.

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CHAPTER FIVE

# The CD200–CD200R1 Inhibitory Signaling Pathway: Immune Regulation and Host–Pathogen Interactions

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## Contents



## Abstract

The CD200:CD200R1 inhibitory signaling pathway has been implicated in playing a prominent role in limiting inflammation in a wide range of inflammatory diseases. CD200R1 signaling inhibits the expression of proinflammatory molecules including tumor necrosis factor, interferons, and inducible nitric oxide synthase in response to selected stimuli. Unsurprisingly, due to the regulatory role that CD200R1 plays in multiple inflammatory pathways, an increasing number of parasitic, bacterial, and viral pathogens exploit this pathway to suppress host defenses. A complete understanding of the pathways regulated by CD200R1 signaling and the diverse mechanisms that pathogens have evolved to manipulate the CD200:CD200R1 pathway can help identify clinical situations where targeting this interaction can be of therapeutic benefit. In this review, we compare CD200R1 to other pathogen-targeted inhibitory receptors and highlight how this signaling pathway is utilized by a diverse number of pathogens and, therefore, may represent a novel targeting strategy for the treatment of infectious diseases.

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## 1. INHIBITORY RECEPTORS

Hosts and pathogens have evolved mechanisms to defeat each other in the battle for control over the host's immune system. A successful infection requires that the pathogen positively regulate its survival, replication, and spread while suppressing the pathogen-specific host immune response. Conversely, it is essential that the host immune response be appropriately controlled to respond to and remove pathogens while avoiding excessive production of cytokines, chemical mediators such as reactive oxygen species (ROS), and the release of proteolytic enzymes all of which can lead to increased tissue damage and morbidity and mortality.

Immune cells express receptors, such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors, which recognize and respond to pathogens with the induction of antivirulence genes and generation of chemical mediators. At the same time these cells express inhibitory receptors that limit the amplitude of the response to prevent immunopathology. The mechanisms by which inhibitory receptors limit the amplitude of proinflammatory responses have been described in detail (Long, 1999; Ravetch & Lanier, 2000). For the purpose of this review, we will focus on members of the inhibitory receptor superfamily that have been targeted by pathogens. Based on the structure of the extracellular domains, there are two major classes within the inhibitory receptor superfamily: the immunoglobulin (Ig) superfamily and the calcium-dependent carbohydrate-binding (C-type) lectin family (Long, 1999) (Fig. 5.1A).

Most members of the inhibitory receptor superfamily have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail of the protein (Vely & Vivier, 1997) (Fig. 5.1). Upon activation of the receptor, phosphorylation of tyrosine residues in the ITIM recruits adaptor proteins such as src homology 2-containing protein tyrosine phosphatases (SHPs) and SH2 domain-containing inositol phosphatase-1 (SHIP-1) (Daeron, Jaeger, Du Pasquier, & Vivier, 2008). This ultimately leads to a decrease in immune functions including cytokine production, calcium release, migration, and proliferation (Ravetch & Lanier, 2000). Many inhibitory receptors also have paired activating receptors, which contain cytoplasmic immunoreceptor tyrosine-based activation motifs and associate with adaptor proteins like DNAX-activating protein of 12 kDa (DAP12) or the FcRγ chain through a positively charged residue in the transmembrane region (McVicar et al., 1998) to induce proinflammatory signaling events (Fig. 5.1).

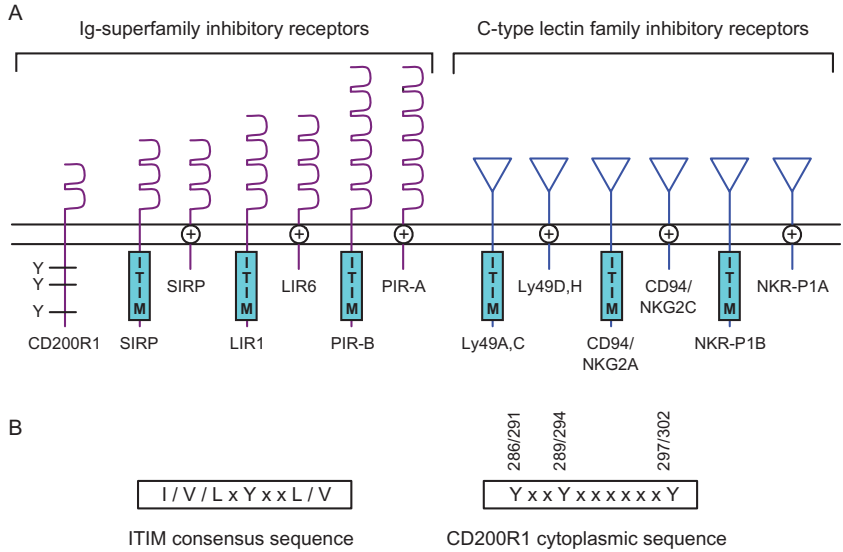


**Figure 5.1** Classes and cytoplasmic signaling domains of the inhibitory receptor superfamily. (A) Classes of inhibitory receptors. Inhibitory receptors are separated into two major classes based on their extracellular domains: the immunoglobulin (Ig) superfamily and the carbohydrate-binding (C-type) lectin family. Many members of these inhibitory receptor families have affiliated activating receptors, which contain a charged residue in the transmembrane region, denoted by a plus sign. (B) Cytoplasmic inhibitory motifs. Most inhibitory receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic region to recruit adaptor proteins upon activation, however the CD200R1 cytoplasmic region contains three tyrosine residues (locations listed as mouse/human), which play a role in adaptor protein interactions upon phosphorylation. LIR, leukocyte inhibitory receptor; PIR, paired Ig-like receptor; SIRP, signal-regulatory protein.

## 1.1. Decoy ligands for inhibitory receptors

Pathogens can express proteins that efficiently bind to a variety of inhibitory receptors that normally distinguish self from nonself. In this way, they avoid recognition and promote persistence in the host. Herpesviruses and poxviruses are exceptionally skilled at avoiding or subverting host immune responses (Table 5.1).

Murine cytomegalovirus (MCMV) expresses m157, which is structurally similar to MHC class I proteins and binds to the inhibitory receptor Ly49I in MCMV-susceptible mouse strains to prevent NK-mediated killing (Arase & Lanier, 2004; Arase et al., 2002). The mouse Ly49 family of molecules is expressed on NK cells that recognize the α1 and α2 subunits of H-2D MHC class I molecules (Karlhofer, Ribaudo, & Yokoyama, 1992).

**Table 5.1** Viral decoy ligands for inhibitory receptors

| Virus | Gene | Cellular homolog | Target receptor | References |
|---|---|---|---|---|
| MCMV | m157 | MHC class I | Ly49I | Arase and Lanier (2004) and Arase, Mocarski, Campbell, Hill, and Lanier (2002) |
| | m144 | MHC class I | ? | Farrell et al. (1997) |
| HCMV | UL18 | MHC class I | LIR-1 | Chapman, Heikeman, and Bjorkman (1999), Cosman et al. (1997), and Reyburn et al. (1997) |
| | UL40 | MHC class I peptide | CD94/NKG2A | Ulbrecht et al. (2000) |
| RCMV | RCTL | Clr-b | NKR-P1B | Voigt et al. (2007) and Voigt, Sandford, Ding, and Burns (2001) |
| Myxoma virus | M128L | CD47 | SIRPα | Arase and Lanier (2004) and Cameron, Barrett, Mann, et al. (2005) |

Interestingly, MCMV-resistant mouse strains, but not MCMV-susceptible strains, express the activating receptor Ly49H, which also binds to m157 but initiates NK killing of the infected cells (Smith et al., 2002). This suggests that virus and host together have evolved to modulate signaling through this receptor. In fact, when MCMV is continuously passaged in Ly49H positive cells in culture, the virus will quickly generate mutations in m157 to avoid binding to the activating receptor (Voigt et al., 2003).

Human cytomegalovirus (HCMV) expresses the protein UL18, a homolog of MHC class I antigens (Cosman et al., 1997; Reyburn et al., 1997). MCMV also expresses m144, which also functions as a MHC class I mimic and is required for efficient viral replication *in vivo* (Farrell et al., 1997). Both UL18 and m144 form the three α domains typical of MHC class I molecules and both can bind to β2M (Farrell et al., 1997; Reyburn et al., 1997). UL18 can bind to both CD94/NKG2 and leukocyte inhibitory receptor (LIR)-1 and it is thought that m144 may interact similarly. The CD94/NKG2 receptors recognize the nonclassical MHC class I molecules human HLA-E and mouse Qa1 (Brooks et al., 1999; Houchins, Lanier, Niemi, Phillips, & Ryan, 1997; Lee et al., 1998; Vance, Kraft, Altman, Jensen, & Raulet, 1998), which are expressed on all cell types except red blood cells (Kuroki, Furukawa, & Maenaka, 2012). Human LIR-1 recognizes epitopes shared by most MHC class

I molecules through interactions with the α3 and β2M domains (Chapman et al., 1999; Willcox, Thomas, & Bjorkman, 2003). In fact, LIR-1 binds to UL18 more tightly than host class I molecules (Chapman et al., 1999), indicating that this receptor may have evolved specifically to bind to UL18. Furthermore, the leader sequence of the HCMV protein UL40 is identical to the MHC class I, HLA-E associated peptide HLA-Cw03. CD94/NKG2A will recognize HCMV-infected cells as self based on presentation of the HLA-E-like peptide and will not kill them (Ulbrecht et al., 2000).

The rat cytomegalovirus (RCMV) C-type lectin-like gene (*rctl*) is characterized as an early gene whose structure closely resembles the mouse and rat C-type lectin related protein Clr-b (Voigt et al., 2001, 2007). The inhibitory receptor NKR-P1B, expressed mainly on NK cells (Voigt et al., 2007), recognizes Clr-b as a ligand, which is expressed on almost all hematopoietic cells (Carlyle et al., 2004). Following infection with RCMV, there is a rapid upregulation of RCTL, which counteracts downregulation of Clr-b expression by host cells in response to infection. Through a non-MHC class I recognition mechanism, RCTL inhibits NK cell-mediated lysis by directly interacting with NKR-P1B. Furthermore, RCTL-deficient virus exhibits decreased virulence and is more easily cleared from the host by NK cells (Voigt et al., 2007). Interestingly, the activation receptor NKR-P1A also recognizes RCTL, indicating that host defenses have evolved to counteract the ability of RCTL to evade recognition.

A variety of poxviruses encode a CD47 mimic (Arase & Lanier, 2004; Cameron, Barrett, Mann, Lucas, & McFadden, 2005), and based on evidence from other paired inhibitory/activation receptors, as described earlier, it has been suggested that these mimics may interact with signal-regulatory protein (SIRP)α to downregulate myeloid cell functions. SIRPα, expressed mainly on myeloid cells and neurons (Adams et al., 1998; Alblas et al., 2005), binds to CD47 to regulate leukocyte chemotaxis and proinflammatory cytokine production (Cameron, Barrett, Mann, et al., 2005). The myxoma virus CD47 mimic, M128L, is required for lethal infections in rabbits and appears to regulate macrophage activation and recruitment, as M128L-deficient virus-infected rabbits exhibit increased inducible nitric oxide synthase (iNOS)-positive cells at infection sites (Cameron, Barrett, Mann, et al., 2005). The activating receptor, SIRPβ does not bind to CD47, and its ligand is unknown, but may have evolved to counteract pathogen infections and/or recognize pathogen-infected cells (Arase & Lanier, 2004; Barclay & Brown, 2006).

In addition to viral decoys, several bacterial strains, including *S. aureus* and *E. coli*, can bind to the mouse paired Ig-like receptors (PIRs) PIR-B

and PIR-A1 and human LIR-1 to suppress macrophage proinflammatory responses (Nakayama et al., 2007). The murine PIRs, which are structurally similar to the human LIRs, recognize MHC class I molecules (Nakamura, Kobayashi, & Takai, 2004) and are expressed on a variety of cell types, including macrophages, dendritic cells, mast cells, and B cells (Kubagawa, Burrows, & Cooper, 1997; Kubagawa et al., 1999). These data suggest that many pathogens can take advantage of host inhibitory receptors to modulate inflammatory responses.

## 2. THE INHIBITORY RECEPTOR CD200R1

CD200R1 is an Ig superfamily transmembrane glycoprotein expressed on the surface of myeloid cells; it can also be induced in certain T-cell subsets (Caserta et al., 2012; Wright et al., 2000, 2003). CD200R1 interacts with CD200, which is also an Ig superfamily transmembrane glycoprotein, to down regulate myeloid cell functions. CD200 is expressed on the surface of a variety of cells including neurons, epithelial cells, endothelial cells, fibroblasts, lymphoid cells, and astrocytes (Caserta et al., 2012; Costello et al., 2011; Hoek et al., 2000; Snelgrove et al., 2008). The regulation of CD200R1 signaling can occur by posttranslational modification—namely, phosphorylation of tyrosines in the CD200R1 cytoplasmic tail—or by the inducible expression or downregulation of either CD200R1 or CD200. Each of these mechanisms can ultimately be exploited by pathogens.

### 2.1. Signaling through the CD200R1 cytoplasmic domain

Unlike most immune inhibitory receptors, CD200R1 does not contain an ITIM (Fig. 5.1). Instead, human CD200R1 contains three cytoplasmic tyrosine residues, Y291, Y294, and Y302 (Y286, Y289, and Y297 in the mouse), one of which, Y302/Y297, is located within a phosphotyrosine binding (PTB) domain recognition motif (NPxY). Stimulation by CD200 leads to the phosphorylation of these tyrosines by Src kinases, which recruit the adapter protein downstream of tyrosine kinase (Dok) 2 through its PTB domain (Mihrshahi, Barclay, & Brown, 2009; Mihrshahi & Brown, 2010; Zhang, Cherwinski, Sedgwick, & Phillips, 2004). Y302/Y297 and to a lesser extent Y291/Y286 are the major tyrosine residues required for CD200R1 association with Dok2 (Mihrshahi et al., 2009; Zhang & Phillips, 2006). Dok2 serves as the major initiator of signaling through CD200R1, beginning with binding to Ras-GTPase activating protein (RasGAP) and is required for CD200R1 function (Mihrshahi et al., 2009).

This is in contrast to ITIM containing inhibitory receptors, which utilize SHPs and SHIP-1 as the major initiator proteins and Dok proteins as secondary modulators of downstream signaling (Daeron et al., 2008; Mihrshahi et al., 2009).

## 2.2. CD200:CD200R1 signaling and infectious diseases

Pathogens have found ways to exploit the CD200:CD200R1 signaling pathway by altering expression of either CD200 or CD200R1, or by expressing a CD200 mimic to engage the host CD200R1 (Table 5.2). In

**Table 5.2** Pathogen susceptibility to CD200:CD200R1 signaling

| Pathogen | Effect on CD200/ CD200R1 expression | Disease severity (type of KO or treatment) | References |
|---|---|---|---|
| *T. gondii* | Increased CD200 and CD200R1 | Decreased (CD200 KO) | Deckert, Sedgwick, Fischer, and Schluter (2006) |
| *L. amazonesis* | Increased CD200 | Decreased (CD200 KO) | Cortez et al. (2011) |
| *N. meningitidis* | Increased CD200 | Increased (CD200 KO) | Mukhopadhyay et al. (2010) |
| *S. masoni* | Increased CD200 and CD200R1 | | Caserta et al. (2012) |
| *S. enterica* | Increased CD200 and CD200R1 | | Caserta et al. (2012) |
| *S. haematobium* | Increased CD200R1 | | Caserta et al. (2012) |
| MHV | | Decreased (CD200 KO) | Karnam et al. (2012) |
| Influenza A | | Increased (CD200 KO); decreased (CD200-Fc) | Karnam et al. (2012) and Snelgrove et al. (2008) |
| | | Decreased (CD200R1 KO) | Goulding et al., 2011 |
| HSV-1 (ocular) | Increased CD200R1 | Decreased (CD200-Fc) | Sarangi, Woo, and Rouse (2009) |
| HSV-1 (brain) | | Decreased (CD200R1 KO) | Soberman et al., 2012 |

certain situations, the greatest threat to the host is the excessive inflammation seen in response to the infectious organism. In these cases, the disruption of the CD200:CD200R1 axis in model systems leads to the death of the host. In the cases of intracellular parasites, this can be deleterious to the pathogen as well, as these organisms benefit from the survival of the host for long-term growth and expansion. In other cases, the subset of antipathogen genes that are suppressed by the engagement of CD200R1 directly allows survival of the pathogen at the expense of the host.

Antipathogen molecules, such as ROS that include nitric oxide (NO), superoxide, and hydroxyl radicals, preformed mediators, and interferons (IFNs) are a subset of proinflammatory genes and mediators. As a protective measure against tissue damage, host macrophages adaptively modify chromatin to allow them to become unresponsive to repetitive or persistent signaling by TLRs (e.g., TLR4 and lipopolysaccharide (LPS) tolerance) (Foster, Hargreaves, & Medzhitov, 2007), leading to decreased pro-inflammatory signaling. Certain antipathogen molecules, however, are not dampened after prolonged TLR signaling because chromatin modification allows antipathogen genes to remain responsive to TLR4 in the presence of ongoing infection (Foster et al., 2007). Pathogens also employ various strategies to engage downregulatory mechanisms to suppress host defenses. These are illustrated by the mechanisms various pathogens use to manipulate the CD200:CD200R1 axis or to manipulate other inhibitory receptors.

### *2.2.1 Bacterial and parasitic pathogens*

#### 2.2.1.1 Toxoplasma

In WT mice, *Toxoplasma gondii* induces increased surface expression of CD200R1 and CD200 in microglia and blood vessel endothelial cells, respectively (Deckert et al., 2006). In CD200 KO mice, microglial cells exhibited increased proliferation, activation, and higher expression of MHC II, tumor necrosis factor (TNFα), and iNOS during infection in chronic *T. gondii* encephalitis. CD200 KO mice also exhibited decreased parasite burden and decreased mortality compared to WT mice following chronic infection. This is likely due to the fact that CD200 KO mice exhibit an increased inflammatory phenotype in response to the TLR ligands, including significantly higher IL-6 and TNFα release and IκBα phosphorylation (Costello et al., 2011). It is known that *T. gondii* stimulation of mouse TLR11 induces IL-12, which is key for the survival of the host (Yarovinsky et al., 2005). TLRs 2 and 4 have also been implicated in the inflammatory response to *T. gondii* (Debierre-Grockiego et al., 2007). These data show

that in the case of Toxoplasmosis, increased inflammatory responses, likely through TLR signaling, are detrimental to the pathogen.

#### 2.2.1.2 Leishmania

*Leishmania amazonensis*, which causes severe disease in both humans and mice, induces CD200 mRNA and protein expression in bone marrow macrophages from WT mice (Cortez et al., 2011). Upregulation of CD200 was essential for replication and development of systemic Leishmaniasis as *L. amazonensis* replication and virulence are significantly decreased in CD200 KO mice. Virulence of *L. amazonensis* can be restored by treatment with soluble CD200-Fc. Not all species of Leishmania have evolved this mechanism, as *L. major*, which causes cutaneous but not systemic disease, does not induce CD200. However, CD200-Fc treatment in *L. major*-infected WT mice shifts its virulence to that of *L. amazonensis* (Cortez et al., 2011). *L. amazonensis* has evolved to utilize CD200 expression as a mechanism for inhibiting both NO production and induction of iNOS during infection. This was confirmed by treatment of macrophages with an iNOS inhibitor, which, in turn, lead to increased replication of *L. major*. Interestingly, *L. amazonensis* increased CD200 expression on macrophages. Macrophages have generally been found to express CD200R1, which can then interact with nonmyeloid cells expressing CD200. These findings suggest that, at least in the case of *L. amazonensis*, macrophages can inhibit neighboring macrophages by expressing both CD200R1 and CD200. Macrophages infected with intracellular pathogens can release exosomes, small vesicles containing various membrane proteins, which can provide signals to naïve macrophages (Bhatnagar, Shinagawa, Castellino, & Schorey, 2007). It may be that these exosomes contain CD200, which can then bind to CD200R1 on nearby macrophages. Whether or how this would occur is not clear, though it is certainly an interesting possibility. Alternatively, macrophages expressing CD200 may interact with activated T-cells expressing CD200R1.

#### 2.2.1.3 Neisseria

CD200 KO mice are more susceptible to infection with *Neisseria meningitidis* than WT mice. While there was no significant difference in bacteremia between WT and CD200 KO mice, CD200 KO mice had higher systemic levels of IL-6 and TNFα, higher numbers of F4/80 + CD11b + macrophages, and expressed higher levels of MHC class II molecules on macrophages (Mukhopadhyay et al., 2010). Furthermore, CD200 expression is upregulated

in bone marrow macrophages following infection with *N. meningitidis*. This is likely due to recognition of Neisserial LPS by TLR4, since TLR ligation can increase CD200 surface expression in macrophages (Mukhopadhyay et al., 2010). These data suggest that in WT mice, CD200:CD200R1 signaling plays a role in regulating the response to *N. meningitidis*, but does not necessarily affect the survival of the pathogen. Therefore, increased mortality in this model is mediated by uncontrolled inflammation, not uncontrolled pathogen replication.

#### 2.2.1.4 Schistosomes and salmonella

Both CD200 and CD200R1 are upregulated and coexpressed in chronically activated CD4 T-cells from mice infected with *Schistosoma mansoni* and *Salmonella enterica*. These cells also lost the ability to generate TNFα and exhibited increased IL-4 secretion. Furthermore, in patients chronically infected with *Schistosoma haematobium*, there was a correlation between CD200R1 expression and parasite load and almost all IL-4 secreting CD4 T-cells were CD200R1 positive. This suggests that chronic infections lead to increased expression of CD200 and CD200R1 and subsequently a decrease in antipathogenic mediators, allowing pathogen persistence.

How pathogens regulate CD200 expression is unclear. However, studies have shown that expression of CD200 is regulated by transcription factors and enhancer elements. Constitutive CD200 expression is regulated by the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) (Chen, Marsden, & Gorczynski, 2006, 2009). Furthermore, there are three enhancer sites (*cis*-elements) upstream of the CD200 transcriptional start site, a NF-κB binding site, an IFNγ-activation site (GAS), and an IFN-stimulatory response element-2, that are important for inducible CD200 expression. NF-κB, STAT1, and IFN regulator factor-1 bind to these enhancer elements, respectively (Chen et al., 2009). Furthermore, it was determined that the NF-κB transcription factor, c-Rel, was required for TLR-induced upregulation of CD200 (Mukhopadhyay et al., 2010). Perhaps pathogens utilize these enhancer sites and transcription factors to induce CD200 expression following TLR recognition. CD200 is also a target of p53 and is upregulated on apoptotic cells to decrease responsiveness to self-antigen (Rosenblum et al., 2004).

The mechanisms that pathogens employ to induce the expression of CD200R1 are also unclear, although their interaction with TLRs is one mechanism (Dentesano et al., 2012; Mukhopadhyay et al., 2010). It has recently been discovered that inducible expression of CD200R1 is regulated

by C/EBPβ (Dentesano et al., 2012). Microglial cells exhibit a significant decrease in CD200R1 mRNA and protein expression following stimulation with LPS, a TLR4 ligand. This decrease is not seen in C/EBPβ KO cells. Additionally, overexpression of C/EBPβ led to a significant decrease in CD200R1 mRNA and protein expression. C/EBPβ directly binds to the CD200R1 promoter to inhibit expression in LPS-treated cells. Furthermore, it was found that histone deacetylase 1 interacts with C/EBPβ to downregulate CD200R1 expression.

### *2.2.2 Viruses*

#### 2.2.2.1 Coronaviruses

Loss of CD200R1 signaling, through use of CD200 KO mice, results in an increase in inflammatory signaling, specifically type I IFN in response to TLR7 ligands, including mouse hepatitis corona virus (MHV). MHV serves as an infection model for the severe acute respiratory syndrome coronavirus (De Albuquerque et al., 2006). Lack of inflammatory signaling control had a positive effect on MHV clearance as CD200 KO mice exhibited decreased viral replication and viral titers (Karnam et al., 2012). Infected CD200 KO mice also had increased levels of IFNα compared to WT mice. These findings indicate that coronavirus infections require a functional CD200:CD200R1 signaling interaction to limit type I IFN production.

#### 2.2.2.2 Influenza virus

The opposite is true for influenza A where CD200 KO mice were highly susceptible to the effects of uncontrolled inflammation in response to pulmonary infection. These mice demonstrated more weight loss and increased mortality in response to influenza than WT mice (Karnam et al., 2012; Snelgrove et al., 2008), even though viral clearance was similar in both strains. CD200 KO mice also had higher levels of NO in lung homogenates, as well as increased levels of IL-6, TNFα, IFNγ, and macrophage inflammatory protein 1α in lavage fluids. Furthermore, the administration of CD200-Fc or anti-CD200R1 agonist was able to partially reverse the phenotype of CD200 KO mice, leading to less weight loss and lower cellularity than untreated CD200 KO mice following infection (Snelgrove et al., 2008). In WT mice, alveolar macrophages exhibit increased expression of CD200R1, which would serve to limit inflammatory responses to the virus, and thus, limit immunopathology (Snelgrove et al., 2008). In this case, the role of the CD200:CD200R1 axis is to protect the host from cytokine storm, which is the major cause of morbidity and mortality.

Influenza-infected CD200R1 KO mice show less bacterial load and exhibit decreased pathogenesis and mortality than WT mice following *S. pneumoniae* superinfection (Goulding et al., 2011). This is thought to occur because during the resolution phase of an influenza infection, apoptotic monocytes/macrophages in the lung express CD200 on their surface while alveolar macrophages upregulate CD200R1 surface expression. This leads to decreased alveolar macrophage responsiveness and increased susceptibility to bacterial superinfections. Interestingly, CD200R1 KO mice exhibit decreased viral pathogenesis and pathology in response to influenza infection (Goulding et al., 2011). These results seem counter-intuitive compared to the previous findings with CD200 KO mice. However, the authors suggest that this may be due to the limited expression of the receptor, compared to the broad expression of the ligand, but further studies need to be performed in order to prove this. Nonetheless, the increased inflammatory response seen in CD200R1 KO mice provides protection to the host in terms of a bacterial superinfection.

#### 2.2.2.3 Herpesviruses

Herpes simplex virus (HSV)-1 mediated keratitis (stromal keratitis) is a chronic infection that causes an influx of CD200R1-expressing cells into the cornea, leading to inflammatory lesions and blindness (Sarangi et al., 2009). A variety of cell types, including myeloid cells, upregulate CD200R1 on their surface following ocular HSV-1 infection (Sarangi et al., 2009). CD200-Fc treatment of ocular HSV-1 infected mice caused decreased CD11b+ immune cells in the cornea, decreased inflammatory lesions, and decreased angiogenesis. These mice also had decreased cellularity in the spleen and draining lymph nodes and this was associated with a decrease in IFNγ-producing T-cells and an increase in FoxP3+ T-regulatory cells both in lymphoid tissue and the cornea. Treatment also mildly reduced lesions in chronically infected mice, though this would need to be combined with another drug to prove efficacious. These results indicate that CD200:CD200R1 signaling plays a key role in modulating inflammation during a viral infection and provides further evidence that decreasing the inflammatory milieu following a viral infection can actually have a beneficial role for unwanted immunopathology.

We have recently examined the role of the CD200:CD200R1 axis in the mouse model of HSV-1 encephalitis (Soberman et al., 2012). A significant component of the morbidity and mortality in this model is the release of cytokines and chemokines triggered by the interaction of HSV-1 with

macrophages and resident microglial cells through TLR2 (Kurt-Jones et al., 2004). Therefore, we predicted that CD200R1 KO mice would show increased morbidity and mortality in response to HSV-1 infection. However, CD200R1 KO mice were markedly protected against infection and exhibited a decrease in viral titers and HSV-1 glycoprotein expression in the brain. Furthermore, the levels of IFNβ were decreased in both the serum and brain, suggesting that the main driving force in survival was decreased viral replication (Soberman et al., 2012). Whether decreased viral titers are due to increased antipathogenic defenses in CD200R1 KO mice or due to a direct effect of CD200R1 on viral replication remains to be determined. When we examined the interaction of HSV-1 with thioglycollate-induced peritoneal macrophages we uncovered a potentially far more complex relationship between CD200R1 and cell signaling by TLR2. Rather than show an amplified generation of IL-6 in response to HSV-1, the cytokine response was blunted by 80%. This was not seen in response to LPS, a TLR4 ligand. Furthermore, the surface expression of TLR2 following HSV-1 infection of macrophages was not upregulated (Soberman et al., 2012).

### 2.2.3 Viral orthologs of CD200

Similar to other inhibitory receptors, several viruses have directly utilized the downregulatory signaling pathways mediated by CD200:CD200R1 interactions for their survival within the host. Members of the herpesviruses and poxviruses have incorporated or evolved orthologs of the host CD200 protein in their genome (Table 5.3).

Perhaps the best characterized is the Kaposi's sarcoma-associated herpesvirus or human herpesvirus 8 or (HHV8) *K14* gene, which encodes a viral ortholog of CD200 (vOX2) that is expressed on the surface of infected cells

**Table 5.3** Viral CD200 orthologs

| Virus | Gene | Binds CD200R1? | References |
|---|---|---|---|
| HHV8 | vOX2 (K14) | Yes | Foster-Cuevas, Wright, Puklavec, Brown, and Barclay (2004), Misstear et al. (2012), and Shiratori et al. (2005) |
| RRV | R15 | ? | Langlais, Jones, Estep, and Wong (2006) |
| Myxoma Virus | M141R | ? | Cameron, Barrett, Liu, et al. (2005) and Zhang et al. (2009) |
| RCMV | e127 | Yes | Foster-Cuevas et al. (2011) |

during the lytic phase (Foster-Cuevas et al., 2004). Although vOX2 shares 36–40% identity with human CD200, both vOX2 and CD200 bind to CD200R1 with equivalent affinity and avidity (Foster-Cuevas et al., 2004; Misstear et al., 2012). *In vitro*, vOX2 can downregulate TNFα, granulocyte colony-stimulating factor (G-CSF), and monocyte chemoattractant protein-1 release from macrophages activated with IFNγ and LPS (Foster-Cuevas et al., 2004). When comparing the function of CD200 and vOX2, Misstear et al. (2012) found that APCs (cell lines that express native HLA-A2 and HLA-B8) transduced to express either CD200 or vOX2 suppressed T-cell IFNγ secretion, ERK1/2 and AKT phosphorylation, and mobilization of CD107a. CD200 and vOX2 also contributes to maintenance of the homeostasis of antigen-specific T-cell responses *in vivo* by negatively regulating their activity in a manner similar to CTLA-4 and PDL-1/2 (Misstear et al., 2012). Human herpesviruses 6 and 7 also express CD200 orthologs that bind to human CD200R1 (Shiratori et al., 2005), though their function is not as well characterized.

Human CD200 and HHV8 vOX2 have also been found to function in downregulating basophil function. Basophils have the highest basal expression level of CD200R1 in human peripheral blood, and activation through FcεR1 engagement, measured by CD11b upregulation and histamine release, was blocked by cross-linking CD200R1 with either human CD200 or vOX2 soluble proteins (Shiratori et al., 2005). Interestingly, this inhibition was not seen when basophils were stimulated with IL-3, suggesting specificity for CD200 inhibitory functions.

Rhesus rhadinovirus (RRV) is a gammaherpesvirus similar to HHV8. RRV expresses a viral CD200 protein, R15, that is expressed on the surface of infected cells and released into the supernatant (Langlais et al., 2006). Similar to other viral CD200 orthologs, R15 decreases TNFα mRNA and cytokine release from PMA-activated THP-1 macrophages as well as primary rhesus monocytes/macrophages. These inhibition levels were similar to that of human CD200.

The myxoma virus CD200 ortholog, M141, can function as a global inhibitor of macrophage and lymphocyte activation, leading to increased pathology and viral spread (Cameron, Barrett, Liu, Lucas, & McFadden, 2005; Zhang et al., 2009). M141 is expressed on the virion of myxoma viruses and contains a single Ig-like domain, similar to the N-terminal region of cellular CD200 (Zhang et al., 2009). M141-deficient virus-infected rabbits exhibited significantly decreased pathology, including decreased lesion size and number as well as increased healing (Cameron, Barrett, Liu, et al., 2005).

There was also a significant increase in the number of iNOS+ cells recruited to sites of infection and activated T-cells in lymph nodes (Cameron, Barrett, Liu, et al., 2005). Additionally, mouse macrophages infected with M141-deficient virus exhibited an activated phenotype, including increased TNFα and G-CSF levels, whereas WT virus-infected macrophages did not, due to decreased NF-κB signaling (Zhang et al., 2009). This is thought to occur through interactions with CD200R1 but has not been proven.

Other viruses express CD200 orthologs, but its direct role in mediating viral fitness is unclear. Such is the case for the e127 CD200-like protein of rat CMV. With approximately 56% identity to the host CD200 protein, e127 binds to CD200R1 with equivalent affinity, however, it does not significantly affect viral replication or myeloid activity *in vitro* or *in vivo* (Foster-Cuevas et al., 2011). This suggests that although CD200 mimics provide an evolutionary advantage to a variety of pathogens, its role may not be entirely the same in each infection and its effect upon binding to CD200R1 may differ.

## 3. PERSPECTIVE

It is clear, based on the number of pathogens that have evolved to exploit CD200 and CD200R1 expression as well as the widespread expression of CD200 mimics in viral genomes, that the CD200:CD200R1 signaling pathway plays a major role in host:pathogen interactions and pathogen survival. Understanding how these infectious agents use the CD200:CD200R1 axis to downregulate host defenses can potentially be exploited in clinical settings. Furthermore, it is important to completely uncover how CD200R1 regulates immune responses, both dependent and independent of CD200 ligation, as this may provide important insight in the development of therapeutics.

An antibody that targets CD200 to block CD200R1 signaling (Kretz-Rommel et al., 2008) is currently in clinical testing for the treatment of cancer (ClinicalTrials.gov identifier: NCT00648739). This drug could also be used to treat pathogenic infections that are impacted by CD200:CD200R1 signaling. In addition to utilizing currently available therapeutics for the treatment of viral infections, it is an intriguing possibility to target viral CD200 orthologs to stimulate viral clearance, as opposed to blocking all signaling through targeting the host CD200 or CD200R1. This virus-specific targeting strategy would allow normal host immune response regulation to

continue, avoiding potential immunopathology, while blocking the ability of a virus to replicate unchecked.

Though the CD200:CD200R1 axis has been implicated to play a role in transplant tolerance (Gorczynski, 2001; Yu, Chen, & Gorczynski, 2013), one can speculate that disrupting this relationship in posttransplant patients, or other immunosuppressed patients may actually have short-term benefit under conditions where viral infections can become an issue. Viral infections in renal transplant recipients, for example, remain a significant problem (Weikert & Blumberg, 2008). In situations where they become difficult to control with antiviral therapy, the major option is to decrease immunosuppressive therapy and restore host antiviral defenses. Viruses most commonly associated with transplant tolerance include CMV, HSV-1, HHV8, Epstein–Barr, Varicella Zoster, BK, and PC viruses (Weikert & Blumberg, 2008). Though pretransplant screening combined with prophylactic treatment with antiviral therapy has been very effective in limiting morbidity caused by these infectious agents, there are times when this is not sufficient, especially with BK and PC viruses. Since some of these viruses target the CD200:CD200R1 signaling pathway, and likely more, blocking the interaction of CD200 with CD200R1 using an antibody or small molecule approach could support more efficient viral clearance while preserving immunosuppression.

There are still many questions about how CD200R1 regulates inflammatory responses in myeloid cells and T-cells. Only a few studies have looked at the signaling molecules within cells that associate with the cytoplasmic domain of CD200R1 following ligation with CD200. Furthermore, our recent findings that CD200R1 plays an immunomodulatory role in TLR2 surface expression and signaling add another level of complexity to an already multifunctional signaling interaction. The concept that inhibitory receptors can be multifunctional and may be required for proinflammatory signaling has emerged. For example, the T- and B-cell coreceptor CD150 contains a motif in its cytoplasmic tail, called the immunoreceptor tyrosine-based switch motif (ITSM), that can recruit either inhibitory or activating molecules (Shlapatska et al., 2001). Additionally, the NK cell receptor 2B4 can induce either inhibitory or activating signals depending on the level of expression, amount of receptor cross-linking, and availability of adaptor molecules (Chlewicki, Velikovsky, Balakrishnan, Mariuzza, & Kumar, 2008). Although CD200R1 has neither an ITIM nor an ITSM domain, it is possible that an alternative domain in the cytoplasmic tail can modulate anti- or proinflammatory signals. This is the case for the inhibitory receptors

CTLA-4, Tim-3, Lag-3, and CD160, none of which contain ITIM or ITSM motifs (Odorizzi & Wherry, 2012).

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CHAPTER SIX

# Immunopathogenesis of Neuromyelitis Optica

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## Contents



## Abstract

Neuromyelitis optica (NMO, Devic's syndrome) is a clinical syndrome characterized by optic neuritis and (mostly longitudinally extensive) myelitis. If untreated, NMO usually takes a relapsing course and often results in blindness and tetra- or paraparesis. The discovery of autoantibodies to aquaporin-4, the most abundant water channel in the CNS, in 70–80% of patients with NMO (termed NMO-IgG or AQP4-Ab) and subsequent

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investigations into the pathogenic impact of this new reactivity have led to the recognition of NMO as an autoimmune condition and as a disease entity in its own right, distinct from classic multiple sclerosis. Here, we comprehensively review the current knowledge on the role of NMO-IgG/AQP4-Ab, B cells, T cells, and the innate immune system in the pathogenesis of NMO.

## 1. INTRODUCTION

Neuromyelitis optica (NMO) is a clinical syndrome characterized by episodes of optic neuritis and longitudinally extensive myelitis. NMO, which mostly takes a relapsing course, was considered a clinical variant of multiple sclerosis (MS) for many decades. Recently, however, the syndrome was shown to be associated with antibodies to aquaporin-4 (AQP4), the most abundant water channel in the CNS, in 70–80% (termed NMO-IgG or AQP4-Ab), which are absent in MS (Lennon, Kryzer, Pittock, Verkman, & Hinson, 2005; Lennon et al., 2004; Jarius & Wildemann, 2013). NMO-IgG/AQP4-Ab-seropositive NMO is now recognized as a disease entity in its own right. In this review, we will discuss the immunopathogenesis of NMO focusing on immune cells and soluble factors that have been implicated in disease initiation and perpetuation.

## 2. AQUAPORIN-4 ANTIBODIES

Recent clinical, neuropathological, and animal studies have provided convincing evidence for a role of AQP4-Ab in the pathogenesis of Neuromyelitis optica (NMO) (Jarius, Paul et al., 2008; Jarius & Wildemann, 2010; Papadopoulos & Verkman, 2012).

### 2.1. Clinical and paraclinical findings

Clinical and paraclinical evidence supporting an immunopathogenic effect of AQP4-Ab can be delineated from studies that have focused on AQP4-Ab titers and, to a large part, from treatment strategies that have been successfully applied in patients with NMO and related syndromes. Moreover, important paraclinical evidence is derived from neuropathological studies.

#### *2.1.1 Evidence from neuropathology*

Human NMO lesions display a marked loss of astrocytic aquaporin-4 (AQP4) immunoreactivity combined with prominent deposits of IgG, IgM, and, as a sign of complement activation, the terminal membrane attack complex surrounding thickened blood vessels at sites of AQP4 loss (Lucchinetti et al., 2002; Misu et al., 2007; Roemer et al., 2007; Sinclair, Kirk, Herron, Fitzgerald, & McQuaid, 2007). As a key feature, the astrocytic

marker glial fibrillary acidic protein (GFAP) is lost in parallel with AQP4 lesions in many lesions, indicating astroglial loss, whereas myelin and neuronal axons are often preserved (Misu et al., 2006, 2007). These findings are consistent with the assumption that the autoimmune response in NMO primarily attacks astrocytes and is initiated by autoantibody-mediated loss of AQP4. Accordingly, as demonstrated by an animal study, the intracerebral administration of IgG from human NMO sera together with human complement promotes a marked loss of AQP4 as the initial event, whereas astrocyte death, demyelination, and neuronal necrosis does not occur until later in NMO lesion evolution (Saadoun et al., 2010). The inflammatory infiltrate in NMO lesions, in addition to macrophages/microglia and a small number of T cells, typically comprises numerous eosinophils and neutrophils, often in conjunction with signs of degranulation (Lucchinetti et al., 2002; Saadoun, Bridges, Verkman, & Papadopoulos, 2012). Interleukin-4 (IL-4), the dominant cytokine secreted by eosinophils, is known to cause a shift toward a type 2 helper T cell cytokine response (Rumbley et al., 1999) and by creating a B-cell-friendly environment could promote humoral autoimmune processes. Neutrophils may be mobilized into acute NMO lesions in response to intrathecal activation of the IL-17/IL-8 axis (Ishizu et al., 2005), and, as suggested by emerging evidence, also act together with eosinophils as direct amplifiers of autoimmune tissue damage during NMO lesion development (discussed in more detail below).

### 2.1.2 Evidence from serological studies

Seropositivity for AQP4-Ab has been demonstrated to predict future relapse and/or later conversion to clinically confirmed NMO in patients with limited forms of NMO such as isolated optic neuritis or longitudinally extensive transverse myelitis (Jarius, Franciotta, et al., 2012; Jarius, Frederikson, Waters, et al., 2010; Matiello, 2007; Weinshenker et al., 2006). Furthermore, AQP4-IgG titers correlated with NMO disease activity or with the severity of opticospinal involvement in several independent studies (Jarius, Franciotta, et al., 2012; Jarius, Abul-Enein et al., 2008; Kim et al., 2012; Takahashi et al., 2007). Although absolute antibody titers varied widely and a disease-triggering threshold value was not apparent, serial assessment performed in those studies showed that clinical attacks were often preceded by a continuous increase in antibody titers, while AQP4-Ab serum concentrations declined during recovery in individual patients and could be stably suppressed in response to various immunosuppressive treatments (Jarius, Abul-Enein et al., 2008; Kim et al., 2012; Takahashi et al., 2007). Recent complementary observations strongly support the impact of AQP4-Ab as an independent prognostic factor acting together with age at onset and the genetic background to determine clinical

phenotype and outcomes (Jarius, Franciotta, et al., 2012; Akman-Demir et al., 2011; Kitley et al., 2012). Indeed, a recent study from Germany stratified 175 patients with definite or limited NMO, all of Caucasian origin, according to AQP4-Ab serostatus and demonstrated that seropositivity is significantly associated with more severe optic and/or motor disability and with more extensive spinal involvement as assessed by MRI (Jarius, Ruprecht, et al., 2012). Interestingly, distinct phenotypic features associated with detectable AQP4-Ab included a relapsing NMO disease course, a pronounced predilection for female gender (10.4:1), and coexisting autoimmunity, whereas seronegative patients more frequently had monophasic NMO and a female-to-male distribution of only 1.9:1. A higher grade of clinical disability in AQP4-Ab-seropositive NMO was also apparent in a smaller survey in Turkey (Akman-Demir et al., 2011) and pediatric NMO was found to follow a milder disease course in a small series of seronegative cases (Huppke et al., 2010).

#### *2.1.3 Evidence from therapies targeting B cells*

Major indirect evidence pointing to a role for AQP4-Ab-related disease mechanisms has been derived from the clear success, in many patients, of therapeutic strategies that selectively target the soluble or cellular parts of the humoral immune response. Thus, anecdotal evidence or results obtained from retrospective studies comprising limited numbers of patients have shown that removal of AQP4-Ab by plasma exchange (Bonnan et al., 2009; Khatri, Kramer, Dukic, Palencia, & Verre, 2012; Kim et al., 2013; Merle et al., 2012; Watanabe, Nakashima, Misu, et al., 2007; Watanabe, Nakashima, Miyazawa, et al., 2007), therapeutic B-cell depletion by rituximab given as a single course or repeated treatment (Bedi et al., 2011; Cree et al., 2005; Jacob et al., 2008; Jarius, Abul-Enein et al., 2008; Kim, Kim, Li, Jung, & Kim, 2011; Pellkofer et al., 2011), or, only recently, counteracting IL-6 as a B-cell growth and differentiation factor (Araki et al., 2012; Ayzenberg et al., 2013; Kieseier et al., 2012), are useful in preventing NMO-related disease activity and disability. NMO patients treated with rituximab were shown to carry a risk of breakthrough attacks, when B cells recur or are incompletely suppressed (Jarius, Paul, et al., 2008; Pellkofer et al., 2011).

### 2.2. Experimental findings

Several lines of experimental evidence have clearly demonstrated that various effector functions elicited by binding of AQP4-Ab to the water channel protein AQP4 expressed within the CNS or, less convincingly, modified target structure function or surface expression contribute to the formation of NMO lesions.

### 2.2.1 AQP4-Ab interaction with AQP4

As a typical feature of pathogenic antibodies, the AQP4-Ab contained in NMO patient sera as well as recombinant AQP4-IgGs generated from cerebrospinal fluid (CSF) plasma cell clones target various extracellular epitopes of surface-bound AQP4 (Crane et al., 2011; Iorio et al., 2012; Nicchia et al., 2009; Pisani et al., 2011; Waters et al., 2008; Yu et al., 2011). Despite the wide variation in binding characteristics, some antibodies were shown to bind with higher affinity to the shorter M23 isoform than to full-length M1-AQP4 (Crane et al., 2011; Nicchia et al., 2009; Mader et al., 2010). It was therefore suggested that AQP4-Ab might partly bind to conformational epitopes linked to the formation of so-called orthogonal arrays of particles (OAPs), which is driven by M23 tetramers in clustered AQP4 expressed in membranes of astrocytic end-feet.

### 2.2.2 Effects of complement-dependent cytotoxicity

Complement activation in response to AQP4-Ab binding to astrocytic AQP4 and subsequent cell lysis likely constitutes the principal pathogenic effector mechanism, as demonstrated in both *ex vivo* and *in vivo* experimental settings. AQP4-IgG comprises mostly IgG1 (Isobe et al., 2012; Jarius, Jacob, Leite, Waters et al., 2008) and via its complement-activating properties has been shown to induce titer-dependent death of AQP4-transfected cell lines *in vitro* (Hinson et al., 2007, 2009; Kalluri et al., 2010). Notably, the percentage of AQP4-transfected HEK cells damaged by complement in the presence of AQP4-Ab-positive serum predicted the severity of clinical relapses (Hinson et al., 2009), underscoring the clinical relevance of this effect and, potentially, of intra-individual antibody titer fluctuations. While a single study reported a correlation between NMO attacks and increased total complement blood levels as determined by elevated CH50 serum levels, which may reflect a systemic inflammatory reaction at relapse (Doi et al., 2009), the demonstration of elevated levels of the anaphylatoxin C5a in the CSF of AQP4-IgG-seropositive patients suggests complement activation in the CNS (Kuroda et al., 2012). A recent study found that complement-dependent cytotoxicity (CDC) mediated by C1q binding to AQP4-Ab is greatly enhanced in M23-AQP4-transfected versus M1-AQP4-expressing cells; this could indicate that AQP4 must be assembled in OAPs to facilitate high-affinity antibody binding and multivalent attachment of the C1q protein to the complement-fixing sites in clustered AQP4-IgGs (Phuan, Ratelade, Rossi, Tradtrantip, & Verkman, 2012; Ratelade & Verkman, 2012). This observation is corroborated by *ex vivo*

and animal models of NMO. In an *ex vivo* approach using cultured mouse spinal cord slices or optic nerves exposure to AQP4-Ab-positive NMO sera or recombinant NMO antibody (generated from clonally expanded CSF plasmablasts of a seropositive NMO patient), but not to control antibody, in the presence of human complement reproduced the loss of AQP4, GFAP, and myelin that characterizes NMO lesions (Zhang, Bennett, & Verkman, 2011). Interestingly, lesion severity was enhanced by adding eosinophils, neutrophils, or macrophages, or various soluble proinflammatory cytokines supporting the potential role of these cofactors in the evolution of NMO lesions (Zhang et al., 2011; Zhang & Verkman, 2013). The crucial importance of CDC in mediating NMO pathology was further established in a rodent model in which the histopathological hallmarks of active NMO lesion pathology were replicated by directly injecting AQP4-Ab containing IgG from NMO sera together with human complement into noninflamed mouse brain, whereas a protective effect was observed when a C1 complement inhibitor was administered together with patient-derived IgG (Saadoun et al., 2010). Verkman and colleagues generated a high-affinity monoclonal antibody (proposed name: aquaporumab) from recombinant monoclonal antibodies derived from AQP4-IgG-positive CSF plasmablasts of a patient with NMO and rendered nonpathogenic by introducing IgG1Fc mutations at locations required for the induction of CDC (Tradtrantip, Zhang, Saadoun, et al., 2012). As a competing antibody, aquaporumab efficiently prevented binding of AQP4-IgG in NMO sera to AQP4-transfected cell lines and abolished target cell killing through CDC in AQP4-expressing astrocyte cultures, spinal cord slice cultures, and mice exposed to intracerebrally injected AQP4-IgG together with human complement. Similar effects were achieved by using AQP4-IgG-binding small molecules (identified by automated high-throughput screening) that sterically block interaction between AQP4-Ab and its target antigen in an idiotype-specific fashion to prevent antibody-dependent cytotoxic effector functions (Phuan, Anderson, et al., 2012; Tradtrantip, Zhang, Anderson, et al., 2012), by cleavage of AQP4-IgG using an IgG-degrading enzyme of Streptococcus pyogenesto (IdeS) to yield Fc and F(ab')2 fragments (Tradtrantip, Asavapanumas, & Verkman, 2013), or by selectively deglycosylating the heavy chain of natural AQP4-IgG with bacteria-derived endoglycosidase S to render it nonpathogenic (Tradtrantip, Ratelade, Zhang, & Verkman, 2013).

Of note, prominent IgM deposition has been demonstrated in NMO lesions (Lucchinetti et al., 2002). Anti-AQP4 serum antibodies of the

IgM class are present in at least 10% of patients with NMO (Jarius, Franciotta, Bergamaschi, Wildemann, & Wandinger, 2010); IgM is generally considered much more efficient in activating complement than is IgG.

### 2.2.3 Effects of cell-mediated cytotoxicity

In contrast to the importance of CDC in generating astrocytic damage in NMO, the *in vivo* relevance of antibody-dependent cell-mediated cytotoxicity (ADCC) as another potential effector consequence of AQP4-Ab is less compelling. ADCC achieves target cell lysis by activating natural killer (NK) cells or, more rarely, other cellular effectors via interaction with Fc-receptors expressed on these cells. Human fetal astrocytes coincubated with NMO serum were efficiently killed through ADCC following exposure to human NK cells (Vincent et al., 2008). Furthermore, when administered together with human NK cells, human recombinant AQP4-IgG has been shown to mediate ADCC in cultured murine astrocytes as well as to elicit loss of AQP4 and GFAP without myelin damage after injection into mouse brain, while coadministration of mutant aquaporumab deficient in ADCC induction prevented these effects (Ratelade et al., 2012). The same study also demonstrated that NK cells amplified the NMO lesion development induced by purified IgG from NMO sera and complement in the aforementioned *ex vivo* spinal cord slice model. In an AQP4-expressing Chinese hamster ovary (CHO) cell culture, Fc fragments generated by IdeS-mediated cleavage of recombinant AQP4-specific IgG effectively reduced AQP4-Ab/NK-cell-mediated ADCC (Tradtrantip, Asavapanumas, et al., 2013). However, a recent histopathological analysis has revealed that Granzyme B+ and Perforin+ NK cells and cytotoxic T cells can only occasionally be detected in active human NMO lesions, thus challenging a relevant *in vivo* role of pathogenic ADCC mechanisms in NMO (Saadoun, Bridges, et al., 2012). Whether the binding of AQP4-IgG to Fc-receptors expressed on macrophages, neutrophils, and eosinophils, which are abundantly present in NMO lesions, contributes to NMO pathology by mediating ADCC remains to be determined, although very recent evidence has shown that eosinophils might be important pathogenic effectors through both complement-dependent and cell-mediated cytotoxicity (Zhang & Verkman, 2013). In addition, the intracerebral or intraperitoneal injection of neutrophil protease inhibitors effectively counteracted neuroinflammation and neural tissue damage in a mouse model of NMO (Saadoun, Waters, et al., 2012), while neutrophilia prompted by inadvertent granulocyte CSF administration induced detrimental disease activity in a

patient with NMO (Jacob et al., 2012). Failure to block granulocytic migration across the blood–brain barrier (BBB) might thus represent a plausible reason why treatment with natalizumab may not be suited to properly prevent NMO relapses (Barnett, Prineas, Buckland, Parratt, & Pollard, 2012; Kleiter et al., 2012).

### 2.2.4 Effects on AQP4 function and surface expression

Other possible consequences of AQP4-Ab interaction with the AQP4 water channel include direct interference with water transport and with surface expression of AQP4 through endocytosis. These two mechanisms have been studied and produced conflicting results. In a study from 2009, AQP4-Ab did not functionally interfere with the water transport capability of AQP4 and this finding was in line with the observation of the same authors that the antibody targets a conformational intertetramer epitope linked to OAP formation rather than an intratetramer region (Nicchia et al., 2009). In astrocyte cultures, short-term exposure to NMO sera did not compromise plasma membrane water permeability as measured by videomicroscopy when AQP4 downregulation was avoided by keeping cultures at a low temperature (Melamud et al., 2012). Concordant with the hypothesis that each AQP4 tetramer comprising four separate water pores can hold only one of the larger AQP4-IgG molecules and therefore is unlikely to become functionally blocked, Rossi and coauthors did not observe significant inhibition of AQP4 water channel function (Ratelade & Verkman, 2012; Rossi, Ratelade, Papadopoulos, Bennett, & Verkman, 2012). Using stopped-flow light scattering to measure osmotic water permeability on plasma membrane vesicles isolated from AQP4-expressing CHO cells, this study found that water transport through M1-AQP4 tetramers and M23-AQP4 clusters exposed to high-titer NMO sera of recombinant AQP4-Ab was preserved. The sequelae of AQP4-Ab binding on water channel function remain, however, controversial since a concurrent study from the Mayo Clinic in 2012 postulated that pooled NMO sera negatively affect water influx in both AQP4 isoforms, as detected in a time-to-lysis assay of AQP4-transfected Xenopus oocytes (Hinson et al., 2012).

Likewise, it is currently a matter of debate as to whether AQP4-Ab binding on astrocytic AQP4 induces internalization of AQP4 or differentially affects the endocytosis of the M1 versus the OAP-forming M23 isoform of AQP (Rossi et al., 2012; Hinson et al., 2012). Overall, the selective cellular endocytosis of AQP4 in response to the specific attachment of

AQP4-Ab that has been independently demonstrated in transfected cell lines and various rodent or human astrocyte culture systems (Hinson et al., 2007, 2008; Vincent et al., 2008; Melamud et al., 2012) was not demonstrated *in* vivo after direct injection of fluorescent AQP4-Ab in mouse brain (Ratelade, Bennett, & Verkman, 2011). The latter approach also failed to confirm coupled depletion of AQP4 and the $Na^+$-dependent excitatory amino acid transporter 2 (EAAT2) from astrocytic membranes, which was shown to impair glutamate uptake and the anti-excitotoxic capability of astrocytic cell cultures and, at least *ex vivo*, appeared to contribute to the unfavorable outcomes attributed to AQP4-Ab binding (Hinson et al., 2008; Ratelade et al., 2011).

## 2.3. Origin of AQP4-Ab within the CNS

How AQP4-Ab enters the CNS and initiates lesion formation is incompletely understood; however, emerging evidence highlights the role of AQP4-specific T cells in rendering the CNS accessible for circulating AQP4-IgG and in fostering antibody-mediated neuroinflammation as discussed below in more detail (see Section 3.1 of this chapter). Although plasma cells secreting pathogenic AQP4-Ab were detected in the CSF of a patient early during the course of NMO (Bennett et al., 2009), two independent studies have assessed parallel CSF and serum samples obtained from seropositive patients with NMO and reported that this autoantibody universally reaches the CSF by passive diffusion from serum and is not, or only exceptionally produced intrathecally (Kalluri et al., 2010; Jarius, Franciotta, Paul, et al., 2010). In line with the assumption that AQP4-IgG mainly originates from peripheral sources, AQP4-Ab-positive plasmablasts are selectively increased in the blood of NMO patients, peak at relapse, and are maintained by increased serum levels of IL-6 (Chihara et al., 2011). Data delineated from several passive transfer studies in which IgG from seropositive patients with NMO was injected in rats with preexisting experimental autoimmune encephalomyelitis (EAE) or pretreated with complete Freund's adjuvant to compromise the integrity of the BBB led to the conclusion that AQP4-Ab requires a background inflammatory environment and a disrupted BBB to become harmful and to induce CNS injury (Bennett et al., 2009; Bradl et al., 2009; Kinoshita et al., 2009, 2010). Several observations in NMO patients are consistent with such prerequisites. Thus, relatively high titers of AQP4-Ab can be detected during remission in many patients (Jarius, Franciotta, et al.,

2012; Jarius, Paul, et al., 2008), and, in rare cases, even many years before NMO onset (Jarius, Franciotta, et al., 2007; Jarius, Paul, et al., 2012; Leite et al., 2012; Nishiyama et al., 2009). Moreover, T-cell activation or cytokine secretion associated with viral infections could operate as a disease trigger in NMO. Signs of viral infections frequently precede the onset of NMO attacks and have been reported in 15–35% of NMO patients (Kitley et al., 2012; Jarius, Ruprecht, et al., 2012; Ghezzi et al., 2004; Wingerchuk, Hogancamp, O'Brien, & Weinshenker, 1999). Some evidence, however, strengthens the probability that accompanying conditions are not always mandatory for AQP4-Ab to exert its pathogenic potential *in vivo*. Thus, brain areas that harbor fenestrated endothelium and therefore lack a proper BBB, such as the area postrema, have been shown to display a selective loss of AQP4 immunoreactivity and, inconsistently, also other typical features of lesions in confirmed NMO cases (Popescu et al., 2011). This important neuropathologic observation indicates that the area postrema might be a site of initial entry of AQP4-Ab in some patients and that at this vulnerable location the autoantibody might act alone to promote NMO disease activity. This hypothesis is supported by reports of intractable nausea or vomiting being the presenting sign or heralding episodes of NMO attacks (Apiwattanakul et al., 2010; Iorio et al., 2013; Patel, Griffith, Blackwood, Dias, & Cordato, 2012; Popescu et al., 2011).

## 2.4. Preferential optic nerve and spinal cord damage

It is presently not known why NMO disease activity, at least in adults, involves the optic nerves and the spinal cord as principal targets. Very interesting, but as yet unpublished findings suggest that the water channel protein AQP4 is expressed at higher levels within the human CNS than in peripheral organs such as kidney and liver, and occurs at maximum concentrations in opticospinal tissues. Moreover, the spinal medulla and the optic nerves appear to contain higher amounts of supramolecular AQP4 aggregates, a feature that might impact the affinity of AQP4-IgG binding to its target (Matiello, Sun, Schaefer-Klein, & Weinshenker, 2012).

In summary, from the evidence on humoral pathology in NMO currently available, it is hypothesized that NMO lesions originate when, via its complement-activating properties, AQP4-IgG causes damage to astrocytes by CDC as soon as it has crossed the BBB and binds AQP4, which is concentrated in a highly polarized fashion along astrocytic end-feet at this and other sites. This initial event is likely to promote subsequent

chemoattraction of neutrophils and eosinophils and release of cytokines, which through further disruption of the BBB and macrophage infiltration then causes oligodendrocyte death, myelin loss, and neuronal injury (Jarius & Wildemann, 2010; Papadopoulos & Verkman, 2012; Jarius, Paul, et al., 2008; Ratelade & Verkman, 2012).

## 3. INNATE IMMUNITY

### 3.1. Myeloid cells and neutrophils

While the encephalitogenicity of AQP4-Ab has been established in experimental models, it is a downstream event in the pathogenesis on NMO. It is likely that the initial immune response against AQP4 in the CNS mediated by CNS-intrinsic myeloid cells, the microglia (Serhan & Savill, 2005; Serhan, 2007). The response is likely a result of astrocyte death and the release of antigen that is not recognized as self. The CNS is a relatively immune-protected organ, and it is conceivable that the systemic immune system is sequestered away from otherwise dominant antigenic determinants. The expression of soluble inflammatory mediators disrupts the BBB during this disease stage, which allows immune competent cells from the periphery to migrate into the brain into the cerebral perivascular spaces. Once there, microglia and systemic myeloid cells phagocytose cell debris, degranulate, release enzymes, cytokines, and chemokines, all of which further amplifies the inflammatory response (Springer, 1994). After a few days, driven by the aforementioned inflammatory cascade and chemoattraction, the second phase of NMO starts, during which cells of the adaptive immune system extravasate into the affected tissue. Myeloid antigen-presenting cells (APCs) process AQP4 and present linearized determinants to $CD4^+$ T cells (Fig. 6.1, Inset 1). During this second stage, neutrophils are also attracted to the CNS (Fig. 6.1, Inset 4). Interleukin-17 (IL-17), expressed mainly, but not exclusively by T-helper cells, might be the main chemoattractant that brings neutrophils into the brain and spinal cord. Neutrophils possess three mechanisms by which they attack foreign or foreign appearing antigen: release of granule protein, phagocytosis, or the generation of neutrophil extracellular traps (NETs) (Segal, 2005).

### 3.2. Complement

The complement system is a component of the innate immune system made up of more than 30 serum proteins whose purpose is to attack foreign pathogens using a combination of cell lysis, chemotaxis, and opsonization

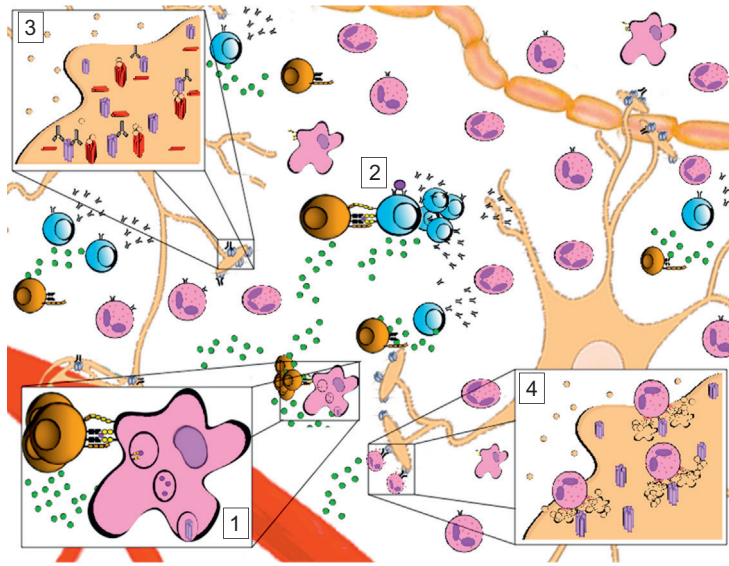


**Figure 6.1** *A pathogenic model of NMO*. The presence of the AQP4-Ab biomarker strongly implicates an adaptive, B-cell-mediated humoral immune response in the final stages of an inflammatory cascade that drives disease activity in patients with NMO. The events that lead to recognitions of the self-antigen AQP4 are currently unknown and the topic of speculation. Two pathomechanistic events might trigger disease onset in susceptible individuals: (1) molecular mimicry or (2) the death of a host cell in a relatively immune-protected compartment that expresses AQP4, an event that leads to an aberrant autoimmune response.

Molecular mimicry could occur in a scenario where a structural or amino acid sequence homology exists between an inoculated pathogen and a host protein (Fujinami & Oldstone, 1985). This structural homology is incomplete, and thereby prevents the host to recognize the antigen as self.

Alternatively, immune sequestration away from certain tissues, including the CNS, may prevent the exposure of dominant antigenic determinants to the systemic immune system. In the case of cellular demise of a cell that expresses such antigen, peripheral immune tolerance may be lost.

If either event occurs within the brain or spinal cord, and the cellular target is an astrocyte that expresses AQP4, an immune response may be initiated, in which CNS-intrinsic myeloid antigen-presenting cells (APC), microglia, phagocytize cell debris, and express chemokines, cytokine, and other soluble inflammatory mediators to attract cells of the adaptive immune system (Insert, 1). Now, microglia, systemic myeloid cells, and B cells process AQP4 and present linearized peptide in the context of major histocompatibility complex (MHC) to T cells with a corresponding T cell receptor (TCR). Activated T cells cross-activate antigen-specific B cells (Insert, 2). The B cells mature into plasma cells and memory B cells, both of which are capable of expressing AQP4-Ab. The antibodies can amplify the inflammation by binding to conformational AQP4 epitopes, and killing the cellular target, the astrocyte, by CDC or ADCC (please see earlier section on pathogenic NMO-IgG; Insert, 3). T cells may also perpetuate the tissue inflammation by

(Sturfelt & Truedsson, 2012). The three arms of the complement cascade include the classical pathway, which is triggered by specific antibody–antigen binding, the alternative pathway triggered by complement protein C3 binding, and the lectin pathway triggered by lectin binding to sugar moieties on the surface of bacteria. All three pathways are mediated by a balance of activating and inhibiting proteins that serve to target foreign bodies and protect against host destruction. In addition to NMO, complement dysregulation plays important roles in the pathogenesis of end-organ damage in lupus (Molina, 2002), lupus nephritis (Sturfelt & Sjoholm, 1984), antiphospholipid antibody syndrome (El-Behi et al., 2011), rheumatoid arthritis (Hedberg, 1970), and Sjogren's Syndrome (Zadura, Theander, Blom, & Trouw, 2009).

The role of the complement system in the pathogenesis of NMO was first highlighted by Lucchinetti et al. (2002) with the demonstration of perivascular complement deposits in NMO lesions. C9neo, the residual component of activated membrane attack complexes, was found in rim and rosette patterns around blood vessels within actively demyelinating NMO lesions in all nine patient samples studied (Lucchinetti et al., 2002). Other complement components were also found deposited in the tissue but in a pattern that suggests a general leaky BBB rather than specific involvement. While this study suggested that complement-mediated destruction was an integral part of the NMO disease process, it was not clear what triggered the complement activation and which complement pathway, classical, alternative, or lectin was implicated.

The discovery of the NMO-IgG biomarker against AQP4 water channels suggested that NMO disease could be triggered by specific antibody–antigen binding in the spinal cord and optic nerve. The theory of a pathogenic NMO antibody (discussed in the first section of this review) suggests that the classical complement pathway is initiated by AQP4-specific antibodies, which triggers complement-mediated destruction in acute NMO lesions (Fig. 6.1, Inset 3). Demonstration of the capacity for NMO-IgG to fix complement upon binding to AQP4 was first shown using

expressing cytokines. In NMO, it is likely that T-helper cells expressing interleukin-17 (IL-17) play a critical role in the disease process, as neutrophils can be detected in abundance in NMO lesions (Lucchinetti et al., 2002). These cells migrate into tissue against an IL-17 gradient. Neutrophils possess three mechanisms by which they attack foreign or foreign appearing antigen: release of granule protein, phagocytosis, or the generation of neutrophil extracellular traps (NETs) (Segal, 2005).

AQP4-HEK cells which would lyse upon exposure to NMO-IgG and active human complement while control cells or control serum did not result in complement-mediated cell death (Hinson et al., 2007). The same group showed that targeting human derived astrocytes, rather than HEK cells, provides limited protection against complement-mediated cell death in culture (Hinson et al., 2008). However, the presence of immune effector cells, such as NK cells, neutrophils and macrophages, or eosinophils could overcome this astrocyte protection (Zhang et al., 2011; Vincent et al., 2008; Zhang, Ratelade, & Verkman, 2012).

Taken together, these studies suggest that antibody–antigen complexes comprised specifically of NMO-IgG and AQP4, respectively, could trigger the classical complement pathway. Further evidence that the classical complement pathway is implicated in human pathology could be shown with staining C4d, a complement component deposited in affected tissue and that is only produced by the classical pathway. Determining which complement pathway is involved in NMO has implications for potential treatment using various complement inhibitors presently being tested in NMO clinical trials (Ricklin & Lambris, 2007).

Animal models have been helpful in confirming the relevance of complement activation in the pathogenesis of NMO. Two rat studies in which passive transfer of the human NMO-IgG exacerbated background neuroinflammation from EAE also found evidence of complement activation within spinal cord lesions (Bradl et al., 2009; Kinoshita et al., 2009). Mouse models using passive transferred NMO-IgG are less likely to fix complement as human IgG1 does not easily fix mouse complement. However, when injected directly into a mouse brain, NMO-IgG along with active human complement can recreate typical appearing NMO lesions (Saadoun et al., 2010).

NMO is not the only neurological disease with complement deposition in affected tissues, as Alzheimer's disease, viral infection with cytomegalovirus or JC virus, multiple sclerosis (MS), and paraneoplastic encephalitis all have evidence of either C3d or C9neo (Barnett, Parratt, Cho, & Prineas, 2009). Complement activation is not a specific marker for NMO or any other disease. However, deposition of complement proteins in these neurologic diseases prompts the question of whether inhibition of complement could ameliorate disease? In NMO, this question has been answered by a phase II, open-label study of eculizumab, a C5a inhibitor, in prevention of NMO relapses. This study of eight treatment-refractory and six naïve subjects who received biweekly eculizumab demonstrated a profound

reduction in relapse rates over 18 months of treatment (Laino, 2012). One of the 14 subjects also developed gram positive bacteremia and sepsis despite vaccination raising concerns over the safety of persistent C5a inhibition. An alternative approach endorsed by the makers of purified human C1-esterase inhibitor (Cinryze®) is to inhibit complement upon hospitalization for acute NMO relapse. This 10-subject safety trial in acute NMO has just started enrolling at the present time (Barnett et al., 2009; Clinicaltrials.gov webpage; Vaknin-Dembinsky, Brill, Orpaz, Abramsky, & Karussis, 2010; Kowarik et al., 2012). Both complement inhibitor approaches provide an alternative mechanism for treatment of NMO to the standard immunosuppressive regimen and they both serve to show that complement is an integral component of the pathogenesis of NMO.

## 4. ADAPTIVE IMMUNITY

### 4.1. T cells

Due to abundant evidence of humoral immunity in the majority of patients with NMO, neurologists have focused on pharmacotherapies that predominantly target the humoral immune system (Cree et al., 2005; Jacob et al., 2008; Kim et al., 2011; Pellkofer et al., 2011). However, there is emerging evidence to suggest a cellular immune response in NMO as well. First, an HLA haplotype analyses of NMO patients recently suggest a positive association with HLA-DRb1*03:01 (HLA-DR17) (Brum et al., 2010; Zephir et al., 2009), a gene that codes for an MHC class II molecule that presents linear antigens of 12–15 amino acid (AA) length to $CD4^+$ T cells (Geluk et al., 1994). Second, NMO-IgG is undetectable in 20–30% of NMO patients (Jarius, Franciotta, et al., 2007), so-called seronegative NMO. Third, in patients with NMO-IgG seropositivity, antibody isotype switching from IgM to IgG can only occur with $CD4^+$ T-helper cell involvement (Cocks, de Waal, Galizzi, de Vries, & Aversa, 1993; Shapira et al., 1991). Finally, $CD3^+$ T cells are abundantly present in NMO lesions (Lucchinetti et al., 2002). It should also be emphasized that B-cell-depleting therapies do not regularly reduce NMO-IgG titers (Cree et al., 2005; Jacob et al., 2008; Kim et al., 2011; Pellkofer et al., 2011) and may act via suppression of T cell activation.

The identification of immunodominant determinants of human AQP4 may have important implications for understanding the etiology of NMO and monitoring disease activity in patients afflicted with this disorder.

Immunodominant determinants are stretches of AQP4 protein that can activate T cells. In different wild-type mouse strains, including C57BL/6 (H-2b) and SJL/J (H-2s) mice, these determinants of the M1 isoform of AQP4 were recently identified (Nelson et al., 2010; Kalluri et al., 2011).

Nelson et al. (2010) and Kalluri et al. (2011) were the first groups to identify critical linear determinants that were capable of stimulating a cellular immune response in mice. By screening overlapping peptides of human and mouse AQP4 in C57BL/6 or SJL/J mice, AQP4 21–40 was identified to be an immunogenic peptide capable of stimulating a cellular immune response in both mouse strains. Specifically, this linear determinant was found to lead to an increase in thymidine incorporation in proliferating T cells in culture as well as production of T cell production of IL-2, IFN-γ, GM-CSF, IL-10, and IL-17a (Nelson et al., 2010; Kalluri et al., 2011).

Immunization with AQP4 determinants can generate an NMO animal model similar to the animal model for MS, EAE. While human and mouse AQP4 have a 93% sequence homology, it is important to note that the immunogenic region of AQP4 21–40, AQP4 22–36, has 100% sequence homology between human AQP4 in the M1 isoform and murine AQP4 in the M1 isoform (Kalluri et al., 2011). Immunization of AQP4 22–36 did not lead to ascending paralysis similar to EAE. Spinal cord and optic nerve T cell infiltration was not observed in AQP4 immunized mice, suggesting that while this peptide is immunogenic in culture, it is not encephalogenic.

Recently, AQP4 was found to have structural homology with the *Escherichia coli* AQPZ protein, a bacterial aquaporin containing three peptides with at least 45% sequence homology to human AQP4: AQPZ 82–101, AQPZ 174–190, and AQPZ 203–220 (Ren et al., 2012). This study found that immunization with AQPZ peptides lead to a recall response in a human AQP4 peptide specific manner. It was also shown that immunization of these three AQPZ peptides into SJL/S mice lead to CNS inflammation with $CD4^+$ T cells and monocytes localizing to the CNS. These data suggest a possible role for molecular mimicry in the initial activation of T cells against AQP4. Perhaps utilizing bacterial proteins homologous to AQP4 will lead to an NMO animal model that will bring forth a better understanding of the important role T cells play in the clinical disease course.

Though this data in C57BL/6 and SJL/J mice identified AQP4 linear determinants that stimulate a cellular immune response in H-2, the C57BL/6 genetic background does not express H-2-IE, the equivalent gene

of the human class II MHC molecule HLA-DR. Therefore, transgenic mice expressing *HLA-DRB1*03:01* were utilized to identify linear dominant determinants of AQP4 M1 (as described in Strauss, Vignali, Schonrich, & Hammerling, 1994). This study specifically aimed to identify immunodominant linear determinants of AQP4 in the context of *HLA-DRB1*03:01*, as this HLA haplotype was recently associated with NMO in several patient cohorts (Arellano et al., 2012; Brum et al., 2010; Zephir et al., 2009). Other studies have shown that blocking of HLA-DR is capable of inhibiting the proliferation of T cells isolated from NMO patients that are AQP4 specific suggesting a crucial role for the MHC II molecule in the activation of AQP4-specific T cells (Varrin-Doyer et al., 2012).

ELISpot assays allowed for determining the frequency of antigen-specific T cells capable of binding AQP4 peptide determinants and to characterize their cytokine profiles. This is relevant, as encephalogenicity of T cells in MS is largely defined by their cytokine phenotype. Historically, Th1 cells, defined by the signature cytokine IFNγ, have been implicated in MS autoimmunity (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). Perhaps the most convincing evidence to support a pathogenic role of IFNγ in patients with MS was inadvertently generated in a clinical study, in which 7 out of 18 patients who received recombinant IFNγ therapy experienced an MS disease exacerbation (Panitch, Hirsch, Haley, & Johnson, 1987). In the last decade, another subclass of pathogenic $CD4^+$ T-helper cells was characterized by the production of IL-17. Th17 cells appear to facilitate the initiation and perpetuation of CNS autoimmune disease (Harrington et al., 2005), and mediate proinflammatory and allergic responses. IL-17 is the critical cytokine needed to localize neutrophils to the sites of infection (Miyamoto et al., 2003). Th17 cells have also been shown to play a critical role in the production of GM-CSF in the periphery and CNS (El-Behi et al., 2011; Codarri et al., 2011). Several investigators recently showed that GM-CSF may play critical role in different models of active and passive EAE (Codarri et al., 2011; Cravens et al., 2011; King, Dickendesher, & Segal, 2009; Ponomarev et al., 2007). GM-CSF is secreted by lymph node cells and splenocytes after antigen restimulation in the presence of IL-12. In an adoptive transfer model of EAE we also showed that GM-CSF is highly expressed by encephalogenic T cells. In the study utilizing HLA-DRB1*0301 transgenic mice, one peptide in this group of 32 overlapping AQP4 peptides, peptide 29 ($AQP4_{281–300}$), was capable of leading to a Th1 and Th17 recall response (Arellano et al., 2012).

Because of their biophysical properties, linear peptides 12–15 AAs in length fit best within the antigen-binding groove of MHC class II molecules (Geluk et al., 1994). Thus, the immunodominant subdeterminants within $AQP4_{281–300}$ within *HLA-DRB1*03:01* were identified by performing IFNγ, IL-17, and GM-CSF ELISpot assays with 15mer peptides spanning $hAQP4_{281–300}$ (Arellano et al., 2012). In lymph node cells and splenocytes from *HLADRB1*03:01* mice immunized with $AQP4_{281–300}$, $AQP4_{284–298}$ induced a significantly higher frequency of IL-17 expressing T cells than other 15mers. $AQP4_{284–298}$ also induced the highest frequency of GM-CSF expressing T cells. CFSE proliferation assays confirmed that the proliferating cells to these peptides are $CD4^+$ T cells.

The identification of $AQP4_{284–298}$ infers that the cellular immune response may play a critical role in NMO disease development and progression due to its ability to stimulate a pronounced $Th_{17}$ immune response in the context of HLA-DRB1*03:01. The levels of IL-17 are increased in patients with NMO during disease relapses (Wang et al., 2011; Wang et al., 2012). This finding would explain the presence of neutrophils at sites of tissue damage (Lucchinetti et al., 2002). It is now recognized that Th17 cells possess substantial plasticity compared to other T-helper cells (Lee et al., 2009). Multiple studies have shown that activation of T cells from NMO patients leads to the secretion of Th17-associated cytokines such as IL-6, IL-21, and IL-17 (Varrin-Doyer et al., 2009; Linhares et al., 2013). In the setting of NMO, the increased levels of IL-6 found in the CSF of NMO patients may also allow for the survival of AQP4-specific Th17 cells, while at the same time inhibiting $FOXp3^+$ T regulatory cells (Goodman et al., 2009; Korn et al., 2008; Uzawa et al., 2010). IL-6 has also shown important in the survival and secretion of NMO-IgG in plasmablasts isolated from NMO patients (Chihara et al., 2011). It is also noteworthy that in a Chinese patient cohort, a polymorphism in the IL-17 gene was recently associated with anti-AQP4 antibody positive NMO (Wang et al., 2012). Thus far, one group of investigators did not find elevated GM-CSF levels in the CSF of NMO patients with active clinical disease (Uzawa et al., 2010). However, the accumulation of eosinophils and granulocytes in NMO lesion may suggest that this cytokine may also play a pathogenic role (Lucchinetti et al., 2002).

T cells, in addition to causing tissue damage themselves through the expression of proinflammatory cytokines and other inflammatory mediators, also are capable of cross-activating B cells (Fig. 6.1, Inset 2).

## 4.2. B cells

B cells may have a dual role in the pathogenesis of NMO. On the one hand, it is clear that AQP4-specific B cells must be the source of differentiating plasma cells that secrete AQP4-specific antibodies. As described in Section 2, emerging evidence suggests that these antibodies may not only serve as a diagnostic marker for NMO, differentiating this disease entity from other neuroinflammatory conditions, such as MS, but may act in a direct pathogenic manner. Therefore, B cells that recognize AQP4 may indirectly contribute to NMO pathogenesis as progenitor cells of this pathogenic humoral response.

In addition, cellular properties of B cells may independently exert pathogenic properties. A recent study indicates that in NMO frequencies of peripheral B cells are shifted from subsets with regulatory properties toward the pool of activated, memory B cells (Quan et al., 2013). Emerging evidence from experimental studies but also from clinical investigations further suggests that the development of an AQP4 antibody response is associated with a T cell response against AQP4, predominantly of Th17 cells (Kalluri et al., 2011; Varrin-Doyer et al., 2012; Vaknin-Dembinsky et al., 2012; Wang et al., 2011). To some extent this is not entirely surprising, as isotype switching and thus the maturation of a robust humoral response requires T-helper function, which can be accomplished by Th17 cells (Mitsdoerffer et al., 2010). In contrast, what is not understood is how this T cell response by itself is generated. In general, antigen-specific activation of cells requires recognition of the antigen, for example, AQP4 in the context of MHC molecules expressed by APCs. Like dendritic cells and macrophages, B cells are professional APCs as defined by a constitutive expression of MHC class II. In contrast to dendritic cells and macrophages, B cells express an antigen-specific B-cell receptor that enables them to recognize, bind, and internalize "their" antigen in a very efficient manner. After this initial step of specific antigen recognition, B cells can then present the processed antigen in the context of MHC class II. As a consequence of these unique cellular features, antigen-specific B cells are very competent in presentation of protein antigen when their B-cell receptor recognizes the same antigen as the responding T cell (Constant, Sant'Angelo, et al., 1995; Constant, Schweitzer, West, Ranney, & Bottomly, 1995; Weber et al., 2010). Thus, besides constituting the source for the potentially pathogenic humoral response, B cells, and in particular AQP4-specific B cells, may serve as potent APCs for activation of AQP4-specific T cells.

Recent findings suggest this B-cell–T-cell interplay may also occur in the target organ of the disease, namely, the CNS. In patients with NMO, the CSF compartment shows suggestive properties; first, B cells found in the CSF of NMO patients display signs of somatic B-cell hypermutation, generally indicative of B-cell antigen recognition within the target organ. Second, and along the same lines, B-cell recruiting and activating factors, such as BAFF or CXCL13 (Kowarik et al., 2012) were found to be increased in the CSF of NMO patients (Quan et al., 2013; Vaknin-Dembinsky et al., 2010). Lastly, CSF samples of NMO patients contain significantly higher amounts of both IL-6 (Uzawa et al., 2009) and IL-17 (Wang et al., 2011). Given that IL-6 together with TGF-beta is the central cytokine for promoted development of Th17 cells, these findings highlight that proinflammatory T cell (re-) activation continues in the CNS itself. Although the cellular source of Th17-polarizing IL-6 remains generally elusive, activated B cells produce substantial amounts of this proinflammatory cytokine and are therefore a promising candidate for its cellular source.

Further evidence for the perception that cellular B-cell properties may independently promote NMO pathogenesis derives from the clinical use of anti-CD20 therapies in NMO, an antibody treatment which potently depletes immature and mature B cells while sparing CD20 negative plasma cells. In two clinical studies, anti-CD20-mediated B-cell depletion prevented relapses and also reduced other parameters of clinical activity (Cree et al., 2005; Jacob et al., 2008); clinical benefit was not restricted to patients experiencing a reduction of serum AQP4 antibody titers. Recent case reports using an antibody neutralizing the effect of IL-6 indicate that this cytokine could be indeed the key factor for progression of NMO. Administration of IL-6 receptor blocking tocilizumab was shown to be clinically beneficial in treatment-resistant NMO patients (Ayzenberg et al., 2013; Kieseier et al., 2012). This finding does suggests that IL-6 and the cells that produce it are involved in the pathogenesis of disease.

In conclusion, several lines of evidence suggest that B cells are important contributors to NMO pathogenesis. AQP4-specific B cells are the source for plasma cells secreting autoreactive AQP4 antibodies. Besides these humoral properties, AQP4-specific B cells could also play a key role as APCs for development of the newly recognized T cells response against AQP4. Lastly, these differentiated T (helper) cells may in return support isotype switching and thereby consolidate the antibody response against AQP4, closing this positive feedback loop of potentially pathogenic B-cell–T-cell interaction in NMO.

## 5. SUMMARY

There is abundant evidence that NMO is an autoimmune disorder of the CNS. The seroprevalence of AQP4-Ab in the majority of patients suggests that AQP4 may be an autoantigen that initiates and perpetuates inflammation in the brain and spinal cord of NMO patients. In the 10 years since AQP4-Ab was identified, there has been tremendous progress in comprehending the pathogenic events underlying this disorder. However, there are still substantial gaps in our knowledge.

For instance, it remains poorly understood why NMO patients who are seronegative for AQP4-Ab behave very similarly to seropositive patients. One seemingly obvious explanation would be that in these patients, another formerly cryptic antigen becomes dominant during the early course of the disease.

It is also unclear why there is an incomplete treatment response to B-cell depleting therapies with anti-CD20 monoclonal antibodies, and why some of the pharmacotherapies that are approved for MS, another inflammatory disorder of the CNS appear to provide no benefit, might even worsen the disease course.

These observations illustrate our incomplete grasp of cellular and cytokine networks that drive and regulate NMO disease activity. The identification of one autoantigen should provide am advantage in elucidating some of these enigmas.

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# INDEX

Note: Page numbers followed by "*f*" indicate figures and "*t*" indicate tables.

## C

## D



## E



## F

## G



## H

## J



## K



## L

## R



## S

# CONTENTS OF RECENT VOLUMES

## Volume 85



## Volume 86



## Volume 87

## Volume 88



## Volume 89



## Volume 90

## Volume 91



## Volume 92

## Volume 93



## Volume 94

## Volume 95



## Volume 96



## Volume 97

## Volume 98



## Volume 99



## Volume 100

## Volume 101



## Volume 102



## Volume 103

## Volume 104



## Volume 105



## Volume 106

## Volume 107



## Volume 108



## Volume 109

## Volume 110



## Volume 111



## Volume 112



## Volume 113

## Volume 114



## Volume 115

## Volume 116



## Volume 117



## Volume 118

## Volume 119



## Volume 120