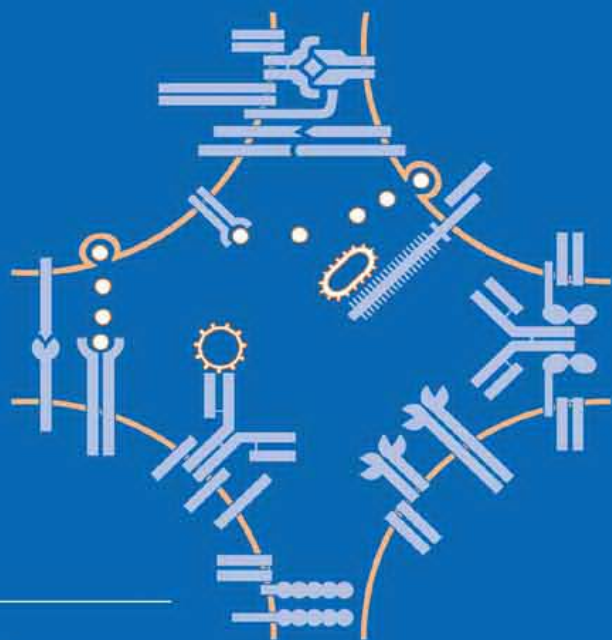


D. Kitamura
Editor



How the Immune System Recognizes Self and Nonself

Immunoreceptors and
Their Signaling

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Preface

How do you discriminate yourself from other people? This question must sound odd to you since you easily recognize others at a glance and, without any effort, would not mistake them for yourself. However, it is not always easy for some people to discriminate themselves from others. For example, patients with schizophrenia often talk with “others” living inside themselves. Thus it is likely that normally your brain actively recognizes and remembers the information belonging to yourself and discriminates it from the information provided by others, although you are not conscious of it. This brain function must have been particularly important for most animals to protect their lives from enemies and for species to survive through evolution. Similarly, higher organisms have also acquired their immune system through evolution that discriminates nonself pathogens and self-body to protect their lives from pathogens such as bacteria or viruses.

The brain system may distinguish integrated images of self and nonself created from many inputs, such as vision, sound, smell, and others. The immune system recognizes and distinguishes a variety of structural features of self and nonself components. The latter actually include almost everything but self: for example, bacteria, viruses, toxins, pollens, chemicals, transplanted organs, and even tumor cells derived from self-tissue. To this end the immune system recruits different kinds of immune cells, such as B and T lymphocytes, natural killer (NK) cells, dendritic cells, and macrophages. These cells have specific functions and are equipped with distinctive sets of receptors to recognize self and/or nonself components. B and T lymphocytes have characteristic antigen receptors whose binding specificities are extremely diverse among individual cells. This diversity is generated through somatic rearrangement of the genes encoding the antigen receptors. The repertoires of the diverse antigen receptors cover a huge variety of self and nonself antigens, which normally results in tolerance to self antigens and the immune responses to the nonself antigens including antibody production, T-cell-mediated cytotoxicity, and inflammation. The antigen recognition signal is critically regulated by activating or inhibitory co-receptors on the same cells. The antigens are also recognized by specific antibodies bound with Fc receptors on various immune cells. NK and NKT cells express collectively what are called NK receptors

composed of various pairs of receptors, one recognizing a specific viral or neoplastic antigen and the other self MHC (major histocompatibility complex) antigen on the self cells, and the pair work together to discriminate a target to kill. In addition to the NK receptors, a growing number of the paired immunoglobulin-like receptors have been identified. Finally, Toll-like receptors expressed on the cells such as dendritic cells and macrophages recognize various structural patterns of pathogens to eliminate them as the first defense and also to regulate the immune response by lymphocytes. Single mutations in some of these receptors or their downstream signaling molecules are known to cause autoimmune diseases in mice or humans, suggesting that functions of those immune cells expressing a different set of receptors are highly integrated and cooperate in an immune system to decide what is self and what is nonself.

In contrast to the extreme diversity of the receptors, the molecules involved in the signaling from such receptors is relatively limited and often is shared among different receptor systems. Therefore, the immune cells may use a common strategy for processing the signals from these receptors and for making a decision about their next action. The aim of this book is to try to clarify how the immune cells recognize through receptors a diversity of targets, being either self or nonself, how they translate this recognition into signals and transmit the signals, and how finally they decide to react or not to play a part in the immune response. For this purpose, an extensive and updated review on each receptor system is described in each chapter by an expert in that area. Although our knowledge is still far from complete, this challenge will give you many clues to discover the principle of the signaling machinery and to understand the complexity of cell interaction through receptor signaling in the immune system. Thus this book will help you to imagine the basic strategy of the immune system to distinguish self and nonself. I believe this challenge is particularly important for basic as well as clinical immunologists who are seeking a breakthrough in the regulation of immune diseases such as autoimmunity and allergy. Also, the concept and strategy of self–nonself discrimination in the immune system might be applied to the brain system as briefly mentioned above, or as a security system of a computer network.

I would like to thank all the authors for their invaluable contributions and their patience.

Daisuke Kitamura
Noda, Japan

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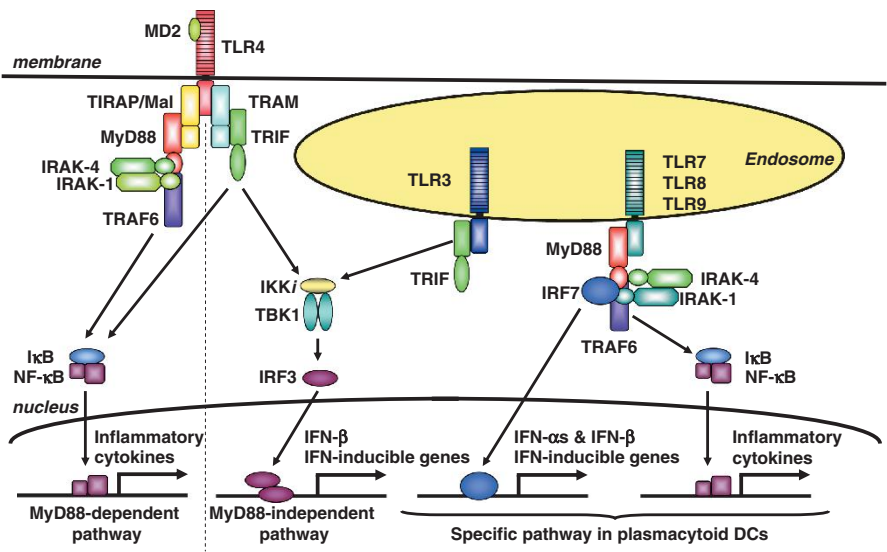
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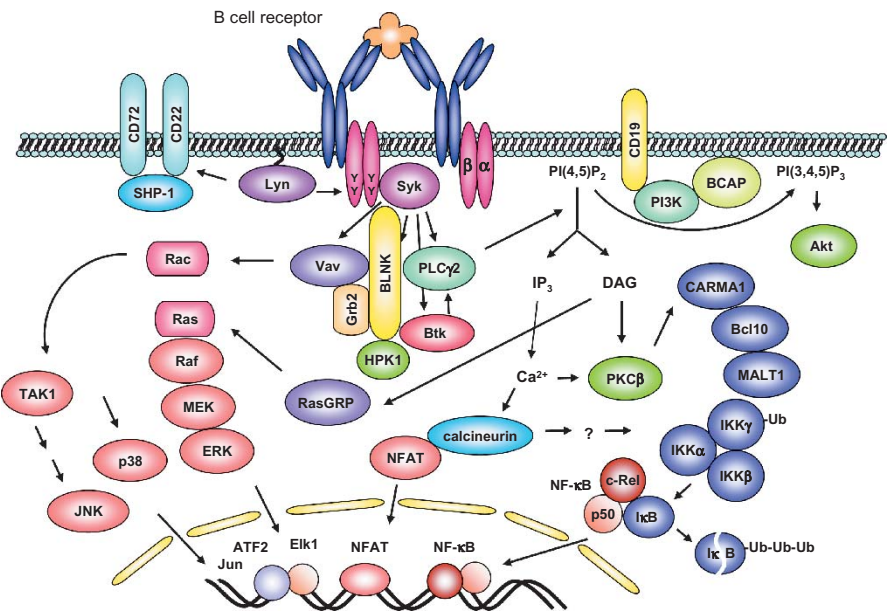
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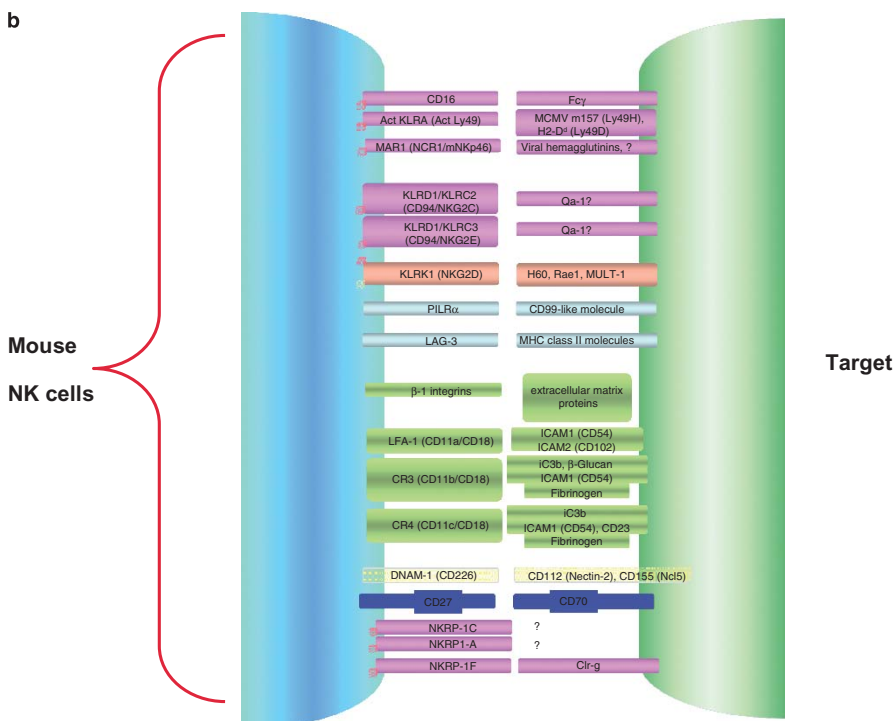
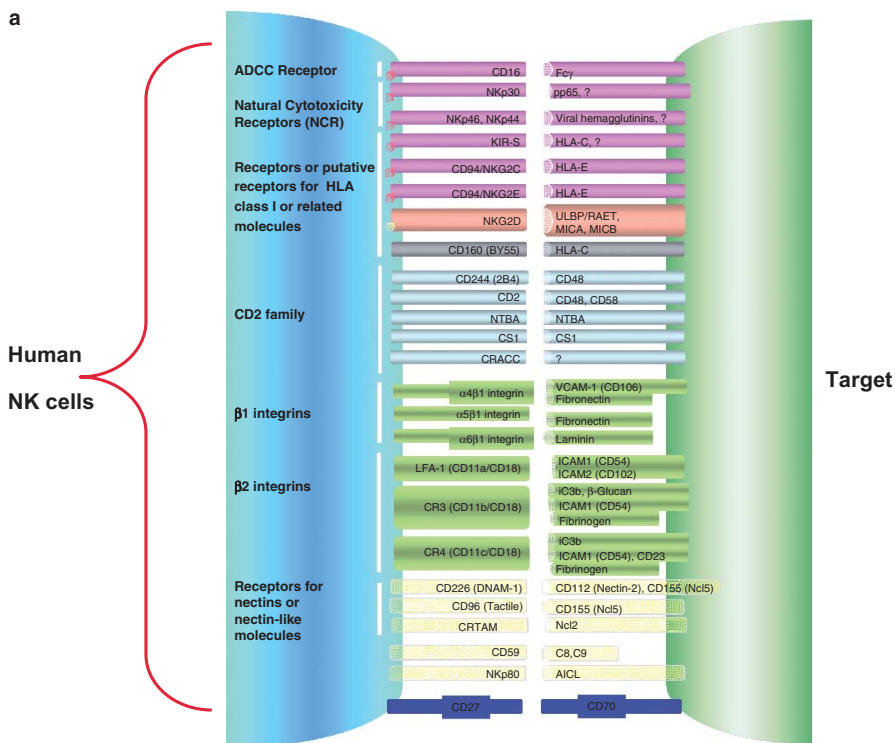
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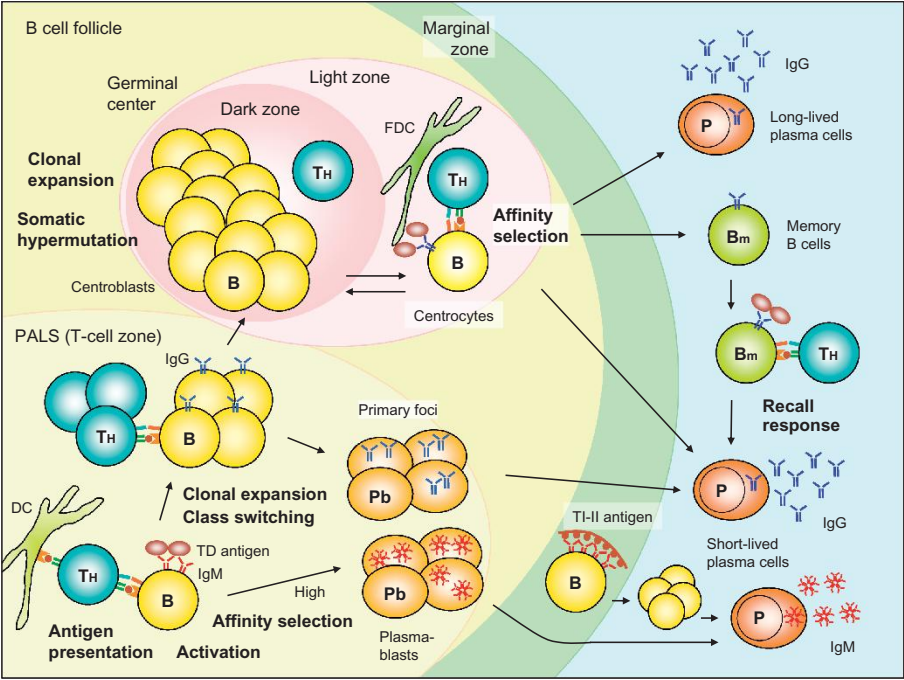
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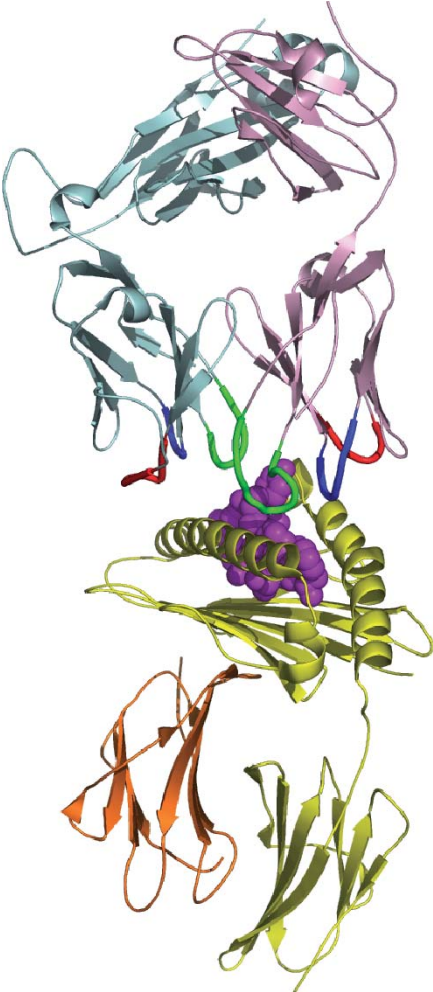
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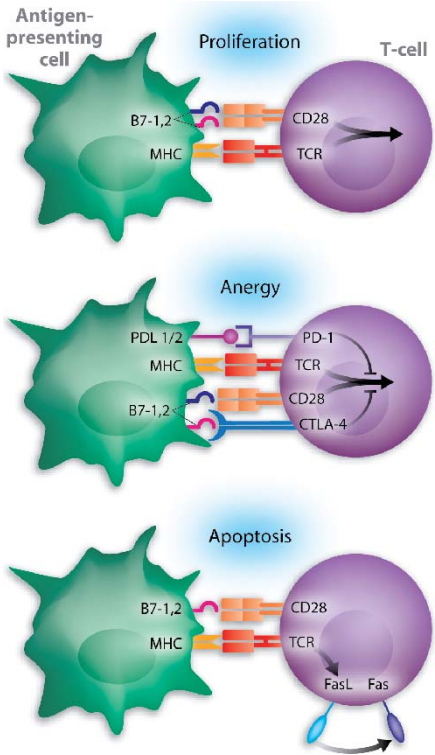
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Recognition of Pathogens: Toll-Like Receptors

Satoshi Uematsu¹ and Shizuo Akira^{1,2}

1.1 Introduction

The innate immune response is the first line of defense against microbial infections. Toll-like receptors (TLRs) are evolutionally conserved and recognize specific components of pathogens including bacteria, fungi, protozoa and viruses. Recognition of microbial components by TLRs triggers activation of signal transduction pathways, which then induces dendritic cell maturation and cytokine production, resulting in development of adaptive immunity. Each TLR has its intrinsic signaling pathway and induces specific biological responses against microorganisms. Here, we discuss the rapidly accumulating members of the TLR family, their functions and signaling mechanisms.

1.2 Innate Immunity

The adaptive immune system uses a random and highly diverse repertoire of receptors encoded by rearranging genes, the T- and B-cell receptors, to recognize a variety of antigens. This mechanism allows the host to generate immunological memory. However, it takes time that specific clones expand and differentiate into effector cells before they can serve for host defense. Therefore, the primary adaptive immune system cannot induce immediate responses to invasive pathogens. To induce immediate responses upon encountering with a pathogen, a host is equipped with innate, nonadaptive defenses that form early barriers against infectious diseases. Although

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the innate immune system was first described by Elie Metchnikoff over a century ago, it has long been ignored as just a nonspecific response to simply kill pathogens, and to present antigens to the cells involved in acquired immunity (Brown 2001). In 1996, an epoch-making study showed that the *Drosophila* protein Toll is required for flies to induce effective immune responses to *Aspergillus fumigatus* (Lemaitre et al. 1996). Since then, accumulating evidence has shown that the innate immune system specifically recognizes invading microorganisms. The targets of innate immune recognition are conserved molecular patterns (PAMPs: pathogen-associated molecular patterns) of microorganisms. Therefore, the receptors in innate immunity are called pattern-recognition receptors (PRRs) (Medzhitov and Janeway 1997). PAMPs are generated by microbes and not by the host, suggesting that PAMPs are good targets for innate immunity to discriminate between self and nonself. Furthermore, PAMPs are essential for microbial survival and are conserved structures among a given class, which allows innate immunity to respond to microorganisms with limited numbers of PRRs. There are many PRRs associated with opsonization, phagocytosis, complement and coagulation cascades, proinflammatory signaling pathways, apoptosis and so on. Among them, Toll receptors and the associated signaling pathways represent the most ancient host defense mechanism found in insects, plants and mammals (Akira 2004). Studies conducted by using the fruit fly have shown that the Toll family is one of the most crucial signaling receptors in innate immunity.

1.2.1 Immune Responses in *Drosophila*

Insects do not have counterparts of mammalian B and T cells, and they cannot induce acquired immune responses based on producing antibodies to pathogenic organisms. Nonetheless, insects can recognize the invasion of various microorganisms and induce antimicrobial responses. Recent studies using a model organism, *Drosophila melanogaster*, have shown that the induction of antimicrobial peptides, which are important for survival after infection, depends on Toll and immune deficiency (Imd) signaling pathways (Tanji and Ip 2005). A transmembrane protein, Toll, originally identified as an essential component in dorsal-ventral embryonic development (Wu and Anderson 1997), is also involved in innate immune responses (Lemaitre et al. 1996). Gram-positive bacterial peptidoglycan might bind directly to extracellular peptidoglycan recognition protein (PGRP)-SA (Michel et al. 2001) and SD (Bischoff et al. 2004), which then stimulate the Toll pathway. Another pattern recognition protein, Gram-negative binding protein-1 (GNBP-1) is also involved in the recognition of Gram-positive bacteria (Gobert et al. 2003; Pili-Floury et al. 2004). Not only Gram-positive bacteria but also fungi stimulate the Toll pathway. Fungi are recognized by a serine protease, Persephone, and a protease inhibitor, Necrotic (Levashina et al. 1999; Ligoxygakis et al. 2002). All upstream cascades lead to the cleavage of pro-Spätzle to Spätzle, and the binding of proteolytically processed Spätzle to Toll induces the dimerization of Toll (Hu et al. 2004; Weber et al. 2003). After the activation of Toll, the adapter proteins MyD88 and Tube, and a serine-threonine kinase, Pelle, are recruited to Toll (Sun et al. 2004).

Then, activated Pelle acts on the Cactus, a *Drosophila* I κ B. Dif and Dorsal are transcription factors of Rel protein family and are retained in the cytoplasm by Cactus. By the stimulation of the Toll pathway, Cactus is degraded and Dorsal and Dif translocate into the nucleus, leading to the induction of antimicrobial peptides (Brennan and Anderson 2004; Hoffmann 2003; Hultmark 2003).

The Imd pathway is responsible for the induction of antimicrobial peptides in response to Gram-negative bacteria (Brennan and Anderson 2004; Hoffmann 2003; Hultmark 2003; Lemaitre 2004). Imd is an adapter protein for this pathway (Georgel et al. 2001). Recent reports show that PGRP-LC (Choe et al. 2002; Gottar et al. 2002) and PGRP-LE (Takehana et al. 2004), which have putative transmembrane domains, are the pattern recognition receptors in this pathway. There are at least three branches downstream of Imd (Brennan and Anderson 2004; Hoffmann 2003; Hultmark 2003; Lemaitre 2004). First is Transforming Growth Factor (TGF)- β -activated kinase 1 (TAK1), which induces the proteolytic cleavage of IKK, followed by activation of the transcription factor, Relish (Lu et al. 2001; Rutschmann et al. 2000; Silverman et al. 2000, 2003; Stoven et al. 2003; Vidal et al. 2001). Second is the Fas-associated death domain (FADD)–Dredd pathway that also activates Relish (Balachandran et al. 2004; Chen et al. 1998; Elrod-Erickson et al. 2000; Georgel et al. 2001; Hu and Yang 2000; Leulier et al. 2000, 2002). Two new components, Sickie and Dnr-1 have been identified: whereas Sickie positively regulates the Relish activation by Dredd, Dnr-1 inhibit this pathway (Foley and O’Farrell 2004; Khush et al. 2002). Third is the JNK pathway that is activated through TAK1. The JNK pathway induces immediate early genes after septic shock, which is negatively regulated by Relish (Boutros et al. 2002; Park et al. 2004a).

As stated above, recent genetic and genomic analyses of *D. melanogaster* have shown that insects have an evolutionally primitive recognition and signaling system (Fig. 1). Collectively, these results provide important insights into the mechanism of pathogen recognition and host responses in mammalian systems.

1.2.2 Discovery of TLR in Mammals

A mammalian homolog of Toll receptor (now termed TLR4) was identified through database searches, and was shown to induce expression of the genes involved in inflammatory responses (Medzhitov et al. 1997). Subsequently, a mutation in the *tlr4* gene was identified in C3H/HeJ mice that were hyporesponsive to lipopolysaccharide (LPS) (Poltorak et al. 1998). The TLR family now consists of 13 mammalian members (Akira 2004). As the cytoplasmic portion of TLRs is similar to that of the interleukin (IL)-1 receptor family, it is called the Toll/IL-1 receptor (TIR) domain. However, the extracellular region of TLRs and IL-1R are markedly different; whereas IL-1R possesses an Ig-like domain, TLRs contain leucine-rich repeats (LRR) in the extracellular domain (Akira 2004). Recent genetic studies have revealed that TLRs play an essential role in the recognition of specific components of pathogens. TLRs are capable of sensing organisms ranging from bacteria to fungi, protozoa and viruses (Table 1).

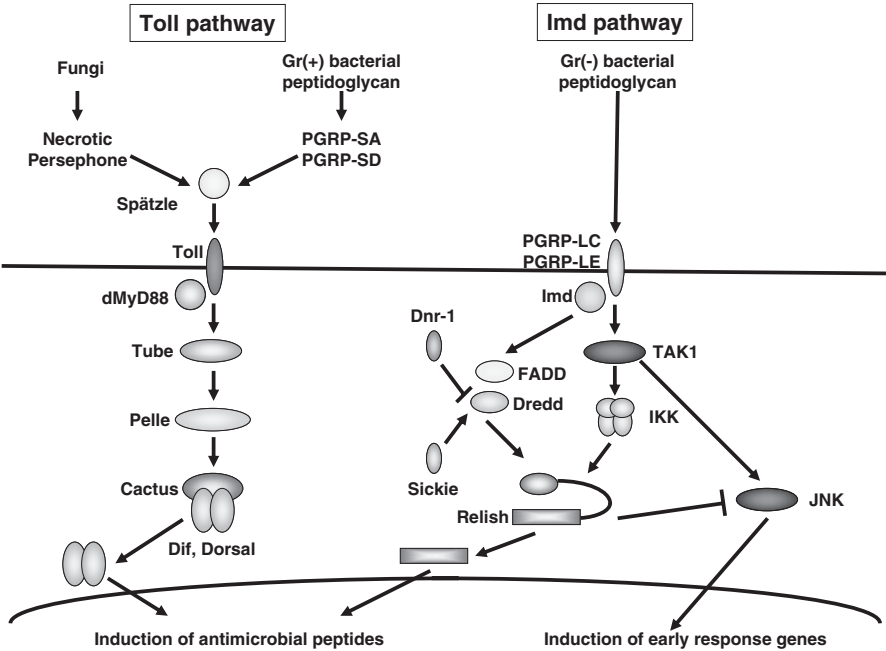


Fig. 1. Toll and immune deficiency (*Imd*) pathways in the *Drosophila* innate immune response. The Toll pathway mediates the response to Fungal and Gr(+) bacterial infection, whereas the Imd pathway mediates the response to Gr(-) bacterial infection. These pathways are similar to the signaling pathway of the mammalian Toll-like receptor, and are essential for *Drosophila* to survive infection. *PGRP*, peptidoglycan recognition protein; *FADD*, Fas-associated death domain; *TAK*, TGF- β -activated kinase; *IKK*, I κ B kinase; *JNK*, Jun N-terminal kinase

Table 1. Toll-like receptors and their ligands

TLR	Ligand
TLR1	Triacyl lipopeptides (bacteria)
TLR2	Peptidoglycan, lipoprotein, lipopeptides, atypical LPS (bacteria), zymosan, phospholipomannan (fungi), GPI anchor (protozoa), envelope protein (virus)
TLR3	Poly(I:C), dsRNA (virus)
TLR4	LPS (bacteria), mannan, glucuronyloxylohexamannan (fungi), glycoinositolphospholipids (protozoa), RSV fusion protein (virus)
TLR5	Flagellin (bacteria)
TLR6	Diacyl lipopeptides (bacteria)
TLR7/TLR8	Synthetic imidazoquinoline-like molecules, ssRNA (virus)
TLR9	CpG DNA (bacteria, protozoa, virus), hemozoin (protozoa)
TLR11	Component of uropathogenic bacteria (bacteria), profolin-like molecule (protozoa)

PLPS, lipopolysaccharide; GPI, glycosyl phosphatidylinositol; RSV, respiratory syncytial virus; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA

1.3 Pathogen Recognition by TLR

1.3.1 Bacteria

Lipopolysaccharide is a cell wall component of Gram-negative bacteria, a strong immunostimulant. As described above, TLR4 is essential for recognition of LPS, which is composed of lipid A (endotoxin), core oligosaccharide and O-antigen. TLR4 recognizes lipid A of LPS; this occurs through the TLR4/MD2/CD14 complex, which is present on various cells such as macrophages and dendritic cells (Shimazu et al. 1999). LPS forms a complex with an accessory protein, LPS-binding protein (LBP) in serum, which converts oligomeric micelles of LPS to a monomer for delivery to CD14, which is a glycosyl phosphatidylinositol (GPI)-anchored, high-affinity membrane protein that can also circulate in a soluble form. CD14 concentrates LPS for binding to the TLR4/MD2 complex (Takeda et al. 2003).

TLR2 recognizes various microbial components, such as lipoproteins/lipopeptides and peptidoglycans from Gram-positive and Gram-negative bacteria, and lipoteichoic acid from Gram-positive bacteria, a phenol-soluble modulin from *Staphylococcus aureus* and glycolipids from *Treponema maltophilum* (Takeda et al. 2003; Takeuchi et al. 1999a). TLR2 is also reported to be involved in the recognition of LPS from nonenterobacteria including *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* (Takeda and Akira 2005). These LPS are atypical LPS, whose structures are different from typical LPS of Gram-negative bacteria (Netea et al. 2002). However, a recent report indicated that lipoproteins contaminated in LPS preparation from *P. gingivalis* stimulated TLR2 and LPS from *P. gingivalis* itself had a poor activity for TLR4 stimulation (Hashimoto et al. 2004). There are also controversial reports regarding peptidoglycan recognition by TLR2. More careful analyses will be needed to exclude any possibility of remaining contaminants.

TLR1 and TLR6 are structurally related to TLR2 (Takeuchi et al. 1999b). TLR2 and TLR1 or TLR6 form a heterodimer, which is involved in the discrimination of subtle changes in lipopeptides from Gram-positive bacteria. TLR6-deficient macrophages do not produce inflammatory cytokines in response to diacyl lipopeptides from mycoplasma; however, they normally produce inflammatory cytokines in response to triacyl lipopeptides derived from a variety of bacteria (Takeuchi et al. 2001). On the contrary, TLR1-deficient macrophages show normal responses to triacyl lipopeptides but not to diacyl lipopeptides (Alexopoulou et al. 2002; Takeuchi et al. 2002). These results suggest that TLR1 and TLR6 functionally associate with TLR2.

CD36 is a member of the class II scavenger family of proteins. A recent report has shown that CD36 serves as a facilitator or co-receptor for diacyl lipopeptide recognition through the TLR2/6 complex (Hoebe et al. 2005).

Bacterial flagellin is a structural protein that forms the major portion of flagella that contribute to virulence through chemotaxis, adhesion to and invasion of host

surfaces. TLR5 is responsible for the recognition of flagellin (Hayashi et al. 2001). Gewirtz et al. reported that TLR5 is expressed on the basolateral surface, but not the apical side of intestinal epithelial cells, suggesting that flagellin is detected when bacteria invade across the epithelium (Gewirtz et al. 2001). A common stop codon polymorphism in the ligand-binding domain of TLR5 (TLR5 392STOP SNP) is unable to mediate flagellin signaling and is associated with susceptibility to pneumonia caused by *Legionella pneumophila* (Hawn et al. 2003). However, researchers in Vietnam have reported that the TLR5 392STOP SNP is not associated with susceptibility to typhoid fever (Dunstan et al. 2005). α and ϵ *Proteobacteria*, including *Helicobacter pylori* and *Campylobacter jejuni*, change the TLR5 recognition site of flagellin without losing flagellar motility (Andersen-Nissen et al. 2005). This modification may contribute to the persistence of these bacteria on mucosal surfaces.

Bacterial DNA, which contains unmethylated CpG motifs, is a potent stimulator of the host immune response. In vertebrates, the frequency of CpG motifs is severely reduced and the cysteine residues of CpG motifs are highly methylated, which leads to abrogation of the immunostimulatory activity. Analysis of TLR9-deficient mice showed that TLR9 is a receptor for CpG DNA (Hemmi et al. 2000).

Mouse TLR11, a relative of TLR5, is expressed abundantly in the kidney and bladder. TLR11-deficient mice are susceptible to uropathogenic bacterial infections, indicating that TLR11 senses the component of uropathogenic bacteria. However, TLR11 is thought to be a pseudogene in human (Zhang et al. 2004).

1.3.2 Fungi

Toll-like receptors have been implicated in the recognition of the fungal pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pneumocystis carinii* (Netea et al. 2004; Takeda et al. 2003). Several components located in the cell wall or cell surface of fungi have been identified as potential ligands. Yeast zymosan activates TLR2/TLR6 heterodimers, whereas mannan derived from *Saccharomyces cerevisiae* and *C. albicans* is detected by TLR4. Phospholipomannan presented in the cell surface of *C. albicans* is recognized by TLR2, while TLR4 mainly interacts with glucuronoxylomannan, the major capsular polysaccharide of *C. neoformans* (Netea et al. 2004).

Dectin-1 is a lectin family receptor for the fungal cell wall component, β -glucan (Brown et al. 2002). Recently, Dectin-1 has been shown to functionally collaborate with TLR2 and induce a strong immune response to yeast via recruitment of the tyrosine kinase, Syk (Gantner et al. 2003; Rogers et al. 2005; Underhill et al. 2005). TLR2 recognizes a variety of microbial products through functional cooperation with several proteins that are either structurally related or not.

1.3.3 Protozoa

Toll-like receptors also sense the components of protozoa including *Trypanosoma cruzi*, *Trypanosoma brucei*, *Toxoplasma gondii*, *Leishmania major* and *Plasmodium falciparum*. Glycosylphosphatidylinositol anchors, glycoinositolphospholipids and genomic DNA derived from *T. cruzi* are recognized by TLR2, TLR4, and TLR9, respectively (Gazzinelli et al. 2004). Murine TLR11 senses the profilin-like molecule of *T. gondii* (Yarovinsky et al. 2005). Malaria parasites digest host hemoglobin into a hydrophobic heme polymer known as hemozoin. Hemozoin from *P. falciparum* stimulates macrophages and DCs to produce inflammatory cytokines and chemokines in a TLR9-dependent manner (Coban et al. 2005). However, it is unclear how TLR9 recognizes both DNA and non-DNA crystals.

1.3.4 Virus

TLR4 recognizes not only bacterial components but also viral envelope proteins. The fusion (F) protein from respiratory syncytial virus (RSV) is sensed by TLR4 (Kurt-Jones et al. 2000). C3H/HeJ mice were sensitive to RSV infection (Haynes et al. 2001). The envelope protein of mouse mammary tumor virus (MMTV) directly activates B cells via TLR4 (Rassa et al. 2002). TLR2 has also been reported to be involved in the recognition of viral components such as Measles virus, human cytomegalovirus and HSV-1 (Bieback et al. 2002; Compton et al. 2003; Kurt-Jones et al. 2004).

Double-stranded (ds) RNA is generated during viral replication. TLR3 is involved in the recognition of a synthetic analog of dsRNA, polyinosine-deoxycytidylic acid (poly I:C), a potent inducer of type I interferons (IFNs) (Alexopoulou et al. 2001; Yamamoto et al. 2003a). Consistent with this result, TLR3-deficient mice were hypersusceptible to mouse cytomegalovirus (Tabeta et al. 2004). Contrarily, TLR3-deficient mice showed more resistance to West Nile virus (WNV) infection. WNV triggers inflammatory responses via TLR3, which results in a disruption of the blood–brain barrier, followed by enhanced brain infection (Wang et al. 2004). These findings suggested that WNV utilizes TLR3 to efficiently enter into the brain.

Mouse splenic DCs are divided into CD11c high B220[−] and CD11c dull B220⁺ cells. The latter contain plasmacytoid DCs (pDCs), which induce large amounts of IFN- α during viral infection. CpG DNA motifs are also found in genomes of DNA viruses, such as Herpes simplex virus type 1 (HSV-1), HSV-2 and murine cytomegalovirus (MCMV). Mouse pDCs produce IFN- α by recognizing CpG DNA of HSV-2 via TLR9 (Lund et al. 2003). TLR9-deficient mice were also shown to be susceptible to MCMV infection, suggesting that TLR9 induces anti-viral responses by sensing CpG DNA of DNA virus (Krug et al. 2004a,b; Tabeta et al. 2004). However, in the case of macrophages, HSV-2-induced IFN- α production is not

dependent on TLRs. Furthermore, mice lacking TLR9 or the adapter molecule MyD88 can still control HSV-1 infection (Hochrein et al. 2004). Thus, TLR9-mediated IFN- α response to DNA virus is limited to pDCs, and the TLR-independent system also plays an important role in DNA viral infection.

TLR7 and TLR8 are structurally highly conserved proteins (Akira 2004). The synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R848) have potent antiviral activities and are used clinically for treatment of viral infections. Analysis of TLR7-deficient mice showed that TLR7 recognizes these synthetic compounds (Hemmi et al. 2002). Human TLR7 and TLR8, but not murine TLR8, recognize imidazoquinoline compounds (Ito et al. 2002). Furthermore, murine TLR7 has also been shown to recognize guanosine analogs such as loxoribine, which has antiviral and antitumor activities (Akira and Hemmi 2003). Since all these compounds are structurally similar to ribonucleic acids, TLR7 and human TLR8 are predicted to recognize a nucleic acid-like structure of a virus. Recently, TLR7 and human TLR8 have been shown to recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV) and influenza virus (Diebold et al. 2004; Heil et al. 2004). Although ssRNA is abundant in hosts, host-derived ssRNA is not usually detected by TLR7 or TLR8. As TLR7 and TLR8 are expressed in the endosome, host-derived ssRNA is not delivered to the endosome and therefore, is not recognized by TLR7 and TLR8.

As well as TLR7 and TLR8, TLR3 and TLR9 are exclusively expressed in endosomal compartments not on cell surfaces (Latz et al. 2004). After phagocytes internalize viruses or virus-infected apoptotic cells, viral nucleic acids are released in phagolysosomes and are recognized by TLRs. However, intracellular localization of TLR9 is not required for ligand recognition but prevents recognition of self DNA. Localization of the nucleic acid-sensing TLRs is critical in discriminating between self and nonself nucleic acids.

1.3.5 Endogenous Ligands

Many reports have suggested that a number of endogenous ligands such as heat shock proteins (hsp) were potent activators of the innate immune system (Tsan and Gao 2004). TLR4 has been shown to be involved in the recognition of endogenous ligands, such as HSPs (HSP60, HSP70 and Gp96) (Asea et al. 2002; Bulut et al. 2002; Dybdahl et al. 2002; Ohashi et al. 2000; Vabulas et al. 2002a,b). Fibrinogens, surfactant protein-A, fibronectin extra domain A, heparan sulfate, β -defensin 2 have since been reported as ligands for TLR4 (Biragyn et al. 2002; Guillot et al. 2002; Johnson et al. 2002; Okamura et al. 2001; Smiley et al. 2001; Termeer et al. 2002). It has also been shown that HSP60, HSP70, gp96 and HMGB1 protein are endogenous ligands for TLR2 and TLR4 (Asea et al. 2002; Park et al. 2004b; Vabulas et al. 2001, 2002b); and that mRNA is an endogenous ligand for TLR3 (Kariko et al. 2004). The extracellular matrix hyaluronan is produced after tissue injury.

A recent study showed that hyaluronan is recognized by both TLR2 and TLR4, and that this interaction regulates both innate inflammatory responses and epithelial cell integrity, which are both crucial for recovery from acute lung injury (Jiang et al. 2005). However, most of these endogenous ligands require very high concentrations to activate TLRs. In addition, it has been shown that the cytokine effect of HSP70 was a result of the contaminating LPS (Wallin et al. 2002). The endogenous ligands used in the previous studies were recombinant products, purified native molecules or purified fragments of macromolecules. As recombinant products are produced by genetically engineered *Escherichia coli*, the final preparations may have been contaminated with bacterial products; similarly, purified preparations are also frequently contaminated with bacterial cell wall products such as LPS and lipoproteins (Tsan and Gao 2004). More careful experiments will be needed to conclude that TLRs recognize these endogenous ligands.

1.4 Toll-Like Receptor Signaling Pathways (Fig. 2)

The engagement of TLRs by microbial components triggers the induction of specific gene profiles that are suited to the removal invading *pathogens*. After ligand binding, TLRs dimerize and undergo the conformational changes required for the recruitment of downstream signaling molecules (Akira 2004). All TLR signals originate from the TIR domain. A crucial role for the TIR domain was first identified in the C3H/HeJ mouse strain, which has a point mutation that results in an amino acid change of the cytoplasmic proline residue at position 712 to histidine (Poltorak et al. 1998). This proline residue in the TIR domain is conserved among all TLRs, except for TLR3, and its substitution to histidine causes a dominant negative effect on TLR-mediated signaling (Akira and Takeda 2004). There are four adapter proteins containing TIR domains, including myeloid differentiation factor 88 (MyD88) (Adachi et al. 1998) (Medzhitov et al. 1998), TIR-associated protein (TIRAP)/MyD88-adapter-like (MAL) (Hornig et al. 2001; Fitzgerald et al. 2001; Hornig et al. 2002; Yamamoto et al. 2002a), TIR-domain-containing adapter protein inducing IFN- β (TRIF)/TIR-domain containing molecule 1 (TICAM1) (Hoebe et al. 2003; Oshiumi et al. 2003a; Yamamoto et al. 2003a,b), and TRIF-related adapter molecule (TRAM)/TICAM2 (Bin et al. 2003; Oshiumi et al. 2003b; Yamamoto et al. 2003b). They are recruited to the TIR domains of TLRs upon ligand stimulation and transduce signals from TIR domains, activating protein kinases and then transcription factors that induce inflammatory responses. Individual TLRs mediate distinctive responses by association with different combinations of these adapters. Nuclear factor (NF)- κ B is the most crucial transcription factor and is universally used by all TLRs. It is also involved in the induction of various genes including proinflammatory cytokines. Recent studies have revealed that specific members of the interferon (IFN)-regulatory factor (IRF) family also play pivotal roles in the induction of TLR-responsive genes such as type I IFNs and interferon inducible genes. Moreover, in TLR7 and TLR9 signaling pathways, a

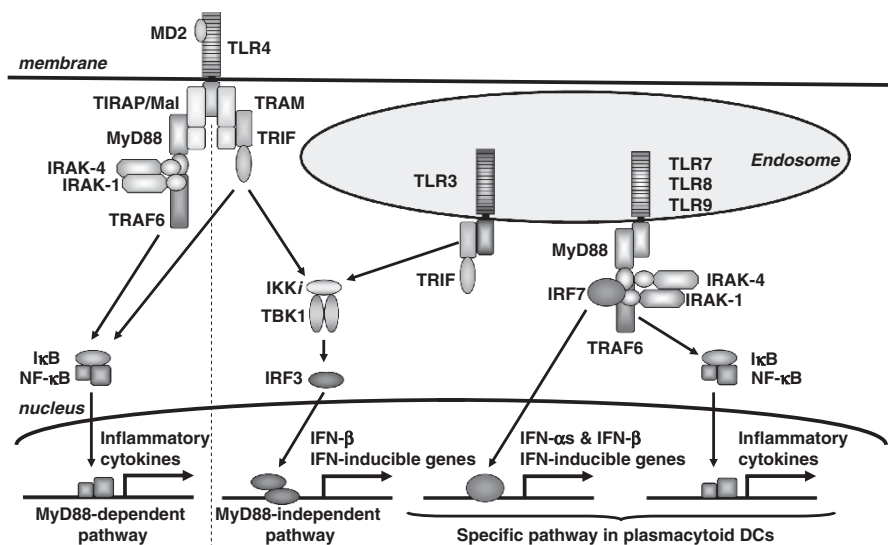


Fig. 2. Toll-like receptor (TLR) signaling pathways. All TLR signals originate from the Toll/interleukin-1 receptor (TIR) domain. There are four adapter proteins containing the TIR domain: MyD88, TIR-associated protein (TIRAP), TIR-domain-containing adapter protein inducing IFN- β (TRIF), and TRIF-related adapter molecule (TRAM). They are recruited to the TIR domains of TLRs upon ligand stimulation and transduce signals from the TIR domains, activating protein kinases and then transcription factors that induce inflammatory responses. Individual TLRs mediate distinctive responses by association with different combinations of these adapters. Nuclear factor κ B (NF- κ B) is the most crucial transcription factor and is universally used by all TLRs, and is also involved in the induction of various genes including proinflammatory cytokines. Specific members of the interferon (IFN)-regulatory factor (IRF) family also play pivotal roles in the induction of TLR-responsive genes such as type I IFNs and interferon-inducible genes. Moreover, in TLR7 and TLR9 signaling pathways, a special subset of dendritic cells (DCs), plasmacytoid DCs (pDCs), have an intrinsic signaling pathway to induce robust IFN- α s. (See Color Plates)

special subset of dendritic cells (DCs), plasmacytoid DCs (pDCs) have an intrinsic signaling pathway to induce robust IFN- α s (Akira and Takeda 2004). In the next sections, we focus on signaling molecules used by TLRs and give an overview of the most recent findings regarding TLR signaling pathways.

1.4.1 Proinflammatory Cytokine Pathways

Toll-like receptor family members use many of the same signaling components as IL-1R since both have a conserved cytoplasmic domain, the TIR domain. The TIR domain-containing adapter MyD88 is utilized by all TLRs, with the exception of TLR3. MyD88 was originally isolated as a gene induced rapidly during IL-6-

stimulated differentiation of M1 cells into macrophages (Lord et al. 1990). MyD88 possesses a TIR domain in its C-terminus and a death domain in its N-terminus. Upon stimulation, MyD88 recruits IL-1 receptor kinases (IRAKs) to TLRs/IL-1Rs through interaction of the death domains (DDs) of the molecules (Akira and Takeda 2004). MyD88 functions as an adapter linking TLRs/IL-1Rs with downstream signaling molecules that have DDs (Adachi et al. 1998) (Medzhitov et al. 1998).

The association of TLRs and MyD88 recruits members of IRAKs (Janssens and Beyaert 2003; Muzio et al. 1997; Wesche et al. 1997). IRAK was originally identified as a serine/threonine kinase associated with the IL-1 receptor, which also harbors a death domain (Li et al. 1999). Four members have so far been identified: IRAK-1, IRAK-2, IRAK-M, and IRAK-4. IRAKs contain an N-terminal DD and a central serine/threonine-kinase domain. IRAK-1 and IRAK-4 have intrinsic kinase activity, whereas IRAK-2 and IRAK-M have none that is detectable. The kinase activity of IRAK-1 strongly increases following TLRs/IL-1Rs stimulation and its kinase domain but not the kinase activity itself is essential for the activation of NF- κ B (Akira and Takeda 2004). However, IRAK-1-deficient mice have a partially impaired response to LPS (Kanakaraj et al. 1998; Swantek et al. 2000; Thomas et al. 1999). Contrarily, overexpression of a kinase-inactive mutant of IRAK-4 inhibits IL-1-mediated NF- κ B activation. Furthermore, a biochemical study indicated that IRAK-4 acts upstream of IRAK-1 and phosphorylates IRAK-1 upon stimulation, suggesting that IRAK-1 is a direct substrate of IRAK-4 but not vice versa (Li et al. 2002). IRAK-4-deficient mice show virtually no response to IL-1, LPS or other bacterial components (Suzuki et al. 2002). Recently, it has been reported that patients with autosomal recessive amorphic mutations in *IRAK-4* have recurrent bacterial infections (Picard et al. 2003). There have now been 10 patients reported with genetically proven IRAK-4 deficiency and three others with probable IRAK-4 deficiency. Taken together, these results show that IRAK-4 is a central molecule in TLR-mediated MyD88-dependent pathways and acts upstream of IRAK-1.

TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family, which comprises two C-terminal TRAF domains (TRAF-N and TRAF-C), an N-terminal RING finger, and a zinc finger domain. The N-terminal domain is essential for the activation of downstream signaling cascades, and the TRAF domain permits self-association and interactions with receptors and other signaling proteins (Akira and Takeda 2004). Upon stimulation of TLRs, TRAF6 is recruited to the receptor complex and activated by IRAK-1. Then the IRAK-1/TRAF6 complex dissociates from the receptor to mediate signals. TRAF-6-deficient mice showed an impaired response to various TLR ligands.

After dissociation, IRAK-1/TRAF6 complex associates with TGF- β -activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2, at the membrane portion. TAK1 is a member of mitogen-activated protein kinase kinase kinase (MAPKKK) family, which was originally identified as a kinase involved in TGF- β signaling (Yamaguchi et al. 1995). TAK1 has been shown to function as an 'upstream' signaling molecule of NF- κ B and MAPKs in IL-1R signaling pathways (Takaesu et al. 2003). Furthermore, TAK1 is activated by TNF- α , LPS and latent

membrane protein 1 from Epstein-Barr virus (Wan et al. 2004). Recently, conditional TAK1-deficient mice were generated, showing that TAK1 is essential for TLR, IL-1R, TNFR, and BCR cellular responses and signaling pathways leading to the activation of JNK and/or NF- κ B (Sato et al. 2005). TAB1 is thought to be an activator of TAK1 because it enhances the kinase activity of TAK1 when co-expressed ectopically (Shibuya et al. 1996). TAB2 was identified as a novel adaptor protein that mediates activation of TAK1 by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway (Takaesu et al. 2000). However, TAB2-deficient embryonic fibroblasts showed no impairment in either IL-1/LPS- or TNF-induced activation of NF- κ B (Sanjo et al. 2003). One possible explanation for this phenotype is redundancy: another molecule can compensate for the loss of TAB2. A mammalian TAB2 homolog, termed TAB3, has been identified recently, and RNAi of both TAB2 and TAB3 have been shown to inhibit both IL-1- and TNF-induced activation of TAK1 and NF- κ B (Ishitani et al. 2003). Thus, TAB2 and TAB3 function redundantly as mediators of TAK1 activation.

It has been shown that ubiquitination has an important role in TAK1 activation and that TRAF6 functions as an E3 ubiquitin ligase (Deng et al. 2000). The complex of TRAF6, TAB1, TAB2 and TAB3 forms a larger complex with other proteins such as the E2 ligase Ubiquitin-conjugating enzyme 13 (Ubc13) and Ubiquitin-conjugating enzyme E2 variant 1 (Uev1A). The Ubc13 and Uev1A complex catalyzes the synthesis of a Lys63-linked polyubiquitin chain of TRAF6 and thereby induces TRAF6-mediated activation of TAK1, which in turn activates the transcription factors nuclear factor NF- κ B and activator protein-1 through the canonical I κ B kinase (IKK) complex and the mitogen-activated protein kinase pathway, respectively (Wang et al. 2001).

NF- κ B proteins are usually sequestered in the cytoplasm in an inactive form by a family of inhibitors of NF- κ B (I κ B). Upon stimulation of the cells by TLR ligands, I κ B is phosphorylated on two serine residues, which triggers its ubiquitination and degradation by the 26S proteasome. NF- κ B is released to enter the nucleus and to activate the transcription of target genes. Activation of NF- κ B through I κ B phosphorylation and degradation depends on the activation of I κ B kinases (IKKs). The IKK complex comprises two catalytic subunits, IKK- α and IKK- β , and the regulatory subunit IKK- γ /NF- κ B essential modulator (NEMO) (Karin and Ben-Neriah 2000). This signaling pathway, mentioned above, is called the “MyD88-dependent pathway” and is essential for the expression of inflammatory cytokine genes, including TNF- α , IL-6, IL-12, and IL-1 β , and co-stimulatory molecules (Akira and Takeda, 2004).

1.4.2 TRIF-Dependent Pathway

In addition to proinflammatory signals, recent studies have shown that some members of the TLR family trigger the induction of type I IFNs. In particular, TLR3 and TLR4 have the ability to induce IFN- β and IFN-inducible genes in MyD88-

deficient cells (Kawai et al. 1999, 2001). Moreover, TLR7, 8 and 9 can also induce type I IFNs in certain dendritic cell subtypes, plasmacytoid DCs (Akira and Hemmi 2003; Hemmi et al. 2003).

In accordance with the loss of inflammatory cytokine production, activation of NF- κ B and JNK by TLR2, TLR7, and TLR9 ligands was abolished in MyD88-deficient mice. However, activation of NF- κ B and JNK in response to LPS was observed with delayed kinetics in MyD88-deficient cells (Kawai et al. 1999). This finding indicated the presence of a MyD88-independent pathway for LPS stimulation. To elucidate this signaling pathway, subtraction analysis was performed using mRNA extracted from unstimulated and LPS-stimulated MyD88-deficient macrophages. This analysis revealed that several IFN-inducible genes, including glucocorticoid-attenuated response gene 16 (GARG-16), immunoresponsive gene 1 (IRG-1), and CXC-chemokine ligand 10 (CXCL10)/IFN- γ -induced 10-kDa protein (IP-10), were induced by LPS in the MyD88-deficient cells. Furthermore, LPS stimulation led to activation of the transcription factor IRF-3 that induced IFN- β in the MyD88-independent pathway. In turn, IFN- β activated Stat1 through IFN- α/β R, leading to the induction of several IFN-inducible genes (Hoshino et al. 2002; Kawai et al. 2001).

In addition to the TLR4 ligand, TLR3 ligand dsRNA has been shown to activate NF- κ B in MyD88-deficient cells. dsRNA also activates IRF-3 and IFN- β as well as IFN-inducible genes. These data indicate that TLR3 also utilizes a MyD88-independent pathway (Akira and Takeda 2004).

A second TIR domain-containing adaptor, TIR-domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like protein (Mal), was identified from a database search (Fitzgerald et al. 2001; Horng et al. 2001). As TIRAP/Mal does not have a death domain, it was initially thought to mediate the MyD88-independent pathway of TLR4-signaling. However, TIRAP/Mal-deficient mice showed the impaired production of inflammatory cytokines in response to LPS, and the LPS-induced MyD88-independent pathway was not impaired. Interestingly, cytokine production in response to TLR2 ligands was also impaired in TIRAP/Mal-deficient mice, suggesting that TIRAP/Mal acts as a bridging adapter binding MyD88 with TLR2 and TLR4 (Yamamoto et al. 2002a) (Horng et al. 2002).

Subsequent studies identified a third adapter TIR-domain-containing adapter protein inducing IFN- β (TRIF)/TIR-domain-containing molecule 1 (TICAM-1) (Oshiumi et al. 2003a; Yamamoto et al. 2002b). TRIF/TICAM-1-deficient mice and *Lps2*-mice with a frame shift mutation of *Trif* gene by the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) failed to show MyD88-independent activation of NF- κ B and are nonresponsive to TLR4 or TLR3 ligand with respect to the expression of IFN-inducible genes (Hoebe et al. 2003; Yamamoto et al. 2003a). Therefore, TRIF is essential for the TLR3- and TLR4-mediated MyD88-independent pathway. Additionally, TRIF-deficient mice displayed defective TLR4-mediated inflammatory cytokine production, although activation of the MyD88-dependent pathway, such as IRAK-1 phosphorylation and early phase NF- κ B activation, was not impaired. Thus, TLR4 requires both MyD88-dependent and MyD88-independent signals to induce the expression of inflammatory cytokines.

TRAF6 is reported to bind TRIF and cooperatively activate NF- κ B (Sato et al. 2003). However, cells doubly deficient in TRAF6 and MyD88 still partially activated NF- κ B in response to LPS, suggesting that TRIF activates NF- κ B through both TRAF6-dependent and -independent pathways in TLR4 signaling (Gohda et al. 2004; Kawai et al. 2001).

Recently, receptor-interacting protein-1 (RIP1) has been implicated in the TLR3-mediated NF- κ B response to dsRNA. RIP1 binds the C-terminus of TRIF/TICAM-1 via a Rip homotypic interaction motif (RHIM). In cells lacking RIP1, TLR3-mediated NF- κ B activation and the subsequent induction of Icam-1 was severely impaired, whereas IFN β induction was intact. By contrast, responses to LPS were normal in the absence of RIP1. However, LPS failed to stimulate NF- κ B activation in *rip^{-/-}MyD88^{-/-}* cells, revealing that RIP1 is also required for the TRIF-dependent TLR4-induced NF- κ B pathway. In the TNF- α pathway, RIP1 interacts with the E3 ubiquitin ligase TRAF2 and is modified by polyubiquitin chain. Upon TLR3 activation, RIP1 is also modified by polyubiquitin chains and is recruited to TLR3 along with TRAF6 and TAK1, suggesting that RIP1 uses a similar ubiquitin-dependent mechanism to activate IKK- β in response to TNF- α and TLR3 ligands (Meylan et al. 2004).

TRIF also binds to RIP3 through its RHIM domain, and RIP3 is likely to negatively regulate the TRIF-RIP1-induced NF- κ B pathway. Because RIP3 deficient mice have yet to be analyzed, the physiological role of RIP3 remains unknown (Meylan et al. 2004).

A fourth TIR-domain-containing adaptor, TRAM (TRIF-related adaptor molecule)/TIRP (TIR domain-containing protein)/TICAM-2 has been identified (Bin et al. 2003; Oshiumi et al. 2003b; Yamamoto et al. 2003b). The analysis of TRAM/TICAM-2-deficient mice demonstrated that TRAM is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway (Yamamoto et al. 2003b). The N terminus of TRAM has a myristoylation site, the mutation of which alters its normal membrane localization. TRAM/TICAM-2 acts as a bridging adaptor between TLR4 and TRIF/TICAM-1 (Oshiumi et al. 2003b).

There is another TIR-domain-containing adaptor, SARM (sterile α and armadillo-motif-containing protein). While an ortholog of mammalian SARM, the *Caenorhabditis elegans* TIR-domain-containing protein 1 (TIR1), has recently been shown to mediate the expression of genes that encodes antimicrobial peptides (Couillault et al. 2004), the function of SARM in mammalian innate immunity remains unknown. All analyses of these adaptor molecules have indicated that TIR domain-containing adaptors regulate the TLR signaling pathways by providing the specificity for each TLR cascade.

Recently, two noncanonical IKKs, inducible IKK (IKKi)/IKK ϵ and TRAF family member-associated NF- κ B activator (TANK)-binding kinase 1 (TBK1)/NF- κ B-activating kinase (NAK)/TRAF2-associated kinase (T2K), have been shown responsible for dsRNA-induced IRF-3 activation (Sharma et al. 2003). These two IKKs are structurally related to IKK α and IKK β , the catalytic subunits of the IKK complex that are essential for phosphorylation of two serine residues in I κ B α . IKKi/IKK ϵ and TBK1/NAK/T2K only induce phosphorylation of one serine residue

in $\text{I}\kappa\text{B}\alpha$, and produce NF- κB activation via different mechanisms from $\text{IKK}\alpha$ and $\text{IKK}\beta$. Overexpression of TBK1/NAK/T2K activates the promoter of IFN- β and IFN-inducible genes (Fitzgerald et al. 2003; McWhirter et al. 2004; Sharma et al. 2003). TBK1/NAK/T2K and $\text{IKK}\alpha/\text{IKK}\epsilon$ were shown to interact with IRF-3 and phosphorylate it using an in vitro kinase assay (Sharma et al. 2003). More recently, $\text{IKK}\alpha/\text{IKK}\epsilon$ -deficient and TBK1/NAK/T2K-deficient mice were generated and analyzed (Hemmi et al. 2004; Perry et al. 2004). TBK1/NAK/T2K and $\text{IKK}\alpha/\text{IKK}\epsilon$ were essential for the induction of IFN- β and IFN-inducible genes via activation of IRF-3 in both TLR-stimulated and virus-infected cells.

1.4.3 TLR7- and TLR9-Mediated Type I IFN Production

pDCs are specialized for producing large amounts of type I IFNs during viral infection (Liu 2005). pDCs highly express TLR7 and TLR9 and produce high levels of IFN- α in response to TLR7 and TLR9 ligands (Akira and Hemmi 2003). It appears that IFN- α induction by TLR7 and TLR9 depends entirely on MyD88 (Hemmi et al. 2003). IRF7 is a transcriptional factor, structurally related to IRF3, which is expressed constitutively in pDCs. Overexpression of IRF7 activates IFN- α - and IFN- β -dependent promoters. IRF7 forms a signaling complex with MyD88 and TRAF6 in the cytoplasm (Honda et al. 2004; Kawai et al. 2004). After ligand stimulation, IRF7 translocates into the nucleus to induce IFN- α s (Honda et al. 2004; Kawai et al. 2004). Similar to IRF3, IRF7 is activated by its phosphorylation. However, in TBK1-deficient cells, TLR9-mediated IFN- α production was still observed (Kawai et al. 2004). Mouse pDCs lacking IRAK-4 failed to produce both inflammatory cytokines and IFN- α s (Honda et al. 2004). Human TLR7-, TLR8- and TLR9-mediated induction of IFN- α/β and - γ was also IRAK-4 dependent (Yang et al. 2005). Recently, IRAK-1 has been shown to serve as an IRF7 kinase. In IRAK-1-deficient mice, TLR7- and TLR9-induced IFN- α production was completely abolished, although inflammatory cytokines were normally produced. Furthermore, IRF7 activation by TLR9 ligand was impaired in IRAK-1-deficient mice, in spite of normal NF- κB activation. IRAK-1 but not IRAK-4 could directly bind and phosphorylate IRF7; thus, IRAK-1 specifically mediates IFN- α induction downstream of MyD88 and IRAK-4 (Uematsu et al. 2005).

IRF8 is involved in TLR9-mediated responses because pDCs lacking IRF8 failed to produce proinflammatory cytokines and IFN- α in response to TLR9 ligand; in IRF8-deficient cells, TLR9-stimulated NF- κB activation was unexpectedly impaired, suggesting that IRF8 mediates NF- κB activation in TLR9 signaling (Tsujimura et al. 2004).

1.4.4 Other Molecules Involved in TLR Signaling

Several other molecules have been implicated in the TLR-mediated signaling pathway. Toll-interacting protein (Tollip) was originally identified through a

yeast-two-hybrid screen using the IL-1R accessory protein (Burns et al. 2000). Tollip is present in a complex with IRAK-1. Upon stimulation with IL-1, the Tollip-IRAK-1 complex is recruited to the IL-1 receptor complex. IRAK-1 is then phosphorylated, which leads to the rapid dissociation of IRAK-1 from Tollip, thereby inducing activation of TRAF6. Tollip has subsequently been shown to negatively regulate the TLR-mediated signaling pathway (Zhang and Ghosh 2002). Overexpression of Tollip inhibited activation of NF- κ B in response to IL-1, the TLR2 and TLR4 ligands. In addition, Tollip expression is elevated in intestinal epithelial cells, which are hyporesponsive to TLR2 ligands (Melmed et al. 2003). A unique C2-like domain in the N-terminus of Tollip has recently identified (Li et al. 2004). C2 domains in other proteins are involved in binding various phospholipids. It has also been shown that Tollip preferentially binds to phosphatidylinositol-3-phosphate and phosphatidylinositol-3,4,5-phosphate. Mutation of a vital lysine residue (K150) to glutamic acid within the C2 domain of Tollip inhibits LPS-induced NF- κ B activation, indicating that its lipid-binding capability is somehow connected with the inhibitory role of Tollip (Liew et al. 2005). However, it remains unclear how Tollip is physiologically involved in TLR signaling.

Pellino was originally identified in *Drosophila* as a molecule that associates with Pelle, a *Drosophila* homolog of IRAK (Grosshans et al. 1999). Three mammalian homologs have so far been identified (Jensen and Whitehead 2003b; Jiang et al. 2003; Yu et al. 2002). Mammalian Pellino1 interacts with IRAK-1, IRAK-4, TRAF6, and TAK1, and is required for the activation of NF- κ B but is not involved in the activation of the MAP kinase pathway (Jiang et al. 2003; Yu et al. 2002). Mammalian Pellino2 also interacts with these same proteins (Yu et al. 2002). There are controversial reports on the involvement of Pellino2 in NF- κ B activation (Liu et al. 2004; Strelow et al. 2003). However, it has recently been shown that Pellino2 promotes activation of ERK1/2 and JNK (Jensen and Whitehead, 2003a). Human Pellino3 interacts with IRAK-1, TRAF6 and TAK1 but is incapable of activating NF- κ B. However, like Pellino2, Pellino3 activates ERK1/2 and JNK (Jensen and Whitehead 2003b). A more recent report has shown that Pellino3 is an upstream regulator of p38 MAPK and activates CREB in a p38-dependent manner (Butler et al. 2005).

ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) has no homology with any known protein and was cloned as a TRAF6-interacting protein by yeast two-hybrid screening (Kopp et al. 1999). ECSIT interacts with the conserved TRAF domain of TRAF6. A *Drosophila* homolog of ECSIT has been identified, and the interaction between TRAF6 and ECSIT is also conserved in *Drosophila*. ECSIT also interacts with MEKK1 (MAPK/ERK (extracellular signal-regulated kinase) kinase kinase 1), which can phosphorylate and activate the IKK complex. Expression of a dominant-negative mutant of ECSIT blocks signaling through TLR4, indicating that ECSIT might transduce TLR signals by bridging TRAF6 and the IKK complex. Furthermore, the inhibition of ECSIT expression, using siRNA in a macrophage cell line, resulted in impaired LPS-induced, but not TNF-induced, NF- κ B activation (Xiao et al. 2003). The physiological function of ECSIT was studied by generating ECSIT-deficient mice, which were found to die

on about embryonic day 7.5 (Xiao et al. 2003). Further characterization showed that ECSIT is an obligatory intermediate in bone morphogenetic protein (BMP) signaling, and therefore ECSIT is an essential component in both the TLR- and BMP-signaling pathways.

Members of the MAPKKK family are implicated in IKK/NF- κ B and MAPK activation. Among them, MEKK3 has been shown to be involved in signaling through TLR4 but not TLR9 (Huang et al. 2004). MEKK3-deficient EFs showed impaired IL-6 production and defective activation of NF- κ B, JNK and p38 MAPK in response to the TLR4 ligand. Stimulation of TLR4 also induced association of MEKK3 with TRAF6. Another member of the MKKK family, TPL2 (tumor-progression locus 2; also known as cancer Osaka thyroid, COT), has been shown to be involved in the TLR4-mediated activation of ERK (Dumitru et al. 2000). In response to TLR4 ligand, TPL2-deficient mice showed impaired TNF- α production and defective activation of ERK.

Recently, IRF5 has been shown to be involved in a MyD88/TRAF6 complex (Schoenemeyer et al. 2005; Takaoka et al. 2005). In IRF5-deficient macrophages and DCs, induction of proinflammatory cytokines in response to various TLR ligands (TLR3, 4, 5, 7 and 9) was severely impaired (Takaoka et al. 2005). Upon TLR ligand stimulation, IRF5 was shown to translocate to the nucleus from the cytoplasm and bind the potential IFN-stimulated response element (ISRE) motifs present in the promoter region of proinflammatory cytokine genes. IRF5 associates with both MyD88 and TRAF6. TLR4 and TLR9 ligands promote nuclear translocation of IRF5 in a MyD88-dependent manner. However, downstream signals of IRF5 are still unclear. Although IRF5 is phosphorylated by TBK1 and IKK- α /IKK- ϵ , this phosphorylation does not induce nuclear translocation of IRF5. The mechanism of TLR3-mediated IRF5 activation is also yet to be clarified as TLR3 does not use MyD88. Furthermore, Schoenemeyer and colleagues showed that IRF5 is not a target of the TLR3 signaling pathway but is activated by TLR7 or TLR8 signaling (Schoenemeyer et al. 2005). Further study is necessary to better characterize the IRF5 pathway.

TRAF3 was identified as a molecule that is required for type I IFN production in response to both TLR activation and viral infection (Hacker et al. 2006; Oganesyan et al. 2006). TRAF3 associates with the TLR adapter TRIF and IRAK-1, as well as downstream IRF3/7 kinases TBK1 and IKK- α /IKK- ϵ , and serves as a critical link between TLR adaptors and downstream regulatory kinases for IRF activation. TRAF3 is also essential for the induction of the anti-inflammatory cytokine, IL-10 (Hacker et al. 2006).

1.5 Negative Regulation of TLR Signaling

Toll-like receptors sense microbial products and induce the expression of immune and proinflammatory genes to eliminate invading pathogens. However, excessive activation of TLRs is harmful for a host since it induces immune-mediated or

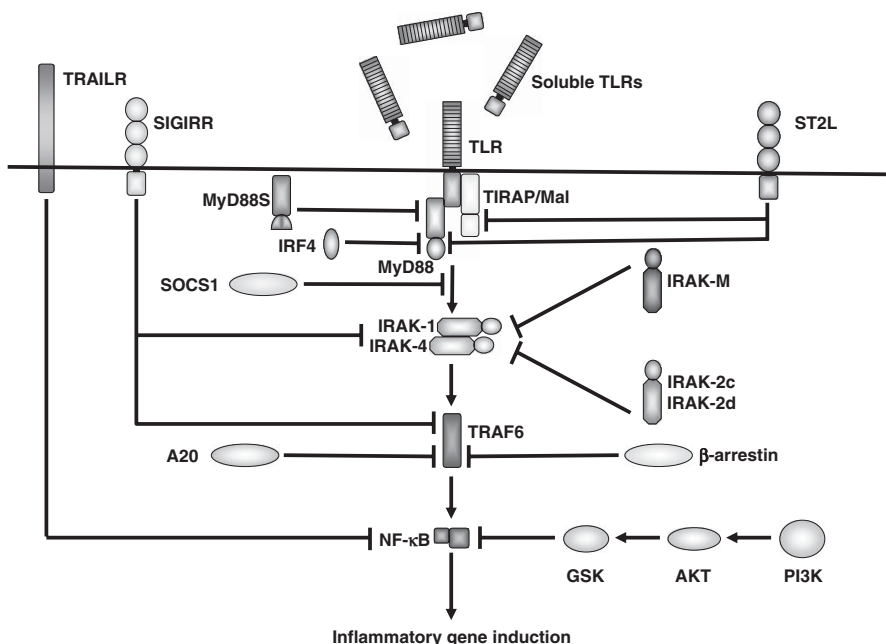


Fig. 3. Negative regulation of TLR signaling. TLR signaling pathways are negatively regulated by several molecules that are induced by TLR stimulations. Soluble forms of TLRs function as the first line of blockade of TLR signaling. Single Ig interleukin-1R-related molecule (*SIGIRR*) and ST2L have been shown to negatively modulate TLR signaling. MyD88S antagonizes MyD88. Interferon regulatory factor 4 (*IRF4*) also participates in the negative feedback regulation of TLR signaling. Inhibitory proteins such as suppressor of cytokine signaling-1 (*SOCS1*), interleukin-1 receptor kinase (*IRAK*)-M, *IRAK*-2c, and *IRAK*-2d selectively suppress *IRAK* functions. Phosphatidylinositol-3-kinase (*PI3K*) negatively regulates some TLR responses through glycogen synthase kinase-3 (*GSK*). A20 and β-arrestin deubiquitylate tumor necrosis factor receptor-associated factor 6 (*TRAF6*). Tumor necrosis factor-related apoptosis-inducing ligand receptor (*TRAILR*) suppresses nuclear factor kappa-B (*NF-κB*) activation during later TLR signaling events

inflammatory disorders. Living organisms have evolved multiple mechanisms of negative regulation to modulate TIR signaling, maintaining the balance between defending pathogens and preventing chronic inflammation and autoimmune diseases (Liew et al. 2005). Here, we discuss the molecules that are thought to negatively regulate TLR signaling (Fig. 3).

There are soluble TLRs (sTLRs) which function as decoys to effectively block TLR signaling. sTLR4 and sTLR2 have been identified in mammals. sTLR4 consists of 122 amino acids, of which 86 are identical to those of the extracellular domain of TLR4, and the remaining 36 amino acids share 70% homology with the N-terminal end of mouse phosphatidylinositol 3-kinase. sTLR4 is thought to block the interaction of LPS with TLR4 and co-receptor complexes, such as MD2 and CD14 (Iwami et al. 2000). sTLR2 was found to be constantly released from blood monocytes (LeBouder et al. 2003). There are six sTLR2 isoforms, which are gener-

ated by the post-translational modification of the transmembrane receptor protein. sTLR2 is present in human milk and plasma, where sTLR co-expresses and physically interacts with sCD14 (Iwaki et al. 2002). sTLRs work as antagonists of TLR2 in monocytes.

MyD88 is composed of three domains that are encoded by five exons (Hardiman et al. 1997). Exon1 encodes the death domain, Exon2 encodes the interdomain and the last three exons encode the TIR domain. MyD88s is an alternative spliced variant of MyD88, which lacks the interdomain (Burns et al. 2003; Janssens et al. 2002). MyD88s is only detected in the spleen but is upregulated in monocytes by LPS stimulation. Unlike MyD88, MyD88s does not interact with IRAK-4, but with IRAK-1. Overexpression of MyD88s does not induce IRAK-1 phosphorylation. MyD88s inhibits LPS-induced NF- κ B activation by blocking the recruitment of IRAK-4 to the receptor complex and the following IRAK-1 phosphorylation (Burns et al. 2003).

Unlike other IRAKs, which are expressed predominantly by peripheral blood leukocytes, the expression of IRAK-M is restricted to monocytes and macrophages and is upregulated following stimulation with TLR ligands (Wesche et al. 1999). IRAK-M-deficient mice showed increased inflammatory responses to various TLR ligands and bacterial infections, and also impaired LPS tolerance (Kobayashi et al., 2002). Biochemical analysis has revealed that IRAK-M prevents the dissociation of the IRAK-1-IRAK-4 complex from MyD88, thereby preventing formation of the IRAK-1-TRAF6 complex. These findings indicated that IRAK-M functions as a negative regulator of TLR signaling.

Four splice variants of the mouse *Irak2* gene, *Irak2a*, *Irak2b*, *Irak2c* and *Irak2d*, have been identified, and are generated by alternative splicing at the 5' end of the gene (Hardy and O'Neill 2004). Overexpression of IRAK-2c and IRAK-2d was shown to inhibit LPS-induced NF- κ B activation. Both IRAK-2c and IRAK-2d lack the death domain which IRAK-2a, IRAK-2b, and full length IRAK-2 contain. Furthermore, LPS induced IRAK-2c but not IRAK-2a in a mouse macrophage cell line RAW264.7, indicating that IRAK-2c and IRAK-2d work as negative regulators in TLR signaling pathways.

SOCS1 is a member of the SOCS (suppressors of cytokine signaling) family, which is known to be important in suppressing cytokine signaling (Yasukawa et al. 2000). Lipopolysaccharide and CpG DNA have been shown to induce the expression of SOCS1 in macrophages (Dalpke et al. 2001; Stoiber et al. 1999). SOCS1-deficient mice overproduced nitric oxide and pro-inflammatory cytokines in response to LPS and CpG DNA, and were hypersensitive to LPS-induced endotoxin shock (Kinjyo et al. 2002; Nakagawa et al. 2002). Furthermore, LPS tolerance was not induced in SOCS1-deficient mice and the ectopic expression of SOCS1 in macrophages inhibited LPS-induced NF- κ B activation, suggesting that SOCS1 directly inhibits TLR signaling pathways. This inhibition seems to occur by targeting IRAK-1, but the precise mechanism of its inhibition remains unclear.

Class IA PI3K (phosphatidylinositol-3-kinase) consists of a p85 regulatory subunit and a p110 catalytic chain. PI3Ks are activated by various TLR ligand stimulations in DCs (Katso et al. 2001). P85-deficient mice show enhanced TLR signaling and a dominant Th1-cell response. PI3K-deficient dendritic cells

overproduce IL-12 in response to the TLR2, TLR4 and TLR9 ligands (Fukao et al. 2002); furthermore, PI3K-deficient mice on a BALB/c background were resistant to *Leishmania major* infection, unlike wild-type mice, because of the skewed Th1 response in these mice. These results indicated that PI3K is an effective negative regulator of TLR signaling. Activation of PI3K can mediate the recruitment and subsequent activation of signaling molecules with pleckstrin homology domains, including serine-threonine kinase Akt (Franke et al. 1997; Lawlor and Alessi 2001; Stokoe et al. 1997). After recruitment, Akt is activated by phosphorylation, subsequently phosphorylating several downstream targets of the PI3K pathway, including the constitutively active serine-threonine kinase glycogen synthase kinase (GSK3)- β . Phosphorylation of GSK3- β results in its inhibition (Cross et al. 1995). Recently, GSK3- β has been shown to differentially regulate TLR-mediated production of pro- and anti-inflammatory cytokines (Martin et al. 2005). Inhibition of GSK3 resulted in profound increases in IL-10 production after TLR2, TLR4, TLR5 or TLR 9 stimulations, whereas the concurrent production of proinflammatory cytokines, including IL-1 β , IL-6, TNF- α , IL-12, and IFN- γ , by human monocytes and peripheral blood mononuclear cells, was substantially reduced. Administration of GSK3 inhibitor protected mice from endotoxin shock. cAMP response element-binding protein (CREB) is an important transcription factor for IL-10 production in human monocytes, whereas NF- κ B regulates many diverse cellular process, including proinflammatory cytokine responses. Both CREB and NF- κ B p65 utilize a nuclear coactivator CREB-binding protein (CBP) to induce their target genes. As nuclear amounts of CBP are limiting, CREB and NF- κ B p65 compete for CBP binding. Without stimulation, GSK3 negatively regulates the activation and DNA-binding activity of CREB. However, when stimulated with TLR ligands, GSK3 is inhibited and increased association of CREB and CBP suppress NF- κ B activity. Thus, GSK3 may regulate inflammatory responses by differentially affecting the nuclear amounts of the transcription factors NF- κ B subunit p65 and CREB interacting with CBP (Martin et al. 2005).

A20 was initially identified as a zinc-finger protein induced by TNF- α , which suppresses TNF-mediated NF- κ B activation (Krikos et al. 1992; Opipari et al. 1990). A20 was reported to be induced by LPS, and macrophages in A20-deficient mice show increased production of inflammatory cytokines in response to various TLR ligands. Mice reconstituted with fetal liver hematopoietic stem cells from A20-deficient mice are hypersensitive to LPS-induced shock but LPS tolerance was not impaired in A20-deficient mice. In vitro study also showed evidence of a role for A20 as a negative regulator in TLR signaling. Overexpression of A20 inhibited TLR-mediated NF- κ B activation, and A20 was shown to be a cysteine protease deubiquitylating enzyme that cleaves the ubiquitin chain of TRAF6 to block TLR signaling (Boone et al. 2004).

ST2 (also known as T1, Fit-1 or DER4), which has been an orphan member of the IL-1 receptor family for a long time, was recently shown to be the receptor of the cytokine, IL-33. IL-33 promoted Th2-type responses via ST2 (Schmitz et al. 2005). ST2 has also been described as a negative regulator of Toll-like receptor-IL-1 receptor signaling (Brint et al. 2004). Two variants of the *St2* gene (ST2L and

sST2) have been identified, and are generated by mRNA splicing (Bergers et al. 1994; Klemenz et al. 1989; Tominaga 1989). ST2L is a type I membrane protein and contains three extracellular immunoglobulin-like domains and an intracellular TIR domain. sST2 is the soluble protein, which consists of the extracellular domains of ST2L with an extra 9 amino acids in the C-terminal (Bergers et al. 1994; Klemenz et al. 1989; Tominaga 1989). ST2-deficient mice (lacking both ST2L and sST2) showed increased production of inflammatory cytokines in response to LPS, CpG DNA and IL-1 but not to the TLR3 ligand, polyI:C. ST2L co-precipitated with MyD88 and TIRAP/MAL through a conserved proline residue in TIR domain, but not with IRAK or TRIF. Taken together, ST2L blocks TLR signaling by sequestering MyD88 and TIRAP/MAL with its TIR domain from TLRs. Although ST2-deficient mice showed impairment in LPS tolerance, ST2-deficient mice were not susceptible to LPS shock. ST2L normally stays in cytoplasm and expresses on the cell surface after at least 4 h of LPS stimulation. The discrepancy of phenotype between LPS shock and LPS tolerance in ST2-deficient mice may due to this time lag (Brint et al. 2004). sST2 is also thought to be a negative regulator of TLR signaling. sST2 expression is upregulated in pro-inflammatory cytokines and LPS (Kumar et al. 1997; Sacconi et al. 1998). It is also present in normal human serum and its levels are increased in various inflammatory diseases. sST2 is thought to enhance cytokine production by binding macrophages through a putative ST2 receptor. There is also another report that sST2 downregulates the expression of TLR4 and TLR1 in LPS-stimulated macrophages. However, the precise mechanism of sST2 inhibition is currently unknown (Sweet et al. 2001).

SIGIRR (single Ig IL-1R-related molecule) is also an orphan receptor, and contains a single extracellular immunoglobulin domain and a cytoplasmic TIR domain (Thomassen et al. 1999). SIGIRR-deficient bone marrow DCs exaggerated responses to LPS and CpG DNA but not to the PolyI:C (Garlanda et al. 2004). Furthermore, SIGIRR-deficient mice were highly susceptible to LPS shock. Following TLR stimulation, SIGIRR has been shown to interact transiently with TLR4, IRAK-1 and TRAF6, thereby negatively regulating TLR signaling (Wald et al. 2003).

TRAILR (tumor necrosis factor-related apoptosis-inducing ligand receptor) belongs to the TNF superfamily and does not have a TIR domain (Wu et al. 1999). TRAIL (Tumor necrosis factor related apoptosis inducing ligand) expression is upregulated by TLR2, TLR3 and TLR4 but not TLR9 ligands. TRAILR-deficient mice showed increased cytokine production in response to TLR ligands. TRAILR seems to inhibit TLR signaling by stabilizing I κ B α , which results in the decreased nuclear translocation of NF- κ B (Diehl et al. 2004).

RP105 is a TLR homolog originally cloned as a B-cell specific molecule (Miyake et al. 1994; Miyake et al. 1995). RP105 has a conserved extracellular leucine-rich repeat domain but lacks a signaling domain. Divanovic et al. recently showed that RP105 is widely expressed and mirrors the expression of TLR4 (Divanovic et al. 2005). RP105 and its helper molecule, MD1, interacted directly with the TLR4/MD2 complex, inhibiting its ability to bind LPS. RP105 has been shown to be a physiological regulator of TLR4 signaling in primary DCs, and of responses to LPS in vivo.

A member of the IRF family, IRF4 has recently been reported as a negative regulator of TLR signaling (Honma et al. 2005; Negishi et al. 2005). IRF4 mRNA is induced by TLR activation and IRF4 competes with IRF5 for interaction with MyD88. IRF4-deficient mice were sensitive to LPS-induced shock, and their macrophages produced high levels of proinflammatory cytokines. Activation of NF- κ B and JNK was enhanced after LPS stimulation in IRF4-deficient macrophages. IRF4 may participate in the negative feedback regulation of TLR signaling.

The arrestin family consists of ubiquitously expressed β -arrestin 1, β -arrestin 2 and two more arrestins expressed exclusively in retinae (Lefkowitz and Whalen 2004). β -arrestins have been shown to directly interact with TRAF6 after TLR-IL-1R activation. Formation of the β -arrestin-TRAF6 complex prevented auto-ubiquitination of TRAF6 and activation of NF- κ B and AP-1. Endotoxin-treated β -arrestin 2-deficient mice had higher expressions of proinflammatory cytokines and were more susceptible to endotoxic shock, suggesting that β -arrestins are essential negative regulators of the innate immune activation via TLR-IL-1R signaling (Wang et al. 2006).

1.6 Conclusion and Future Prospects

Following the discovery of Toll, the molecular mechanisms of pathogen recognition by TLRs have been elucidated over the past few years. Toll-like receptors have now been identified as important components of the innate immune system. However, it is reported that viruses and intracellular bacteria also induce innate immune responses through a TLR-independent mechanism. For example, intracellular administration of polyI:C induces type I IFNs from DCs lacking TLR3 (Hemmi et al. 2004; Yamamoto et al. 2003a). The DExD/H box RNA helicase RIG-I (retinoic acid inducible gene I) has recently been identified as a cytoplasmic sensor for dsRNA (Kato et al. 2005; Yoneyama et al. 2004). Another DExD/H box RNA helicase, Mda-5 (Melanoma differentiation associated gene 5), which is closely related to RIG-I, has been suggested to be receptor-like molecule for viral RNA (Andrejeva et al. 2004; Kang et al. 2004). Recently, the RIG-I/Mda5-associated adapter IPS-1 (interferon-beta promoter stimulator-1)/MAVS (mitochondrial antiviral signaling)/VISA (virus-induced signaling adaptor)/CARDIF (CARD adaptor inducing interferon-beta) was identified, and is required for IFN- β and NF- κ B activation (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005). Furthermore, DNA, including host DNA, can be recognized independently of TLR9 (Boule et al. 2004; Decker et al. 2005). DNaseII in macrophages is important for cleaving DNA of engulfed apoptotic cells or debris (Kawane et al. 2003). Without cleaning off DNA in phagosomes, macrophages produced IFN- β in a TLR-independent manner, indicating that there is a TLR-independent, endogenous DNA-sensing mechanism to activate innate immunity (Okabe et al. 2005; Yoshida et al. 2005). Recently, Ishii et al. reported that intracellular administration of double stranded B-form DNA (B-DNA) but not Z-DNA triggered antiviral responses

including production of type I IFNs and chemokines independently of TLR. B-DNA-mediated signaling might utilize a similar pathway of RIG-I or Mda-5 (Ishii et al. 2006).

The newly discovered CATERPILLER gene family regulates inflammatory and apoptotic responses, and some act as sensors to detect pathogen products (Ting and Davis 2005). Among them, NOD1 (nucleotide-binding oligomerization domain) and NOD2, which contain N-terminal CARD (caspase recruiting domain) domains, a central NACHT (neuronal apoptosis inhibitory protein (NAIP), MHC class II transcription activator (CIITA), incompatibility locus protein from *Podospora anserina* (HET-E), and telomerase-associated protein (TP1)) domain and C-terminal LRRs, recognize γ -D-glutamyl-*meso*-diaminopimelic acid (ie-DAP), a cell wall derivative from Gram-negative bacteria, and muramyl dipeptide (MDP), found in both Gram-positive and Gram-negative bacteria, respectively (Chamaillard et al. 2003; Girardin et al. 2003). Mutation in the human *nod2* gene is reportedly associated with the pathogenesis of Crohn's disease and Blau syndrome (Hugot et al. 2001; Ogura et al. 2001; Philpott and Girardin 2004). NAIP (neuronal apoptosis inhibitor protein) is a member of the inhibitor of apoptosis (IAP) family, which contain a BIR (baculovirus inhibitor of apoptosis protein repeat) domain in their N-termini. NAIP5 has also been implicated in host responses to *Legionella* infection (Diez et al. 2003).

As stated above, recent reports have brought rapid clarification of TLR-independent pathogen recognition. As TLRs recognize pathogens at cell surfaces or in phagosomes, they cannot sense pathogens such as intracellular bacteria and viruses that replicate in the cytoplasm. Hence, hosts have developed other mechanisms to sense and exclude them. To more comprehensively understand innate immune responses, further studies of both TLR-dependent and TLR-independent mechanism will be needed.

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Strategies of Natural Killer (NK) Cell Recognition and Their Roles in Tumor Immunosurveillance

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2.1 Introduction: An Interesting Epistemological Case

Natural Killer cells (NK cells) represent an interesting epistemological example in Immunology. First considered as “background noise” in T-cell cytolytic assays, Natural Killer (NK) cells were characterized more than 30 years ago as cytotoxic effectors of the innate immune system (Kiessling et al. 1975). Later, NK cells were recognized as a peculiar type of large granular lymphocytes that are widespread throughout the body (Lanier et al. 1986), being present in both lymphoid organs and non-lymphoid peripheral tissues (Cooper et al. 2004; Ferlazzo and Munz 2004). Their specificity for a variety of tumor cells, virus-infected cells or allogeneic cells along with their lack of antigen-specific receptors, have puzzled immunologists for many years. Since this time, a series of discoveries have shed light on the mechanisms of NK cell effector function and have simultaneously broadened our views on immune detection strategies (Carayannopoulos and Yokoyama 2004; Lanier 2005; Moretta et al. 2002; Stewart et al. 2006; Vivier and Biron 2002). Such discoveries include “missing-self recognition” (via major histocompatibility complex [MHC] class I) (Kärre et al. 1986), the identification of inhibitory cell surface receptors that modulate NK cell activation (via Immunoreceptor Tyrosine-based Inhibition Motifs: ITIM) (Vély and Vivier 1997) or the “stress-induced self recognition” (via NKG2D) (Raulet 2003) (Fig. 1). The involvement of NK cells in the control of viral and parasitic infections, in auto-immunity, in reproduction as well

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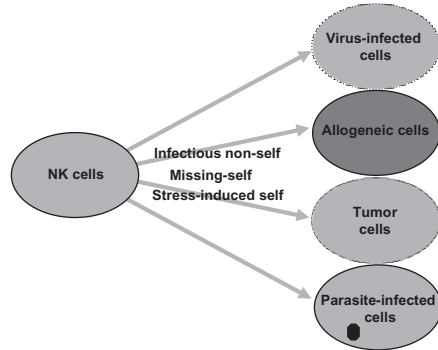
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Fig. 1. Natural killer cell recognition strategies. Schematic representation of the mode of NK cell interaction with partner cells (see text for details)



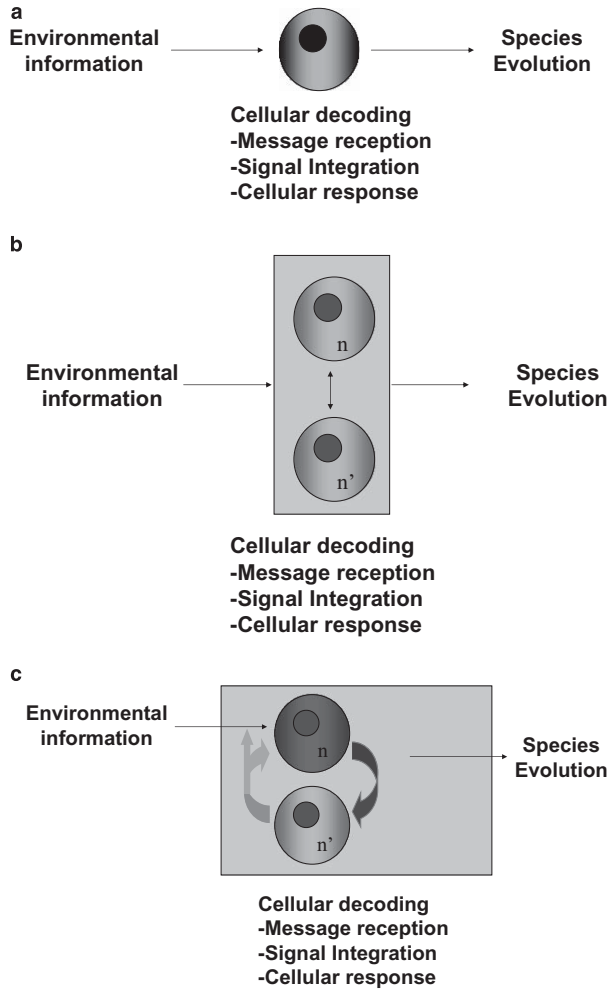
as in the clinical outcome of hematopoietic transplants has been reviewed recently (Carayannopoulos and Yokoyama 2004; Johansson et al. 2005; Korbel et al. 2004; Lodoen and Lanier 2005; Orange and Ballas 2006; Parham 2005; Ruggeri et al. 2005; Zhang et al. 2005). Similarly, extensive reviews on NK cell signaling and developmental pathways and the interaction between NK cells and other innate immune sentinels such as dendritic cells (DC) have been published (Degli-Esposti and Smyth 2005; Di Santo 2006; MacFarlane and Campbell 2005; Moretta et al. 2005; Vivier et al. 2004; Walzer et al. 2005). Herein, we focus on reviewing the molecular devices by which NK cells discriminate “resistant” cells from “target” cells, and address how these recognition strategies may govern the involvement of NK cells in cancer immunosurveillance processes.

2.2 Natural Killer Cells in Innate Immunity

The appearance of multicellular organisms is an outstanding step in the evolution of life. Multicellularity was accompanied by specialization of cells which allowed the acquisition of novel biological functions. All cells have an autonomous capacity to respond to various forms of stress, such as physical stress (UV, heat shock, etc.), chemical stress (pH, osmolarity, etc.) or microbial stress. Multicellularity permitted the emergence of cells specialized in recognition of pathological stress. These cells form the immune system. They form an extrinsic system that complements autonomous stress responses and shares the burden of stress-recognition and stress-resolution with affected cells. Cells of the immune system act at the organism-level to facilitate controlled, measured, appropriate, and memorable responses (Fig. 2).

The immune response is organized in two complementary systems: the innate immune system and the adaptive immune system (Vivier and Malissen 2005). Adaptive immunity in higher vertebrates is due to T and B lymphocytes, which recognize a colossal antigenic repertoire via specific receptors that are the products of combinatorial gene segment rearrangements. Alternative forms of adaptive

Fig. 2a–c. The immune system in multicellular organisms. **(a)** Driving forces in the evolution of unicellular organisms. **(b)** Complexity of multicellular organisms. **(c)** Schematic representation of the immune system, as a group of cells (n') that are alerted by cells in distress (n), without having been exposed to the stress themselves. This mode of operation distinguishes a “stress response” that every cell can exert in every organism from an immune response



immunity have also recently been described in lower vertebrates (Pancer and Cooper 2006). The innate immune system is present in all metazoans and is characterized by a functional duality: On one hand, innate immune cells can directly exert their effector function against the “aggressors” of the organism (e.g. via the production of type I interferons, via cell cytotoxicity), and on the other hand, they also initiate and orient the adaptive immune response (e.g. via the antigenic presentation, via the production of cytokines and chemokines).

The innate immune system is fundamental in antimicrobial responses. Indeed, a variety of receptors have been identified, which confer to innate immune cells the capacity to recognize microbial products (e.g. Toll-like receptors). These receptors are characterized by their phylogenetic conservation, which bears witness to their evolutionary selection (Beutler 2004; Janeway and Medzhitov 2002). However, the roles of innate immunity in the defense mechanisms against tumors, in

allogeneic reactions and in other processes are still poorly understood. Amongst cells of the innate immune system, NK cells represent an interesting and important case as these lymphocytes were initially characterized for their antitumoral and anti-allogeneic cytolytic functions.

2.3 Natural Killer Cell Recognition of “Missing-Self”: An Array of Inhibitory Cell Surface Receptors

2.3.1 MHC Class I-Specific Inhibitory Receptors

The demonstration that NK cell cytolytic activity inversely correlates with the level of MHC class I expression on target cells provided the first evidence for missing-self recognition by NK cells (Kärre et al. 1986). Part of the intellectual appeal of the missing-self hypothesis in MHC class I recognition is how it complements the adaptive immune system, providing a means of detecting cells that might otherwise escape immune recognition through loss of MHC class I (Kärre 1997). The widespread presence of MHC class I on normal tissues supports the argument that missing-self recognition prevents NK cell auto-immune reactions. This is also true for missing-self recognition mediated by other processes including for example, complement activation. The alternative pathway of complement activation involves spontaneous hydrolysis of the labile complement component C3. The complement regulatory proteins CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), and CD35 (complement receptor type 1, CR1) are expressed by most normal cells and coordinately reduce the creation and accelerate the decay of C3 and C5 convertases (Kim and Song 2006). Without adaptation to mimic host cells, microbes lack these regulators and are susceptible to complement attack.

Multiple MHC class I receptors have been identified that inhibit cell activation through recruitment of the protein tyrosine phosphatases (PTPs) SHP-1 and/or SHP-2 via their cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs; I/V/S/LxYxxL/V, where x can be any amino-acid) (Burshtyn et al. 1996; Long 1999; Olcese et al. 1996; Vivier and Daëron 1997). The receptors can be classified into the C-type lectin CD94/NKG2 heterodimers, the C-type lectin Ly49 family and the immunoglobulin (Ig)-like receptors including the killer Ig-like receptors (KIR) and Ig-like transcripts (ILT) (Fig. 3a,b).

The CD94/NKG2 receptors are composed of C-type lectin type II transmembrane proteins. These recognize the nonclassical MHC class I molecules human leukocyte antigen (HLA)-E (in human) and Qa-1^b (in mouse) (Braud et al. 1998; Borrego et al. 1998; Lee et al. 1998; Vance et al. 1998). Of the NKG2 family members that associate with CD94 (including NKG2A, C, and E), NKG2A contains ITIMs in its cytoplasmic domain conferring inhibitory function. Recognition of HLA-E or Qa-1^b by CD94/NKG2 requires the presence of peptides in their peptide binding grooves. These peptides are frequently derived from the leader sequences

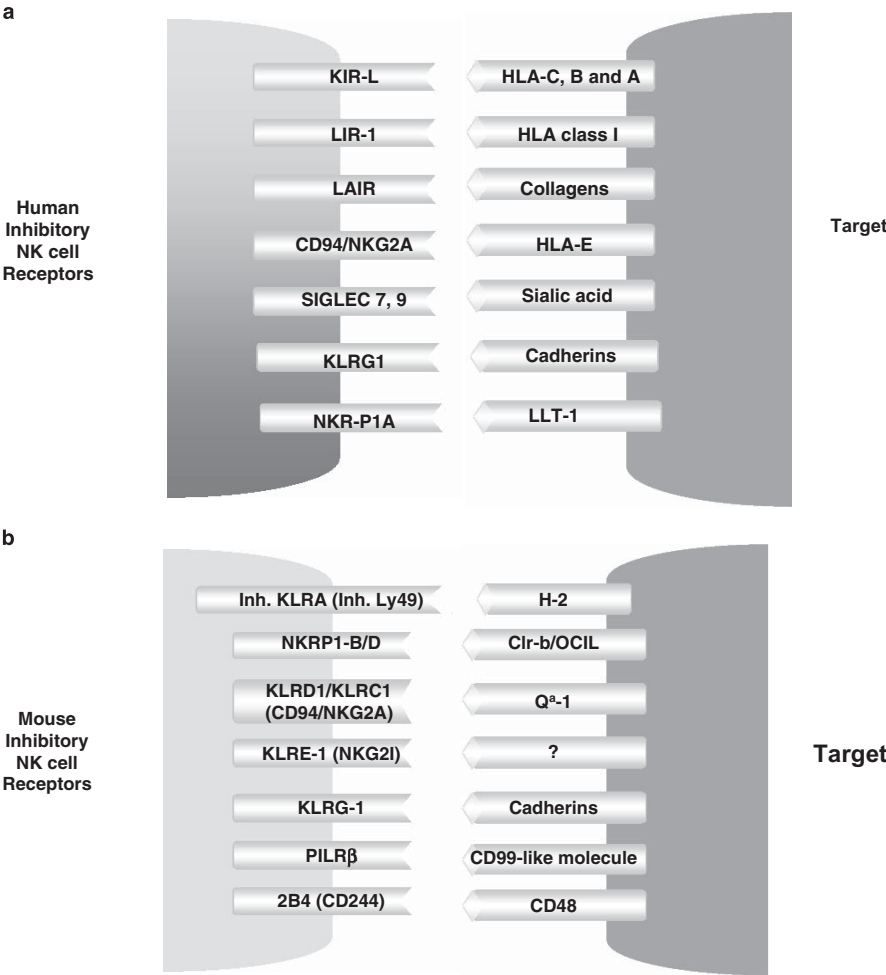


Fig. 3a,b. The “inhibitory NK cell zipper.” Inhibitory cell surface receptors and ligands for human (a) and mouse (b) NK cells. *KIR*, killer Ig-like receptors; *LIR*, immunoglobulin-like transcript; *LAIR*, leukocyte-associated immunoglobulin-like receptor; *SIGLEC*, sialic acid binding Ig-like lectins; *KLRG*, killer cell lectin-like receptor; *NKR-P1A*, NK cell receptor protein 1; *HLA*, human leukocyte antigen; *LLT*, lectin-like transcript

of classical MHC class I molecules (Braud et al. 1997; Kraft et al. 2000; Lopez-Botet et al. 1998). As a result, CD94/NKG2A is a sensor of active MHC class I biosynthesis and presentation. CD94/NKG2 is unique in both its high evolutionary conservation and its means of recognizing classical MHC class I as a “proxy” sensor.

Members of the other NK cell MHC class I receptor families directly recognize classical MHC class I molecules. Natural killer cell recognition of subsets of MHC class I allotypes is mediated by members of the Ly49 family in the mouse and rat.

In contrast, human NK cells use immunoglobulin domain-containing type I transmembrane proteins for the same function. Immunoglobulin-like transcript 2 (ILT2; or LIR1 or CD85j) recognizes a broad range of both classical and non-classical MHC class I molecules (Chapman et al. 1999; Colonna et al. 1997), whilst the KIR family members (or CD158 molecules) are specific for subsets of MHC allotypes. Primate KIR receptors therefore appear to be functional homologues of rodent Ly49 despite their independent evolutionary origins, thus providing an interesting example of convergent evolution. A feature of both KIR and Ly49 recognition of MHC class I is sensitivity to peptides bound in the MHC class I groove. Many KIR and some Ly49 receptors are sensitive to peptide changes, although clearly less sensitive than the TCR (Franksson et al. 1999; Hanke et al. 1999; Hansasuta et al. 2004; Natarajan et al. 2002; Peruzzi et al. 1996; Rajagopalan and Long 1997; Stewart et al. 2005; Zappacosta et al. 1997).

Despite the specificity of KIR and Ly49 receptors for restricted groups of MHC class I allotypes, these receptors endow human and mouse NK cells with a panoramic recognition system of MHC class I. The breadth of this system arises from a number of features. In any individual, multiple *KIR* genes mean that receptors are present for many different types of MHC class I: KIR2DL1 recognizes HLA-C allotypes with a lysine at position 80, KIR2DL2/3 recognize HLA-C allotypes with asparagine at position 80, KIR3DL1 recognizes HLA-Bw4 allotypes, and KIR3DL2 binds HLA-A3 and HLA-A11 molecules (Boyington and Sun 2002; Hansasuta et al. 2004; Parham 2005). Each of these inhibitory receptors is present in most humans (Middleton et al. 2005), meaning that the NK cell pool of most individuals can detect alterations in the expression of almost all HLA-C allotypes, a large proportion of HLA-B allotypes and some HLA-A allotypes. The capacity of the NK cell pool to detect changes in expression of these HLA molecules is also founded on the variegated expression of individual NK cell receptors and resulting repertoire of specificities within the NK cell pool (Anderson 2006; Raulet et al. 2001).

2.3.2 Inhibitory Receptors for Non-MHC Class I Molecules

ITIM-based inhibitory signaling is not restricted to MHC class I recognition. ITIM-bearing receptors recognizing many different molecules are found on cells across the spectrum of hematopoiesis. On NK cells, such inhibitory receptors include carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1), certain sialic acid binding Ig-like lectins (Siglecs), inhibitory NK cell receptor protein 1 (NKR-P1) molecules, and killer cell lectin-like receptor G1 (KLRG1) (Fig. 3a,b). The ligands for some of these receptors are widely expressed, like MHC class I molecules themselves, leading to proposals that these receptors have missing-self function (Kumar and McNerney 2005; Plougastel and Yokoyama 2006).

2.3.2.1 Siglecs

Siglec-7 (p75/AIRM1) is a member of the CD33-related Siglec family that has an ITIM and an ITIM-like sequence in its cytoplasmic tail enabling the binding of SHP-1 and SHP-2 upon tyrosine phosphorylation (Crocker 2005; Falco et al. 1999; Nicoll et al. 1999). Siglec-7 is expressed by all NK cells, all monocytes, and some CD8⁺ T cells (Falco et al. 1999; Nicoll et al. 1999). Activation of NK cytotoxicity and other forms of cellular activation are inhibited upon ligation of Siglec-7 with specific antibody (Crocker 2005; Falco et al. 1999). Siglec-7 has a ligand preference for α 2,8-linked disialic acids that are found on certain gangliosides including GD3 (Ito et al. 2001b; Yamaji et al. 2002). GD3 is expressed at high levels on neurons but also on melanoma and renal cell carcinoma cells (Ito et al. 2001a; Urmacher et al. 1989), potentially making such cells resistant to monocyte and NK cell responses. However, the understanding of Siglec biology is complicated by “masking” interactions in *cis* that can occur between Siglecs and multiple sialylated ligands on the same cell. In this way, NK cells require sialidase treatment, and the consequential unmasking of Siglec-7, for their cytolytic activity to be inhibited by recognition of Siglec-7 ligand, ganglioside GD3, on P815 target cells (Nicoll et al. 2003). Intriguingly, in the presence of masked Siglec-7, the over-expression of ganglioside GD3 on P815 cells rendered them more sensitive to lysis by interleukin-2 (IL-2) activated peripheral blood lymphocytes, suggesting that GD3 may also act as an activating ligand for a currently unknown receptor.

2.3.2.2 KLRG1

Killer cell lectin-like receptor G1 (KLRG1) was originally identified as mast cell function-associated antigen (MAFA). Resting human NK cells (~50%), mouse NK cells (30%) and lower numbers of CD4 and CD8 T cells express KLRG1. A large increase in expression is seen on these cell populations following activation, for example that occurring during viral infection (Blaser et al. 1998; Hanke et al. 1998; Robbins et al. 2002, 2004; Voehringer et al. 2002). KLRG1 expression on human peripheral blood T cells occurs on antigen-experienced T cells with an effector memory phenotype (Voehringer et al. 2001, 2002). Recently the ligands for mouse KLRG1 have been identified as the E-, N- and R-cadherins, of which E-cadherin shows the strongest binding (Grundemann et al. 2006; Ito et al. 2006). Cadherins are ubiquitously expressed on solid tissues, and are localized at tight junctions in epithelia. Many epithelial cancers are known to down-regulate E-cadherin, thus facilitating invasion and metastasis (Cavallaro and Christofori 2004). In addition to their role as cell adhesion molecules involved in homophilic interactions, E-cadherin also serves as a ligand for the integrin α_E (CD103) β 7. α_E (CD103) β 7 is expressed by very low frequencies of blood cells, instead being found on

intraepithelial lymphocytes, mucosal mast cells, mucosal dendritic cell and CD25⁺ T regulatory cell populations (Andrew et al. 1996; Kilshaw and Higgins 2002; Lehmann et al. 2002). Ligation of KLRG1 inhibits activation of rat basophilic leukemia cells and inhibits cytotoxicity of NK cells in some studies (Guthmann et al. 1995; Ito et al. 2006; Robbins et al. 2002). However, in other NK cell studies the ligation of KLRG1 by antibody or by its E-cadherin ligand did not block cytotoxicity, but did reduce proliferation of T cells in response to antigen stimulation (Grundemann et al. 2006; Hanke et al. 1998).

2.4 Complexity of the “NK Cell Zipper”

The mere lack of MHC class I surface expression is not sufficient to ensure sensitivity to NK cell attack. This is true for red blood cells, but also for MHC class I-deficient lymphocytes that are classically used as *in vitro* NK targets only during a short time window after mitogenic treatment (2–3 days after ConA treatment for mouse splenocytes or after PHA treatment for human peripheral blood cells). In addition, some MHC class I-positive cells can be sensitive NK cell targets. These observations have prompted the search and the discovery of other NK cell receptors whose engagement with target cell ligands triggers NK cell cytolytic programs, as well as cytokine and/or chemokine secretion. Some of these activating cell surface receptors initiate protein tyrosine kinase (PTK)-dependent pathways through non-covalent association with transmembrane signaling adaptors that harbor ITAMs (immunoreceptor tyrosine-based activation motifs; YxxL/Ix₆₋₈YXXL/I). Additional cell surface receptors that are not directly coupled to ITAMs also participate in NK cell activation, including NKG2D which is non-covalently associated with the DAP10 transmembrane signaling adaptor, as well as integrins and cytokine receptors. A variety of activating NK cell receptors (in particular those that are dependent upon ITAMs) can be antagonized by the aforementioned ITIM-bearing inhibitory cell surface receptors. The tyrosine phosphorylation status of several signaling components, that are substrates for both PTKs and PTPs, is therefore key to the propagation of the NK cell effector pathways (Long 1999; MacFarlane and Campbell 2005; Tomasello et al. 2000; Vely and Vivier 2005a,b; Vivier et al. 2004). Consequentially, NK cell activation is controlled by a dynamic balance between activating and inhibitory pathways. A very complex “zipper” thus forms between NK cells and their interacting partner, involving complementary as well as antagonist pathways (Fig. 3a,b, Color Plate 2a,b). Dissecting the integration of these multiple signals represents a current and future challenge in the understanding and manipulation of NK cell effector signaling pathways. Natural killer cells have been instrumental in revealing the function of ITIM-bearing molecules and their dynamic equilibrium with activating receptors. However, it is important to note that these notions are in no way limited to NK cells, but are widely described in a variety of (if not all) hematopoietic cells, as well as some non-hematopoietic cells.

2.4.1 Natural Cytotoxicity Receptors

The natural cytotoxicity receptors (NCR) are NK cell receptors that are critically involved in the human natural cytotoxicity against a broad panel of target cell types *in vitro*. Members of the NCR, NKp46, NKp44 and NKp30 (also known as NCR1, NCR2, and NCR3 respectively), were identified through the screening of antibodies raised against human NK cell molecules whose cross-linking resulted in redirected NK cytotoxicity against $\text{Fc}\gamma\text{R}^+$ P815 target cells (Moretta et al. 2001; Pende et al. 1999; Sivori et al. 1997; Vitale et al. 1998). All three NCR are members of the immunoglobulin superfamily, NKp46 containing two C2-type Ig-like domains and both NKp44 and NKp30 containing one V-type Ig-like domain. NKp46 and NKp30 are expressed by all human NK cells and associate with the $\text{Fc}\gamma\text{R}$ and $\text{CD}3\zeta$ adaptors. NKp46 or NKp30 cross-linking by antibodies results in a strong NK cell activation, including calcium flux, cytotoxicity, and production of cytokines (Bottino et al. 2005). NKp44 is not expressed by freshly isolated NK cells, but is induced following culture with IL-2. Natural cytotoxicity receptors are mostly NK cell-specific. However, NKp46 has been detected on subsets of activated intraepithelial T lymphocytes (Jabri et al. 2000), and NKp44 has been also found on rare $\gamma\delta$ T-cell clones (Vitale et al. 1998). Recently, NKp44 has also been described on a subset of plasmacytoid DC (Fuchs et al. 2005). In contrast to its strong stimulatory function on NK cells, NKp44 ligation inhibits $\text{IFN-}\alpha$ in response to cytosine-phosphate-guanosine oligonucleotides (Fuchs et al. 2005), prompting the extensive dissection of ITAM-mediated inhibition of immune responses seen also in other models (Hamerman and Lanier 2006). Indeed, NKp44 signaling is based on its association with the ITAM-containing KARAP/DAP12 adaptor (Tomasello and Vivier 2005).

Natural cytotoxicity receptors have heterogeneous expression levels among individuals. The ability to discriminate NK clones based upon NCR expression level was first described for NKp46 (Sivori et al. 1999) and it was later found for NKp30 and NKp44 expression levels (Pende et al. 1999). The $\text{NCR}^{\text{bright}}$ and NCR^{dull} phenotypes vary in proportion between the NK populations of different individuals and are unchanged upon culture of NK cells with different cytokines including IL-2, IL-15, IL-12 and IL-18. The $\text{NCR}^{\text{bright}}$ or NCR^{dull} phenotype correlates with the ability of NK populations and NK clones to perform natural cytotoxicity. In addition, antibodies blocking studies indicate that $\text{NCR}^{\text{bright}}$ and NCR^{dull} cells have a high and low capacities for NCR mediated natural cytotoxicity respectively (Moretta et al. 2001; Sivori et al. 1999).

The involvement of NCR in NK recognition of cells of both tumoral and non-tumoral origin is demonstrated by the blocking of NK cell activation using antibodies recognizing one or combinations of NCR (Moretta et al. 2001). However, a number of obstacles prevent a complete understanding of NCR biology. Firstly, there is currently only one murine model for NCR function, the NKp46-deficient mice (Gazit et al. 2006). Indeed, there is no known murine homologue of NKp44, and the *Ncr3* gene for mouse NKp30 is a pseudogene in many mouse strains (Hollyoake et al. 2005). Secondly, the tumor ligands recognized by NCR have not yet been

conclusively determined. NKp46 and NKp44 have been reported to recognize the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus (Arnon et al. 2001; Mandelboim et al. 2001). This interaction requires the sialylation of NKp46 and NKp44 (Arnon et al. 2004). These findings are supported by the mortality of Ncr1(NKp46)-deficient mice upon influenza virus infection (Gazit et al. 2006). In addition, NKp30 has been reported to interact with the pp65 major tegument protein of human cytomegalovirus (Arnon et al. 2005). Heparan sulfate proteoglycans have been described as tumoral ligands for NKp30 and NKp46 (Bloushtain et al. 2004; Zilka et al. 2005). However, this finding was not reproducible in other experimental systems (Warren et al. 2005). Therefore the molecular basis of NCR-based NK target recognition remains an open and important question.

2.4.2 *NKG2D*

NKG2D is a receptor for self ligands that are typically found at low levels on normal cells but at higher levels on infected, stressed or transformed cells (Gonzalez et al. 2006; Raulet 2003). The association of induced NKG2D ligand expression with cellular “stress” has led to the hypothesis that NKG2D responds to “danger” signals associated with tumorigenesis. Indeed, the NKG2D ligands, MICA, MICB and Raet1 are often up-regulated by tumor cells (Cerwenka et al. 2000; Diefenbach et al. 2000; Groh et al. 1999). “Danger signals” that are included in the “infectious nonself” mode of immune recognition have been extensively characterized and include the detection of pathogen associated molecular pattern (PAMP) by Pattern Recognition Receptors, such as the Toll-like receptors (TLR) (Janeway and Medzhitov 2002). However, the molecular definition of “danger signals” that are not microbial molecules has been controversial (Gallucci and Matzinger 2001). Agents inducing DNA damage have recently been shown to result in NKG2D ligand expression (Gasser et al. 2005), identifying a non-microbial “danger signal” that results in “stress-induced self recognition.” This mechanism stands not only as a new paradigm in NK cell recognition (Vivier et al. 2002), but more generally as a novel strategy of immune detection.

NKG2D is a member of the NKG2 family of C-type lectins encoded in the human and mouse natural killer gene complexes (NKC) (Trowsdale et al. 2001). Unlike other NKG2 family members, NKG2D forms homodimers and does not associate with CD94. In addition to NK cells, large populations of CD8⁺ $\alpha\beta$ T cells, $\gamma\delta$ T cells and NK1.1⁺ T cells also express NKG2D (Bauer et al. 1999; Diefenbach et al. 2000). NKG2D-based stimulation of NK cells leads to strong activation (Bauer et al. 1999; Cerwenka et al. 2000; Diefenbach et al. 2000; Diefenbach et al. 2001). However, NKG2D gives co-stimulatory signals to most CD8⁺ $\alpha\beta$ T cells and $\gamma\delta$ T cells as effector functions are not induced without a primary activating signal coming from the TCR (Das et al. 2001; Groh et al. 2001; Roberts et al. 2001). Distinct NKG2D-based stimulatory and co-stimulatory activities may be partially due to alternative signaling mechanisms in different cell populations. Human NKG2D and the long splice-variant form of mouse NKG2D (NKG2D-L)

associate with the signaling adaptor, DAP10 (Andre et al. 2004; Diefenbach et al. 2002; Gilfillan et al. 2002; Rosen et al. 2004). DAP10 contains a YxxM motif that binds the p85 regulatory subunit of phosphatidylinositol-3 kinase and Grb2 following phosphorylation (Chang et al. 1999; Wu et al. 1999). In mouse, an additional short splice-variant form of NKG2D (NKG2D-S) is able to associate with both DAP10 and KARAP/DAP12, therefore also triggering ITAM-mediated activation of the Syk-family protein tyrosine kinases. However, most murine T cells lack KARAP/DAP12 expression, thus restricting NKG2D activation to the DAP10 pathway in T cells (Diefenbach et al. 2002; Gilfillan et al. 2002). Ectopic expression of KARAP/DAP12 in murine T cells confers direct stimulatory capacity on NKG2D (Diefenbach et al. 2002; Teng et al. 2005), demonstrating that it is the lack of KARAP/DAP12 expression that prevents full T-cell stimulation through NKG2D. In human, pre-activated T cells, such as IL-15-stimulated T cells, are fully activated by NKG2D ligation in absence of KARAP/DAP12 association, maybe because of very high levels of DAP10 in these cells (Meresse et al. 2004).

The numerous NKG2D ligands have structural homology to MHC class I molecules. In humans, these include MICA, MICB and various RAET1/ULBP molecules, whilst murine H60, MULT1 and Raet1 molecules (also known as Rae1) are mouse NKG2D ligands (Cerwenka et al. 2000; Diefenbach et al. 2000; Groh et al. 1999). Human MICA and MICB molecules are encoded within the MHC on chromosome 6. Human RAET1 (or ULBP) molecules are also encoded on chromosome 6, but outside the MHC. The genes for all the known murine NKG2D ligands, the Raet1 family members (Rae1 molecules), H60 and MULT1, are linked on a region of chromosome 10 that is syntenic with the human RAET1 gene cluster (Radosavljevic et al. 2002; Sutherland et al. 2001). Mouse Raet1 molecules and some human RAET1 molecules have no transmembrane domain, but are instead anchored to the cell surface through glycosylphosphatidylinositol (GPI) linkage (Bacon et al. 2004; Cerwenka et al. 2000; Cosman et al. 2001). All NKG2D ligands have $\alpha 1$ and $\alpha 2$ domains that form an MHC class I-like fold. MICA and MICB also have an MHC class I $\alpha 3$ -like domain, but they do not interact with $\beta 2$ -microglobulin (Li et al. 1999). The crystal structures of single and NKG2D-complexed ligands suggest that NKG2D ligands are unlikely to have peptide-binding grooves (Holmes et al. 2002; Li et al. 1999, 2001, 2002; Radaev et al. 2001). Despite the large molecular differences between NKG2D ligands, in all cases NKG2D binding occurs over their α -helical surfaces in a manner analogous to TCR-MHC class I/peptide interactions (Li et al. 2001, 2002; Radaev et al. 2001).

2.4.3 CD16

CD16 (Fc γ RIII) is the low-affinity IgG Fc receptor involved in antibody-dependent cellular cytotoxicity (ADCC) of antibody coated (opsonized) target cells. The gene for CD16 expressed by human NK cells (FCGR3A) is also expressed by subsets of mononuclear phagocytes and by $\gamma\delta$ T-cell subsets, whilst polymorphonuclear neutrophils (PMN) express *FCGR3B*. Peripheral blood human NK cells can be

divided into CD56^{bright} and CD56^{dim} subsets comprising around 5% and 95% of the circulating NK cell pool. The large majority of blood CD56^{dim} NK cells expresses CD16 and CD56^{bright} cells exhibit low level or negative staining (Cooper et al. 2001). A CD16 positive phenotype is therefore predominant in the peripheral blood NK cell population, but this situation is not necessarily reflected in other tissues. For example, most decidual, lymph node, and skin NK cells lack the expression of CD16 (Ebert et al. 2006; Ferlazzo et al. 2004; King et al. 1997).

FcγRIIIA/CD16, like NKp46 and NKp30, associates with FcRγ and CD3ζ and uses these ITAM-bearing adaptors for signal transduction. As with other NK activating receptors including the NCR, NKG2D and activating MHC class I receptors, triggering of CD16 on IL-2 activated NK cells leads to cytotoxicity against target cells (Bryceson et al. 2005b). Fresh NK cells are also activated by anti-CD16 antibody stimulation, resulting in calcium flux or production of the cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ upon CD16 cross-linking, or cytotoxic granule degranulation and cytotoxicity in the context of a cellular target (Bryceson et al. 2005b). Interestingly, the triggering of CD16 in apparent isolation by insect cells (which are not thought to express ligands for mammalian NK receptors) coated with rabbit immunoglobulin, leads to degranulation of fresh, otherwise unstimulated, human NK cells (Bryceson et al. 2005a). The high reactivity of CD16 may reflect a physiological requirement of IgG antibodies that have been produced in accordance with rigorous tolerance mechanisms of the adaptive immune system (Bryceson et al. 2005b).

Animals deficient for FcγRIII lack NK cells that can perform ADCC. Other deficiencies in phagocytosis and mast cell degranulation also exist in these mice, and the immunological effects of this deficiency are not purely NK cell mediated (Hazenbos et al. 1996). A number of polymorphisms of FCGR3A exist in the human population (Orange 2002). The L48→H polymorphism has been linked in a few cases to susceptibility to herpes virus infections, though NK cells from individuals homozygous for the 48H allele have normal NK cell ADCC (de Vries et al. 1996; Jawahar et al. 1996). The affinity of 48H CD16 for IgG is frequently higher than the 48L form due to linkage of this polymorphism with another 158V/F FCGR3A polymorphism that directly influences IgG binding (de Haas et al. 1996; Koene et al. 1997). The 158V/F polymorphism also influences the clinical sensitivity to cell-depleting therapeutic antibodies, implicating NK cell ADCC as a potentially important mechanism of their action (Dall'Ozzo et al. 2004).

2.4.4 Activating Homologues of Inhibitory MHC Class I Receptors

The KIR, Ly49, and NKG2 families have activating members that lack the ITIM in the cytoplasmic tail and instead associate with the ITAM-containing signaling adaptor, KARAP/DAP12. These activating receptors are often highly homologous

to particular inhibitory receptors in their extracellular domains. The existence of these activating and inhibitory isoforms is a feature of most (if not all) ITIM-bearing receptors (Vély and Vivier 1997). Direct binding of the “activating homologue” to the inhibitory receptor’s MHC class I ligand has been demonstrated for certain KIR and CD94/NKG2C (Biaassoni et al. 1997; Stewart et al. 2005; Vales-Gomez et al. 1998, 1999, 2000). In addition, the stimulatory murine receptor Ly49D gives activating signals in response to H-2D (George et al. 1999). Phylogenetic analysis of activating *KIR* and *Ly49* suggests that these genes are constantly evolving from the extracellular domains of inhibitory homologues which, through processes of gene duplication and recombination, combine with “activating” transmembrane and cytoplasmic domains (Abi-Rached and Parham 2005). Notably, the affinities of activating interactions are lower than those of inhibitory counterparts. The murine activating Ly49 molecules Ly49H and Ly49P have roles specific to infection with murine cytomegalovirus (MCMV). Ly49H directly binds the product of the MCMV gene m157, and control of infection in some strains of mice is critically dependent upon Ly49H (Arase et al. 2002; Brown et al. 2001; Lee et al. 2001, 2003b; Smith et al. 2002). Ly49P is also involved in recognition of MCMV. However, the recognition of MCMV-infected cells by Ly49P is MHC-restricted, being dependent upon the presence of H-2Dk molecules on the host cell (Desrosiers et al. 2005). The human activating receptor KIR2DS4 has also been reported to bind a non-MHC class I ligand expressed by melanoma cells (Katz et al. 2004).

Except in the context of mouse infection with MCMV infection, the functions of the numerous activating Ly49 and KIR are still incompletely understood. Genetic epidemiologic studies have given some clues. *KIR* and *Ly49* are highly polymorphic. *KIR* haplotypes contain variable numbers of genes with most of this variation relating to the activating *KIR* genes present (Hsu et al. 2002). Individual *KIR* genes are also highly polymorphic with certain allelic polymorphisms are known to affect ligand binding (Carr et al. 2005; Gardiner et al. 2001). A growing number of genetic disease-association studies show roles for *KIR* genes in susceptibility or resistance to disease (Carrington and Martin 2006; Rajagopalan and Long 2005). In particular, activating *KIR* genes have been linked to susceptibility to a number of autoimmune conditions, including psoriatic arthritis, psoriasis, rheumatoid arthritis, scleroderma and type I diabetes (Martin et al. 2002b; Momot et al. 2004; Nelson et al. 2004; Suzuki et al. 2004; van der Slik et al. 2003; Williams et al. 2005; Yen et al. 2001). In addition, activating *KIR* might be beneficial against some microbial infections, as the presence of *KIR3DS1* and *HLA-Bw4* genes is associated to better clinical evolution in HIV-infected individuals (Martin et al. 2002a).

2.4.5 Natural Killer Cell Co-stimulatory and Adhesion Molecules

In addition to cell surface molecules that are coupled to specialized transduction subunits, NK cells express a number of cell surface molecules that are included in the “NK cell zipper” and participate in adhesion and co-stimulatory functions (Fig. 4a, b).

2.4.5.1 The CD2 Family

The CD2 family is a family of immunoglobulin-superfamily molecules with homology to CD2 that are broadly expressed on cells of the immune system. Natural killer cells express CD2, CD48, CD58 (LFA-3), 2B4 (CD244), CD229 (Ly9), CS1 (CD2 subset 1; CRACC, CD2-like receptor activating cytotoxic cells), and NTB-A (NK-T-B antigen) (McNerney and Kumar 2006; Nichols et al. 2005). A small population of NK cells expresses CD150 (or SLAM, signaling lymphocyte activation molecule) following activation (Sayos et al. 2000). All CD2 family members have an N-terminal Ig V-type domain followed by a C-terminal Ig C2-type domain. For most, these are single copy domains and are followed by a transmembrane domain. However, CD48 and one form of CD58 are GPI-linked and CD229 has two pairs of Ig V-type and Ig C2-type domains (McNerney and Kumar 2006; Nichols et al. 2005).

Members of the CD2 family perform signaling functions through a variety of mechanisms. CD2 has a large proline-rich cytoplasmic domain that interacts directly with src family kinases. Members of the CD150 subfamily of CD2 family members (including 2B4, CD229, CS1, NTB-A, and CD150) have a number of copies of the TxYxxV/I immunoreceptor tyrosine-based switch motif (ITSM) in their cytoplasmic tails. ITSM can perform either stimulatory or inhibitory functions. They can associate with signaling molecules classically involved in ITAM and ITIM-based signaling, but they also associate with the Src homology 2 (SH2) containing proteins SH2 domain-containing gene 1A (SH2D1A; also SAP, SLAM-associated protein), and Ewing sarcoma-activated transcript-2 (EAT-2) (Veillette and Latour 2003). Importantly, mutations in SH2D1A cause X-linked lymphoproliferative disorder (XLP). XLP is a fatal progressive variable combined immunodeficiency in which symptoms associated with aberrant proliferation of lymphocytes and macrophages appear upon Epstein–Barr virus (EBV) infection. Disease manifestations include fulminant infectious mononucleosis, lymphomas and hypogammaglobulinemia (Nichols et al. 2005). The other CD2 family members, CD48 and CD58, are believed to have principally ligand function. However, CD48 engagement by 2B4 has been reported to stimulate NK activity and signaling may occur through the GPI-linkage-based association of this molecule with lipid microdomains (Assarsson et al. 2004; Messmer et al. 2006).

CD2 binds CD58 in humans and CD48 in mice (McNerney and Kumar 2006). The CD2-CD58 interaction is involved in activating human NK cells. Transfection of targets with CD58 induces killing by human NK clones and NK redirected lysis can be induced using antibody against CD2. Conversely, activation of NK cells can be blocked using antibodies against CD58 (Lanier et al. 1997). However, CD2-deficient mice have no major defect in NK cell function, possibly due to a redundancy in receptor function (McNerney and Kumar 2006).

2B4 (CD244) binds CD48. The expression of CD48 on target cells and its recognition by 2B4 leads to inhibition of NK cytotoxicity (Lee et al. 2004b). On the contrary, 2B4–CD48 interactions between cells of the same population, i.e. NK cell–NK cell interactions or T cell–T cell interactions, leads to enhanced activation

(Lee et al. 2003a). The bimodal function of 2B4 may be linked to its use of the adaptor SH2D1A, as this is required for NK cell activation and in its absence, 2B4 can mediate inhibitory signaling (McNerney and Kumar 2006). In the mouse, CD48 is the shared ligand of both CD2 and 2B4. 2B4 and CD48 are both important in IL-2 driven proliferation of NK cells along with NK cytotoxicity and cytokine production, whereas CD2 is apparently redundant (Lee et al. 2006), suggesting that 2B4/CD48 interaction is the more physiologically important.

The other CD2 family members expressed by NK cells, CD229, CS1 and NTB-A, all perform homophilic interactions and activate human NK cell cytotoxicity (McNerney and Kumar 2006; Stark and Watzl 2006).

2.4.5.2 Nectin and Nectin-Like Receptors

DNAM-1 (CD226), Tactile (CD96) and class I-restricted T-cell associated molecule (CRTAM) are a group of Ig-family NK cell receptors that bind Nectin and Nectin-like (Necl) adhesion molecules. DNAM-1 is expressed by most NK cells, T cells, monocyte, platelets and some B cells (Kojima et al. 2003; Shibuya et al. 1996), and recognizes Necl-5 (poliovirus receptor, PVR; CD155) and Nectin-2 (CD112) (Bottino et al. 2003; Tahara-Hanaoka et al. 2004). Tactile is expressed by NK cells, T cells and some B cells and binds Necl-5 (Fuchs et al. 2004). CRTAM is expressed by NK cells and T cells following activation and recognizes Necl-2 (also tumor suppressor in lung cancer-1, TSLC1) (Boles et al. 2005).

DNAM-1 triggers NK cell cytotoxicity and its interaction with Necl-5 and Nectin-2 may be critical in regulation of NK cell lysis and adhesion to target cells (Bottino et al. 2003; Shibuya et al. 1996). Tactile and CRTAM also mediate strong adhesion and stimulate cytotoxicity (Boles et al. 2005; Fuchs et al. 2004). Nectins and Necls are expressed by epithelial cells, mediating cell-cell adhesion (Sakisaka and Takai 2004), and also by APCs (Boles et al. 2005; Pende et al. 2006). Additionally, DNAM-1 ligands are over-expressed by certain types of tumor providing a possible recognition structure for NK cell tumor recognition (Castriconi et al. 2004; Pende et al. 2005; Tahara-Hanaoka et al. 2006).

2.4.5.3 Integrins

Mature NK cells express both $\beta 1$ and $\beta 2$ integrins. These are critical in NK cell-target adhesion, NK cell-matrix interactions and NK cell effector functions (Helander and Timonen 1998). The engagement of the $\beta 2$ integrin LFA-1 (CD11a/CD18) on NK cells by antibody cross-linking leads to src tyrosine kinase activation, Vav1 phosphorylation, and MAP kinase activation (Perez et al. 2004). The ligand for LFA-1, ICAM-1 (CD54), either plate-bound or expressed by *Drosophila* insect cells, permits binding of fresh, unstimulated NK cells in a signaling-dependent manner. This binding through the interaction of LFA-1 with ICAM-1 is enhanced following brief activation of NK cells with IL-2 or IL-15 or in concert with CD2/

CD58 or 2B4/CD48 interaction (Barber and Long 2003). In addition to adhesion function, LFA-1-based signals lead to cytotoxic granule polarization in resting NK cells and cytolytic activity of activated NK cells against ICAM-1 expressing insect cells (Barber et al. 2004; Bryceson et al. 2005a). Therefore $\beta 2$ integrin signaling plays an important and complex role by mediating multiple aspects of NK cell adhesion and effector functions.

2.5 Coordination of NK Cell Activating and Inhibitory Signals in Cancer

2.5.1 Cancer Immunosurveillance

The cancer immunosurveillance hypothesis, as originally proposed by Ehrlich in 1909 and later extended by Burnet and Thomas, stated that the immune system acts to recognize and remove newly arising transformed cells. Though initially focusing on a role for the adaptive immune system in preventing carcinogenesis, the hypothesis has been extended to encompass the concepts of innate immune recognition and immune tolerance mechanisms (Smyth et al. 2001b). The validity of the cancer immunosurveillance hypothesis has been and still remains contested (Dunn et al. 2002; Willmsky and Blankenstein 2005). However, a wide range of data from genetic models to clinical observations supports a role for the immune system in cancer regulation.

Carcinogenesis is a multi-step process and multiple natural regulatory mechanisms can prevent the generation of cancer (de Visser et al. 2006; Jakobisiak et al. 2003). The six “hallmarks of cancer” that distinguish tumors from normal cell populations are “self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis” (Hanahan and Weinberg 2000). Each of these features represents a cellular or physiological tumor-defense mechanism that has been breached. It has been estimated that in humans at least four to six mutations or epigenetic changes are required to bypass these checks (Hahn and Weinberg 2002). It is therefore important to consider any role for the immune system in the regulation of cancer within this framework of carcinogenesis, both with respect to other mechanisms that prevent tumorigenesis, and in how the immune system might recognize tumors.

Definitive evidence for cancer immunosurveillance is difficult to obtain because observation of successful cancer immunosurveillance of newly arising tumors is almost invisible. Evidence therefore comes from immunocompromised animals in which immune defects can be correlated with increased incidences of both spontaneous and carcinogen-induced tumors. These studies have the caveat that indirect effects of the immunosuppression, in particular those caused by pathogen infection, e.g., *Helicobacter pylori*, may be influencing cancer incidence. Important additional evidence therefore comes from the higher immunogenicity (and therefore lower

success upon tumor transfer) of tumors originating in immunocompromised animals than in wild-type animals, suggesting that such tumors would be subject to immune control in a wild-type situation (Dunn et al. 2002).

Recently the concept of tumor immunosurveillance has been extended to include the capacities of the immune system to shape or modulate, ignore, or even assist in stages of tumor development. The “cancer immunoediting” hypothesis proposed by Dunn and colleagues highlights three potential interactions between a tumor and the immune system: elimination, equilibrium and escape (Dunn et al. 2004a). Elimination represents the original concept of cancer immunosurveillance, whereas equilibrium and escape respectively describe an immune system’s ability to control but not destroy certain tumors and a tumor’s capacity to finally evade this control. Support for an equilibrium phase comes from a few transplantation cases. In these cases, a tumor was believed to have been eliminated from an organ donor, but the same tumor then reappeared in an organ recipient undergoing pharmacological immunosuppression, suggesting that the tumor had been controlled in a latent state before transplant (Dunn et al. 2004b). Escape is thought to occur through the loss of molecules on which immune control is based. Alternatively, an immune system may be tolerant to a tumor. In a model of spontaneous tumorigenesis in immunocompetent mice, Willmsky and Blankenstein found that highly immunogenic tumors developed, despite the induction of tumor antigen-specific CD8⁺ T cells and antibody responses, due to immune tolerance associated with non-responsive cytotoxic T cells (CTL), high serum levels of tolerogenic TGF- β and low serum levels of pro-inflammatory IFN- γ (Willmsky and Blankenstein 2005). Furthermore, under some conditions, the actions of the immune system may exacerbate carcinogenesis (de Visser et al. 2006). Agents causing chronic inflammation are known to predispose to cancer. For example, *Helicobacter pylori* is linked to gastric cancer and the hepatitis B and hepatitis C viruses are linked to chronic hepatitis and hepatocellular carcinoma (Balkwill et al. 2005; O’Byrne and Dalglish 2001). Amongst other factors, the inflammatory environment provides growth factors, reactive oxygen species that promote DNA-damage and cyclooxygenase enzymes that promote angiogenesis, all of which could contribute to carcinogenesis. The interplays between the immune system and carcinogenesis are therefore multiple and complex.

2.5.2 Natural Killer Cell Recognition of Transplanted Tumors

In addition to their ability to kill tumor cells in vitro, NK cells are involved in the in vivo rejection of certain transplanted tumors dependent upon the NK ligands expressed by the tumor. The best-known experiment involving NK cell elimination of transplanted tumor cells was Kärre and colleagues demonstration of missing-self recognition (Kärre et al. 1986). This experiment demonstrated that mutagenized lymphoma cells (RMA), but not derivatives selected for the loss of MHC expression (RMA-S), are able to grow following sub-cutaneous injection in syngeneic

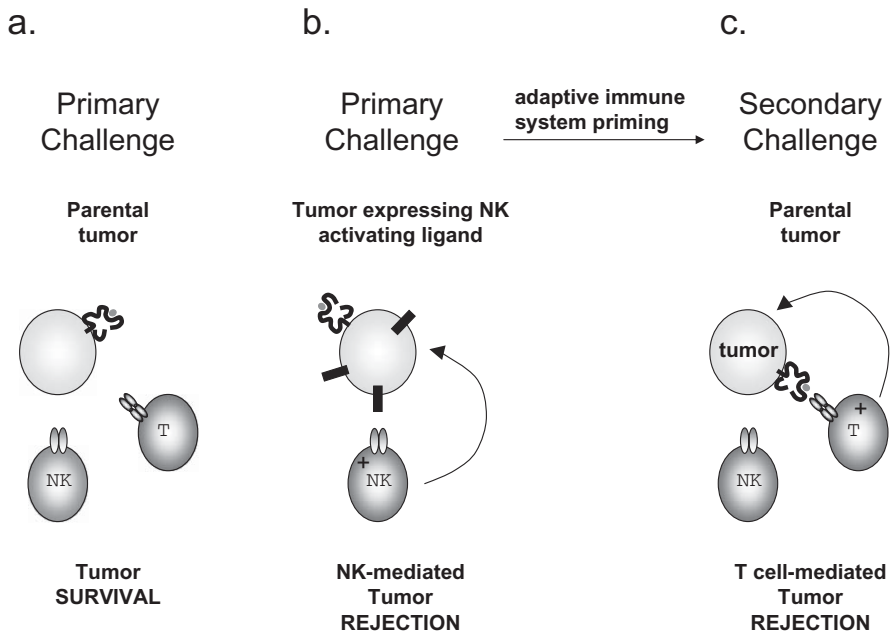


Fig. 5a–c. Natural killer recognition of tumors leads to tumor rejection and adaptive immunity against the tumor cells. (a) A parental tumor cell line is resistant to NK and T cells. (b) Expression of an activating NK cell ligand on the tumor leads to tumor rejection. (c) Following rejection of the tumor in (b), hosts are able to reject the parental tumor line through T cell-mediated adaptive immunity

mice. The selection of RMA for loss of MHC expression had rendered these cells sensitive to NK cell mediated cytotoxicity. As detailed previously, the molecular details of this missing-self recognition are now well established.

The roles of a range of NK activating receptors in tumor rejection have been studied in similar tumor-transplant systems. Amongst these, NKG2D is the only “primary” activating receptor studied to date. Ectopic expression of multiple NKG2D ligands, including Rae1 β , H60, Rae1 δ and Rae1 γ , on RMA tumor cells facilitates their rejection from syngeneic mice (Fig. 5) (Cerwenka et al. 2001; Diefenbach et al. 2001). This NKG2D ligand-mediated tumor response occurs for other cell types including the B16-BL6 melanoma cell line, and in addition to preventing subcutaneous or intraperitoneal tumor growth, it can reduce the number and/or growth of lung metastases (Diefenbach et al. 2001). As NKG2D is expressed by $\gamma\delta$ T cells and CD8 $^{+}$ $\alpha\beta$ T cells in addition to NK cells (Bauer et al. 1999; Diefenbach et al. 2000), any NK-mediated antitumor effect required precision. Diefenbach and colleagues showed that in RAG-deficient animals lacking T cells, NKG2D ligand-expressing tumors were still rejected, but this rejection was prevented by NK cell-depletion using anti-NK1.1 antibodies. However, the growth of

NKG2D ligand-expressing tumors in these NK cell and T cell-deficient animals was still retarded compared with controls, possibly due to incomplete NK cell depletion or alternatively due to the action of other cells such as macrophages (Diefenbach et al. 2001). Cerwenka and colleagues' approach to confirm the role of NK cells was similar in its use of anti-NK1.1 antibodies, additionally showing that certain NK1.1 expressing T-cell populations (invariant NKT cells) were not relevant by the use of animals deficient in CD1 (against which these T cells are restricted) (Cerwenka et al. 2001). In addition, under some experimental conditions (Diefenbach et al. 2001; Westwood et al. 2004), but not others (Cerwenka et al. 2001), the rejection of NKG2D ligand-expressing tumors leads to antitumor immunity against the NKG2D ligand-negative parental cell lines (see Fig. 5). In summary, these studies effectively demonstrate that expression of NKG2D ligands can contribute to NK cell-mediated innate immune responses against transplanted tumors.

NKG2D may not be sufficient to mediate antitumor innate immune responses. The EL4 and RMA tumor lines used in these studies are precisely those tumors used in the demonstration of missing-self recognition (Kärre et al. 1986), indicating that these tumors express stimulatory ligands for NK cells. As the lack of staining of these cells with recombinant NKG2D (Cerwenka et al. 2001; Diefenbach et al. 2001) indicates they do not express high levels of NKG2D ligands, their rejection in the context of missing-self is probably due to other NK activating receptors. Additionally, there may be other receptors for NKG2D ligands (Kriegeskorte et al. 2005).

A number of co-receptor and ligand pairs have been studied in similar tumor rejection systems, using NK or T-cell depletion to confirm roles for NK cells. The expression of the DNAM-1 ligands, CD155 and CD112, by RMA tumor cells leads to increased rejection and better survival of transplanted animals. Depletion of CD8⁺ cells or NK1.1⁺ cells with antibodies implicated both cell types in this DNAM-1-mediated rejection (Tahara-Hanaoka et al. 2005). In other models, expression of either CD70 or CD80 on tumor cells results in more efficient rejection of the tumor by syngeneic mice (Kelly et al. 2002a,b). CD70 is recognized by CD27 that is constitutively expressed by most murine NK cells, whilst CD80 (also known as B7.1) is recognized by CD28 and CD152 (CTLA-4). However, on human NK cells, a receptor other than CD28 may be involved in recognition of CD80 (Wilson et al. 1999). Tumor rejection in these models is dependent upon NK cells as suggested by anti-asialo-GM1 antibody-depletion of NK cells (Kelly et al. 2002a,b). An additional T cell-dependent component of rejection is shown by the higher tumor incidence in RAG-deficient animals. Support for direct interaction between tumor-expressed CD70 or CD80 and NK cells and its influence on tumor rejection is given by the NK cell-dependent reduced growth of CD70-transduced or CD80-transduced tumors in RAG-deficient animals. Interestingly, the immune responses against RMA-S expressing either CD70 or CD80 led to adaptive immunity against the parental RMA tumor line in a similar manner to that seen upon NKG2D ligand transduction (see Fig. 5) (Diefenbach et al. 2001; Kelly et al. 2002a,b). The priming of adaptive immunity was blocked if NK cell or IFN- γ depletion was performed

by antibody administration, demonstrating a crucial role for NK cells in T-cell priming (Kelly et al. 2002a,b).

2.5.3 Natural Killer Cell Recognition of Spontaneous and Induced Tumors

Roles of NK cells as mediators of cellular innate immunity and regulators of adaptive immune responses make them pertinent candidates as effectors of cancer immunosurveillance. In human, an 11-year follow-up epidemiologic survey conducted on 3625 individuals has shown that the level of NK cell activity in peripheral blood is associated with reduced cancer risk, whereas low activity is associated with increased cancer risk (Imai et al. 2000). This important study suggests a role for NK cells against cancer in human. In the mouse, initial evidence for a role of NK cells in tumor immunosurveillance came from beige mice that are deficient in natural cytotoxicity (Talmadge et al. 1980). In these mice, higher levels of spontaneous and carcinogen-induced tumors are observed (Haliotis et al. 1985). However, the beige defect results from a generalized defect in the lysosomal secretory pathway and is not restricted to the NK cell compartment. Though alternative models of NK cell deficiency have been reported, a clean model that results in selective deficiency of the NK compartment has been lacking (Kim et al. 2000, 2005; Yokota et al. 1999). The recent innovative use of the NKp46 promoter to selectively target NK cells for depletion will provide an important tool for future studies (Walzer et al. 2007).

Owing to the lack of a selective NK-deficiency model, many studies elucidating the role of NK cells in spontaneous or induced carcinogenesis rely on antibody-mediated depletion of NK cells. Antibodies directed against either NK1.1 or the glycolipid asialo-GM1 are typically used for selective depletion of NK cells, but a number of side effects may also occur. Depletion of NK cells with anti-NK1.1 antibody (PK136) may also affect populations of invariant-TCR ($V\alpha 14J\alpha 281$) NKT cells and other NK1.1⁺ T-cell populations, making interpretation of depletion experiments difficult. For example, in a model of methylcholanthrene (MCA)-induced fibrosarcoma the incidence of tumors in $J\alpha 281$ -deficient mice (which lack NKT cells) was not altered by depletion of NK1.1⁺ cells. This contrasts with wild-type mice in which depletion of NK1.1⁺ cells leads to an increase in fibrosarcoma incidence, suggesting that NK1.1⁺ cell-based tumor immunosurveillance requires NKT cells and may be NK cell independent (Smyth et al. 2000a). In some models, depletion using antibody against asialo-GM1 may be a more selective means of NK cell depletion. Expression of asialo-GM1 is higher on NK cells than NKT or T cells and NK cell depletion with antibody against asialo-GM1 does not always drastically affect the size or function of the NKT-cell population (Smyth et al. 2001a). Using this calibrated approach of NK depletion Smyth and colleagues showed that both NK cells and NKT cells are important in control of MCA-induced fibrosarcoma. Antibody-mediated depletion of asialo-GM1⁺ (selective for NK) cells, NK1.1⁺ (NK and NKT) cells, or genetic deficiency in NKT cells ($J\alpha 281$ -deficient mice) or T cells (RAG1-deficient mice), all lead to increased incidence of

induced tumors (Smyth et al. 2001a). Although asialo-GM1 expression may be selective for NK cells over invariant NKT cells, other cell populations including activated macrophages and both naïve and virus specific CD8⁺ T cells also express asialo-GM1 (Lee et al. 1996; Slifka et al. 2000). Caution is therefore required when interpreting studies based upon antibody depletion both because of the lack of specificity of antibody treatment against NK populations and because the depletion process itself, through cell surface molecule targeting and destruction of cell populations, may have other effects on the immune system.

In addition to a generic role for NK cells in tumor recognition, the roles of individual NK cell activation mechanisms and effector functions in protection against spontaneous and induced tumors have been addressed using a number of genetic or depletion models. A role for NK cell-mediated cytokine production is supported by analysis of IFN- γ signaling-deficient animals, one for direct NK cell cytotoxicity is supported by perforin-deficient mice and a role for inducers of apoptosis is suggested by models of TRAIL deficiency. In addition, means of NK cell activation through the NKG2D receptor and through type I IFN are also implicated as pathways of NK cell immunosurveillance.

2.5.3.1 Interferon- γ

NK cells and T cells are major producers of IFN- γ , and IFN- γ produced by NK cells and NKT cells plays an important role in antitumor immunity (Hayakawa et al. 2001). Selective deficiency in either IFN- γ or the α chain of the IFN- γ receptor (IFNGR1) results in increased incidences of MCA carcinogen-induced tumors or spontaneous tumors in a number of different models (Kaplan et al. 1998; Shankaran et al. 2001; Street et al. 2001, 2002). Street and colleagues demonstrated that C57BL/6 (B6) mice deficient in IFN- γ (IFN- γ ^{-/-}) or perforin (pfp^{-/-}), but not mice deficient in the cytokines IL-12, IL-18, or TNF, have a higher incidence of spontaneous tumor development than wild-type mice (Street et al. 2002). 50% (16/32) of IFN- γ ^{-/-} B6 mice developed tumors over a 750-day observation period. Whilst most of these tumors from B6 mice were disseminated lymphomas, a similar experiment in BALB/c IFN- γ ^{-/-} mice resulted in the development of spontaneous lung adenocarcinomas and other tumors, but not lymphomas. A previous study by the same group (Street et al. 2001) showed an increased incidence of MCA-induced fibrosarcoma in B6 mice deficient for perforin, IFN- γ , or J α 281 compared with wild-type. Interestingly, the speed of tumor growth was faster when the mice were deficient for IFN- γ , but not in animals deficient for perforin. However, this increased tumor growth was also observed in NKT cell-deficient animals (J α 281^{-/-}) suggesting that NKT cells are mediators of this IFN- γ -dependent growth inhibition. Schreiber's group has investigated the mechanism of IFN- γ -mediated tumor suppression. 129/Sv/Ev p53-deficient animals that are also deficient for the IFN- γ receptor α -chain (p53^{-/-}IFNGR1^{-/-}) develop tumors more rapidly than p53^{-/-} mice. Additionally, a different spectrum of tumor types occurs in these animals. Transplantation of a selection of MCA-induced tumor lines into wild-type or IFN- γ ^{-/-} animals led to equivalent growth of these tumors, suggesting that the IFN- γ signaling deficiency was at the level of the tumor cell and not components of the immune

system. Furthermore, reconstituted expression of the IFN- γ R α chain in one tumor line led to de novo rejection of this line from wild-type mice, suggesting a direct effect of IFN- γ in controlling tumor cell growth (Kaplan et al. 1998). Rejection of the same tumor line could also be induced by over-expression of TAP1 in the tumor cell (Shankaran et al. 2001), suggesting that deficiency in IFN- γ -induction of the MHC class I processing and presentation pathway allowed this tumor to avoid immune surveillance. Tumor rejection was T-cell dependent and led to immunity against the parental tumor cell line. These results suggest that one role of IFN- γ in tumor immunosurveillance is to induce MHC class I expression on the tumor cell itself, supporting the following model: MHC class I-negative tumor cells \rightarrow IFN- γ production by NK cells \rightarrow MHC class I-positive tumors \rightarrow tumor cell recognition by T cells. However, this role is one of many in tumor immunosurveillance, as IFN- γ also induces other tumor suppressor effects, including sensitization to apoptosis, and additional stimulation of immune responses (Farrar and Schreiber 1993; Schroder et al. 2004; Tanaka and Taniguchi 2000).

2.5.3.2 Perforin

Expression of perforin is more restricted than that of IFN- γ , being limited to NK cell and T-cell subsets. An increased incidence of MCA-induced or spontaneous tumor has been well documented for perforin (pfp)-deficient animals (Smyth et al. 2000a,b; Street et al. 2001; van den Broek et al. 1996). Van den Broek and colleagues first described an increased frequency and faster occurrence of MCA-induced fibrosarcomas in pfp-deficient mice (van den Broek et al. 1996). An increased fibrosarcoma incidence was not observed in CD8 $^{-/-}$ animals, suggesting that CD8-negative perforin-expressing cells were responsible for the protection. Importantly, perforin deficiency had no effect on incidence of skin papillomas induced by the application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 7,12-dimethylbenzanthracene (DMBA) to the skin. A tumor-specific susceptibility of perforin-deficient mice was also found with spontaneous tumors occurring in pfp $^{-/-}$ animals (Smyth et al. 2000b). 10 of 20 pfp $^{-/-}$ animals developed lymphomas during late life compared to 1 in 36 of perforin-sufficient controls. An additional experiment using mice deficient in p53 or doubly deficient in both p53 and perforin showed that the effect of perforin deficiency is largely restricted to the control of disseminated lymphomas. p53-deficient animals developed a wide range of tumors, including disseminated lymphomas, sarcomas and thymic lymphomas. Only in the case of disseminated lymphomas was the age of tumor morbidity lower for mice additionally deficient for perforin. The same group later studied the immune responses mediated against such lymphomas upon transfer to tumor-free mice (Street et al. 2004). Primary B-cell lymphomas were derived from C57BL/6 mice deficient in both perforin and β 2-microglobulin. These tumors were rejected from wild-type but not pfp-deficient mice. Additionally, mice deficient for TRAIL, TNF, FasL, IFN- γ , IL-12, or IL-18 rejected these B-cell lymphomas. Through a combination of knockout animals and antibody depletion, the rejection of lymphoma was shown to be mediated by either $\gamma\delta$ T cells or NK1.1 $^{+}$ cells, suggesting that $\gamma\delta$ T

cells and NK cells are each capable of this rejection. Importantly, this experiment was performed using lymphoma cells lacking $\beta 2m$, meaning that CD1d-restricted NKT cells were not involved in the rejection, but also introducing a potential for missing-self recognition by NK cells due to the lack of MHC class I by the lymphomas. Whether the rejection afforded by NK cells was due to missing-self recognition was not addressed.

2.5.3.3 Other Cytolytic Molecules

The granzyme serine proteases found within cytotoxic granules are released into target cells during cytolysis. The prototypical granzymes A and B cooperate with perforin to induce apoptosis associated with DNA-fragmentation and granzyme B leads to caspase activation (Russell and Ley 2002). The functions of multiple other known granzymes have yet to be fully determined. Though these effectors are variously important in the control of virus infections, there is currently no evidence that they are required in tumor immunosurveillance processes (Smyth et al. 2003a).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptotic cell death in various target cells. TRAIL is not generally detectable on freshly isolated NK cells, T cells, NKT cells, B cells, DCs or monocytes. However, a population of mouse liver NK cells, but not human liver NK cells (Ishiyama et al. 2006), expresses TRAIL under normal conditions. Stimulation of NK cells, T cells, DCs or monocytes with various cytokines leads to up-regulation of TRAIL expression (Smyth et al. 2003b). Importantly, NK cells activated through IL-2 or IL-15 treatment (LAK cells) are capable of killing through perforin, FasL and TRAIL-dependent pathways (Kayagaki et al. 1999). TRAIL-deficient mice are more susceptible to MCA-induced fibrosarcoma (Cretney et al. 2002). An increase in MCA-induced fibrosarcomas is similarly observed upon treatment of mice with anti-TRAIL antibodies (Takeda et al. 2002). In $p53^{-/+}$ animals, treatment with either anti-TRAIL antibodies or anti-asialo-GM1 antibodies during MCA-induced tumorigenesis leads to the outgrowth of tumor cells that are more susceptible to TRAIL-mediated lysis than those from mock-treated animals, supporting a role for NK cells in TRAIL-mediated tumor immunosurveillance. Animals treated with both anti-asialo-GM1 and anti-TRAIL antibodies were even more susceptible to MCA-induced tumorigenesis, suggesting that NK cells can also use other effector pathways. However, anti-TRAIL treatment did not affect tumorigenesis in IFN- γ -deficient mice, indicating a requirement for IFN- γ in the protection afforded by TRAIL (Takeda et al. 2002).

2.5.3.4 NKG2D

A role for NK cells in the direct detection of neoplasia is best exemplified by the role of NKG2D in carcinogen-induced neoplasia. C57BL/6 and BALB/c mice

treated with nondepleting blocking antibody against NKG2D have a higher incidence of MCA-induced fibrosarcoma than wild-type controls (Smyth et al. 2005). Perforin-deficient animals were unaffected by anti-NKG2D antibody treatment, indicating that NKG2D function in prevention of neoplasia is dependent upon perforin. However, IFN- γ - and TRAIL-deficient animals were more susceptible to MCA if treated with anti-NKG2D antibody. A similar dependence of NKG2D-mediated antitumor effects on perforin but not IFN- γ or TRAIL was previously demonstrated in models of metastasis affected by cytokine treatment (Smyth et al. 2004). Strong support that NKG2D mediates its effects in direct recognition of tumor cells was provided through examination of NKG2D ligand expression on tumors derived from MCA-treated mice. Most tumors derived from wild-type mice were Rae1 negative (10/16), whereas all tumors (7/7) from perforin-deficient animals all had medium to high expression of Rae1 (Smyth et al. 2005). Furthermore, transfer of a Rae1 positive tumor to recipients resulted in rejection that was dependent upon NKG2D and perforin, suggesting that direct NKG2D-based recognition of tumor cells acts as a protective mechanism against cancer. A simple role for NK cells in this NKG2D-based recognition was not confirmed as blocking NKG2D did not affect the incidence of MCA-induced fibrosarcoma in T-cell-deficient animals (RAG-1-deficient). However, fibrosarcoma incidence in NK cell-deficient animals (anti-asialo-GM1-treated) was also refractory to anti-NKG2D antibody treatment (Smyth et al. 2005). It is therefore possible that both NK cells and T cells play non-redundant roles in NKG2D-mediated tumor-suppression.

Roles for other NK recognition pathways are less well understood. NKG2D is not the only murine receptor involved in triggering NK cytotoxicity of targets as some NK-susceptible cell lines, including RMA-S, do not express NKG2D ligand. In addition, NK cytotoxicity of NKG2D ligand-positive cells is not completely abrogated by the addition of antibody against NKG2D (Jamieson et al. 2002). The expression of NKG2D ligand by multiple different tumors indicates that this receptor is not sufficient to mediate antitumor immunity. Type I IFNs may play an important role as these cytokines can be produced by nonhematopoietic cells and they induce activation of NK cytotoxic activity. Deficiency in type I IFN signaling results in increased susceptibility to MCA-induced fibrosarcoma, with hematopoietic cells rather than cancer cells being the relevant targets of type I IFN in protection against tumors (Dunn et al. 2005).

2.5.4 Other Antitumor Innate Effectors

Together, the higher incidence of spontaneous or carcinogen-induced neoplasia in multiple models of NK cell-deficiencies, and the higher immunogenicity of tumors derived within these models, strongly support a role for NK cells in protection against neoplastic disease. Redundancy may also be in place both within pathways of NK recognition through multiple different receptors, and in the NK effector

mechanisms used. For example, RMA-S cells are not killed by perforin-deficient NK cells in short 4 hour assays, but up-regulation of death receptors including Fas on RMA-S occurs during longer incubations and results in killing (Screpanti et al. 2001). Alternatively, different newly arising tumors may be sensitive to different effector mechanisms (Smyth et al. 2004). Models of selective deficiency in the NK cell population are required in order to understand the full extent of NK cell-based control of spontaneous or carcinogen-induced neoplasia. Yet, non-NK cell populations also play a major role in tumor elimination, and in particular in NKG2D-mediated tumor recognition. For example, induction of skin tumors through initiation with DMBA and promotion with TPA is enhanced in mice deficient for $\gamma\delta$ T cells. This tumorigenic treatment with TPA induces expression of the NKG2D ligands H60 and Rae1 both 24 hours after treatment and in resulting papillomas and carcinomas. Additionally, $\gamma\delta$ T-cell killing is sensitive to blocking of NKG2D (Girardi et al. 2001). Unlike MCA-induced tumorigenesis, perforin-deficient animals are not more sensitive to DMBA/TPA-treatment than wild-type mice (van den Broek et al. 1996). Therefore, depending upon the cancer and its tissue location, different immune cell types and different effector mechanisms may be important in immunosurveillance.

Very recently, a population of hematopoietic cells with NK and DC features was described in the mouse (Chan et al. 2006; Taieb et al. 2006). These IFN-producing killer dendritic cells (IKDCs; MHC class II⁺ Ly6C⁻ B220⁺ NK1.1⁺) produce type I IFN, IL-12 and IFN- γ . They kill typical NK target cells using NK-activating receptors such as NKG2D and can present antigen to T cells. Although these cells are still an enigma, their antitumoral function prompts further characterization in mouse and man.

2.6 Programming a “Natural” Killer: Steady-State and Situation-Specific Regulation of NK Effector Functions

Despite their fame as mediators of spontaneous “natural” cytotoxicity (Herberman et al. 1975; Kiessling et al. 1975), fresh unstimulated NK cells from specific pathogen-free mice or healthy human donors are limited in their capacity to kill classical NK targets. For this reason, mouse studies often use polyinosinic polycytidylic acid (poly(I:C)) effector NK cells or lymphokine activated killer (LAK) cells produced by NK culture with IL-2. Similarly, human studies frequently employ IL-2 to observe responses of primed, activated NK cells. These types of stimulation can produce large and divergent changes in NK cell responsiveness and phenotype (Bryceson et al. 2005b; Chiesa et al. 2006). In addition to soluble mediators, direct interactions of NK cells with other cell populations such as DCs, results in major phenotypic changes (Degli-Esposti and Smyth 2005; Moretta et al. 2005; Walzer et al. 2005). The power of cytokine stimulation to activate NK cells suggests that priming may be an important regulator of NK cells in vivo and illustrates that their “natural” effector functions remain highly sensitive to environmental control.

2.6.1 Natural Killer Responsiveness to MHC Class I and Missing-Self

Inhibitory MHC class I receptors have been recently shown to contribute to NK cell education via the recognition of self-MHC class I molecules. As described above, missing-self recognition of MHC class I is mediated by multiple inhibitory receptors that are expressed in a variegated fashion by the NK cell population leading a repertoire of specificity for MHC class I (Raulet et al. 2001). This NK repertoire must remain tolerant to the MHC class I molecules expressed by self cells. One proposed mechanism for self-tolerance was that all NK cells express “at least one” inhibitory receptor for self-MHC class I. This hypothesis was supported by Valiante et al. who found that virtually all NK clones grown from two human donors were inhibited by individual self-MHC class I molecules (Valiante et al. 1997). However, using combinations of antibodies, populations of fresh murine or human NK cells can be identified that do not express any known inhibitory NK receptor (Anfossi et al. 2006; Fernandez et al. 2005). Additionally, in mice and humans who lack normal cell surface expression of MHC class I, NK cells remain tolerant to self, whilst the fraction of the NK pool expressing individual Ly49 or KIR receptors is largely unchanged (Furukawa et al. 1999; Salcedo et al. 1998; Zimmer et al. 1998). The existence of NK cells lacking inhibitory MHC class I receptor expression argues against the “at least one” hypothesis and suggests that Valiante et al.’s results may be peculiar to cloned NK cells (Valiante et al. 1997).

Coincident with their self-tolerance, the NK cells from mice and humans lacking normal MHC class I expression (due to deficiencies in β 2-microglobulin or in TAP) are hyporesponsive to the classical NK cell targets such as YAC-1 and K562 respectively (Furukawa et al. 1999; Liao et al. 1991; Zimmer et al. 1998). However, there is no evidence that this hyporesponsiveness is due to the decreased expression of activating receptors or the increased expression of other inhibitory receptors (Vitale et al. 2002). Additionally, the NK cell hyporesponsiveness can be (at least partially) overcome in vitro by long term stimulation of NK cells with cytokines. Thus, activation of human TAP-deficient NK cells with IL-2, IL-12, or IL-15 renders them responsive against classical NK targets (Furukawa et al. 1999; Zimmer et al. 1998). In addition, IL-2 treatment of TAP2-deficient NK cells allows them to kill autologous B-lymphoblastoid cell lines (B-LCL) or IFN-treated fibroblasts, both cell types being protected from lysis under normal circumstances due to expression of MHC class I (Zimmer et al. 1998, 1999). This break of tolerance might not, however, be universal; as autologous PHA-blasts were not susceptible to lysis by IL-2 activated TAP2-deficient NK cells (Vitale et al. 2002). A break in hyporesponsiveness and/or tolerance can also be achieved through treatment of murine NK cells with IL-2. Natural killer cells from mosaic mice expressing an MHC class I transgene (Dd/Ld) on a subpopulation of hematopoietic cells are tolerant to cells lacking the MHC class I determinant. However, separation of NK cells into Dd/Ld-positive and Dd/Ld-negative populations and culture of these in IL-2, endows the Dd/Ld-positive population of NK cells with reactivity against Dd/Ld-negative

targets (Johansson et al. 1997). Therefore tolerance to an MHC class I-determinant can be broken in its absence.

It has recently been shown that the mechanism resulting in NK cell tolerance to self-MHC class I affects the responsiveness of NK cells *per se*. Specifically, the stimulation of NK cells in a target-cell free system shows a generalized hyporesponsiveness of NK cells that do not express a self-MHC class I-specific inhibitory receptor (Anfossi et al. 2006; Fernandez et al. 2005; Kim et al. 2005a,b). Stimulation of fresh mouse NK cells with plate-bound antibody against NK1.1 (the activating receptor Nkrp1c) led to IFN- γ production by B6 NK cells that express Ly49C, which recognizes H-2K^b present in B6 mice, but not B6 NK cells that express Ly49A, which has no known B6 ligand. The higher responsiveness of NK cells expressing a self-MHC class I specific inhibitory receptor was termed “licensing” and was found to be dependent upon the presence of the correct MHC class I determinant and the ITIM of the Ly49 receptor, but not the PTP SHP-1 (Kim et al. 2005a,b). Similarly, murine NK cells lacking the expression of all known MHC class I-specific inhibitory receptors are hyporesponsive to stimulation with antibody against NKG2D or Ly49D (Fernandez et al. 2005). This phenomenon also extends to human NK cells. Stimulation with antibody against CD16 results in a higher response from NK cells expressing a self-specific inhibitory KIR than NK cells expressing a KIR with no self-ligand (Anfossi et al. 2006). In all these systems, the phenomenon of licensing or education applies to NK cell responses against a variety of cellular targets and other stimuli (Anfossi et al. 2006; Fernandez et al. 2005; Kim et al. 2005). However, the presence of this phenomenon in the absence of target cells rules out the possibility of other inhibitory receptors compensating for the lack of MHC class I-inhibitory receptor interaction. It further suggests that this mechanism of tolerance acts directly on the “programming” of individual NK cells.

Education of NK cells on their MHC class I background may serve to optimize missing-self responsiveness (see Fig. 6). Maturation of NK cells in the context of inhibitory KIR or Ly49 ligation would increase the potential of activating signaling circuits. Natural killer cells would therefore be fully responsive upon subsequent encounter of MHC class I^{dim/-} cells. HLA class I ligands were recently reported to increase the frequencies of NK cells expressing cognate KIR (Yawata et al. 2006), demonstrating an amplification of MHC class I-inhibitory receptor bearing NK cells during a still unknown stage of NK cell maturation. Therefore selective proliferation of educated NK cells could be an additional mechanism providing optimal missing-self responsiveness. Furthermore, the hyporesponsiveness of “uneducated” or “unlicensed” NK cells does not prevent NK function in all circumstances as NK cells from MHC class I-deficient mice are fully protective against mouse cytomegalovirus infection (Tay et al. 1995).

The heightened activation state of MHC class I educated NK cells has implications for our understanding of NK biology in the context of autoimmunity and immunotherapies. Genetic epidemiologic studies have revealed a role for KIR and HLA in the susceptibility or resistance to a variety of pathologies, including autoimmune syndromes, cancer, and infectious diseases (Carrington and Martin 2006).

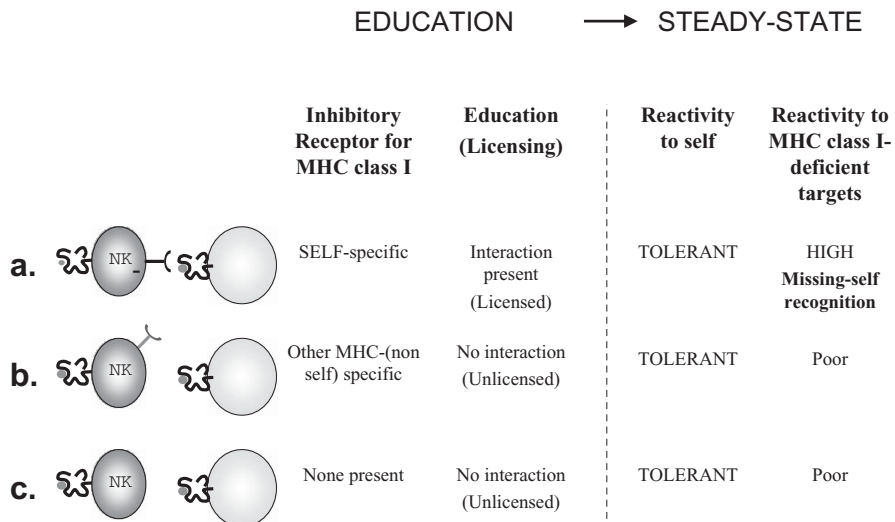


Fig. 6a–c. Natural killer cell (NK) Education (or Licensing) enables effective missing-self recognition. Natural killer cells that express a self-specific inhibitory major histocompatibility (MHC) class I receptor (**a**) are educated to be reactive to cells lacking this particular MHC class I molecule. Natural killer cells lacking self-specific inhibitory MHC class I receptors (**b,c**) do not gain this reactivity during their education and are hyporesponsive. Education processes therefore maintain self-tolerance. It is not yet clear whether NK cell education is a continuous process in vivo, or whether it only occurs at specific stages of NK cell development

The current interpretation of these associations relies on the role of KIR-HLA interaction in the modulation of NK cell effector function during interaction with target cells. One can now suggest that the association between KIR, HLA and human disease might also be the consequence of the role of KIR-HLA in NK cell education. Similarly, pioneering work has shown that donor-versus-recipient NK cell alloreactivity can eliminate leukemia relapse and graft rejection, while protecting patients against graft-versus-host disease (Ruggeri et al. 2002). In this regime and model, it appears that during the first few months post-hematopoietic transplant, donor NK cells develop in the recipient in the same way as they would develop in the donor (Parham 2005). Under certain KIR-HLA donor/recipient combinations, some NK cells become alloreactive to the recipient. If these alloreactive NK cells were instead educated on the MHC class I profile of the recipient, they would be hyporesponsive. The instruction of NK cell education may be dependent upon the recipient’s conditioning regimen as well as the dose of donor hematopoietic progenitors (i.e. the likelihood of interaction with donor or recipient MHC class I molecules), providing a possible basis for the variable outcome of these MHC-mismatched hematopoietic transplantation protocols (Parham 2005).

It is likely that other inhibitory receptors expressed by NK cells contribute to their education through MHC class I-dependent or -independent mechanisms. As

discussed above, a variety of other inhibitory receptors have been described on hematopoietic cells, several of them being expressed at early stages of cell differentiation (Daeron and Vivier 1999; Long 1999; Ravetch and Lanier 2000). Thus, inhibitory receptors, other than MHC class I-specific receptors, might also educate a wider array of hematopoietic cells to discriminate between interacting cells that do, or do not, express self-ligands.

2.6.2 Natural Killer Responsiveness to Activating NK Ligands

Pathways of NK cell activation are also sensitive to tolerogenic mechanisms that may affect their responsiveness in tumor settings. The sensitivity of the NKG2D recognition system is modulated through a number of mechanisms. In addition to membrane bound forms of NKG2D ligands that can stimulate immune system cells, tumor cells may also produce soluble or membrane forms of these ligands that promote the down-regulation of NKG2D on NK and T cells (Coudert et al. 2006; Groh et al. 2002; Salih et al. 2003). Alternatively, transforming growth factor β (TGF- β) can induce down-regulation of the NKG2D receptor (Castriconi et al. 2003; Lee et al. 2004a). TGF- β is produced by some cancers, but is also an important product and mediator of CD4⁺CD25⁺ regulatory T-cell populations.

In vivo, the effect of altered NKG2D responsiveness due to tolerogenic mechanisms is best illustrated by transgenic mice ectopically expressing the Rae1 ϵ NKG2D ligand (Oppenheim et al. 2005). In these mice, ectopic expression of Rae1 ϵ on all cells (conferred by a β -actin promoter) or selectively on epithelial cells (using an involucrin promoter) resulted in lower expression levels of the NKG2D receptor by NK cells and T cells, a reduced capacity of NK cells to kill Rae1 bearing targets, and a higher susceptibility to DMBA and TPA-induced tumors. The in vivo killing of MHC class I-deficient RMA-S targets was also reduced in transgenic mice, suggesting that NKG2D ligand-induced tolerance produces a wider suppression of NK responsiveness. Furthermore, the suppression of NK functions could be partially overcome by poly(I:C) treatment of transgenic mice (Oppenheim et al. 2005).

The suppression of NK cell effector functions by CD4⁺CD25⁺ regulatory T cells (T regs) and TGF- β has been recently described by a number of groups (Barao et al. 2006; Ghiringhelli et al. 2005; Laouar et al. 2005; Smyth et al. 2006). Scurfy mice, which lack the Foxp3 transcription factor necessary for development of T regs, have NK cells that have higher basal activation and are more cytolytic against YAC-1 targets (Ghiringhelli et al. 2005). In addition, the killing of NKG2D ligand-bearing targets is regulated by T reg cells in a TGF- β dependent fashion (Ghiringhelli et al. 2005; Smyth et al. 2006). Interestingly, the presence of T regs in tumors may prevent in vivo rejection of tumors by NK cells, as depletion of T regs can reveal potent NK cell-dependent cytotoxicity against autologous tumor cells (Ghiringhelli et al. 2005).

2.7 Conclusions and Perspectives: NK Cells, “Self Versus Nonself,” and Cancer

How do NK cells discriminate “self” from “nonself”? NK cells can recognize infectious nonself ligands, stress-induced self ligands, and missing-self (Fig. 1). We have discussed above how the recognition of stress-induced ligands and missing-self might be involved in innate immunosuppression mechanisms to control cancer formation. We have also discussed how these NK cell recognition pathways can be modulated by other homeostatic processes such as the effect of T reg cells. Definitive proof of NK cell involvement in cancer immunosurveillance has not yet been obtained due to inherent difficulties. However, the mass of correlative evidence discussed here suggests that NK cells are important mediators of cancer resistance, reinforcing a need for fine dissection of their biology and highlighting potential therapeutic application.

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3

Recent Progress on Paired Immunoglobulin-Like Receptors

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3.1 Introduction

Almost 10 years ago the Takai and Kubagawa laboratories independently identified the paired immunoglobulin-like receptors (PIRs) in mice based on limited homology with the human Fc α receptor/CD89 (Hayami et al. 1997; Kubagawa et al. 1997). Two PIR isoforms were identified on the basis of their signaling properties as activating (PIR-A) and inhibitory (PIR-B) types. *Pir* is a multigene family located on the proximal end of mouse chromosome 7 (Kubagawa et al. 1997; Tun et al. 2003; Yamashita et al. 1998a), in a region syntenic with the human chromosome 19q13 where a cluster of structurally related gene families called the leukocyte receptor complex resides. Among these human genes are the closest PIR homologs, the immunoglobulin (Ig)-like transcripts (ILTs) [also called leukocyte Ig-like receptors (LIRs), monocyte/macrophage Ig-like receptors or CD85; see the new LILR nomenclature at www.gene.ucl.ac.uk/nomenclature/genefamily/lilr.html] (Arm et al. 1997; Barten et al. 2001; Colonna et al. 1999; Cosman et al. 1999; Long 1999; Martin et al. 2002; Wagtmann et al. 1997). Paired immunoglobulin-like receptor-A and PIR-B are cell surface glycoproteins with very similar extracellular regions (>92% homology) containing six Ig-like domains, but with structurally and functionally distinct transmembrane and cytoplasmic regions (see Fig. 1). There are multiple PIR-A isoforms (>6), each encoded by a different *Pira* gene. Paired

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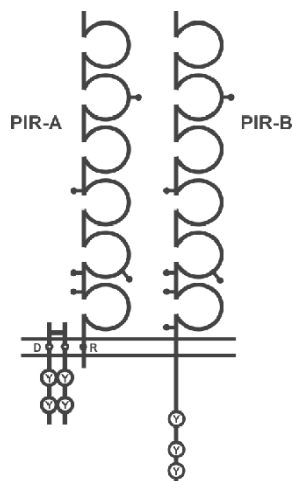
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Fig. 1. Schematic presentation of paired immunoglobulin-like receptor (PIR)-A and PIR-B. Both PIR-A and PIR-B cDNAs encode type I transmembrane proteins consisting of similar extracellular regions with six Ig-like domains, but having distinctive trans-membrane and cytoplasmic regions. The ectodomain has five or six potential sites for N-linked glycosylation (*bars with closed circles*). The predicted PIR-A has a short cytoplasmic tail and a positively charged arginine (R) residue in the transmembrane domain of the disulfide-linked homodimer of the Fc receptor common γ chain ($FcR\gamma$) carrying immunoreceptor tyrosine-based activation motifs (ITAMs). In contrast, the PIR-B protein has a typical uncharged transmembrane region and a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIMs).



immunoglobulin-like receptor-As associate non-covalently with the Fc receptor common γ chain ($FcR\gamma$), a transmembrane signal transducer that contains immunoreceptor tyrosine-based *activation* motif (ITAM) “D/ExxYxxL/Ix₆₋₈YxxL/I” (single amino acid code, where x represents any amino acid) in the cytoplasmic tail, to form a cell activation complex (Kubagawa et al. 1999a; Maeda et al. 1998b; Ono et al. 1999; Taylor and McVicar 1999). In contrast, PIR-B is encoded by a single gene and contains three functional immunoreceptor tyrosine-based *inhibitory* motifs (ITIM) “I/VxYxxL/V” in its cytoplasmic tail, thereby negatively regulating cellular activity via the SHP-1 and SHP-2 tyrosine phosphatases (Bléry et al. 1998; Maeda et al. 1998a; Uehara et al. 2001; Yamashita et al. 1998b). It has been suggested recently that PIR-B may also have an additional SH2-binding motif called the immunoreceptor tyrosine-based *switch* motif (ITSM) “TxYxxV/I”, as characterized in the signaling lymphocytic activation molecule or CD150 protein family (Siderenko and Clark 2003). Paired immunoglobulin-like receptor-A and PIR-B are expressed by many hematopoietic cell types. These include B lymphocytes, dendritic cells (DCs), monocyte/macrophages, granulocytes, mast cells, and megakaryocyte/platelets. Paired immunoglobulin-like receptors are not expressed by T lymphocytes, natural killer (NK) cells or erythrocytes (Kubagawa et al. 1999a), a feature that distinguishes PIRs from human ILTs/LIRs some of which are expressed by T lymphocytes and NK cells as well (Colonna et al. 1999; Cosman et al. 1999; Long 1999; Arm et al. 1997). The cell surface levels of PIR often increase as a function of cellular differentiation, suggesting that PIR is involved primarily in mature cell function. Our recent findings, however, suggest that PIR is also expressed by early hematopoietic progenitors. In addition, several interesting results regarding PIR-B ligands and functions have recently been reported. Since several review articles describing PIR are now available (Kubagawa et al. 1999b; Takai 2005a,b; Takai and Ono 2001), we will focus this discourse on recent findings.

3.2 PIR Expression by Early Hematopoietic Cells

To determine whether PIR is expressed by early hematopoietic progenitors, we examined progenitor populations that were enriched from adult bone marrow based on the lack of expression of lineage markers (Lin^-) as defined by monoclonal antibodies (mAbs) against Ter119, B220, Mac-1, Gr1, CD3, CD4, CD8, and DX5 antigens and the expression of c-kit and Sca-1 antigens. The $\text{c-kit}^+/\text{Sca-1}^+/\text{Lin}^-$ cells, which represent $\sim 0.05\%$ of the mononuclear bone marrow cells, were found to express PIR proteins at variable levels on their cell surface (Fig. 2). Since mAbs discriminating PIR-A and PIR-B are unavailable, the levels of cell surface PIR-A and PIR-B proteins were then determined by comparing the PIR staining intensity on cells among mice deficient for PIR-B ($\text{PIR-B}^{-/-}$) or $\text{Fc}\gamma\text{R}$ ($\text{Fc}\gamma\text{R}^{-/-}$) and wild-type control mice. Since $\text{Fc}\gamma\text{R}$ has been shown to be prerequisite for cell surface expression of most PIR-A family members, $\text{Fc}\gamma\text{R}^{-/-}$ mice are deficient in cell surface PIR-A. The surface PIR intensity on the $\text{c-kit}^+/\text{Sca-1}^+/\text{Lin}^-$ cells was found to be greatly reduced in $\text{PIR-B}^{-/-}$ mice, but not in $\text{Fc}\gamma\text{R}^{-/-}$ mice when compared with wild-type control mice, suggesting the predominance of PIR-B isoform on those early hematopoietic cells. This data is supported by the results of reverse transcription–polymerase chain reaction (RT-PCR) analysis which demonstrated higher levels of PIR-B transcripts in the $\text{c-kit}^+/\text{Sca-1}^+/\text{Lin}^-$ cells. The predominance of inhibitory activity seems a general rule for other pairs of activating and

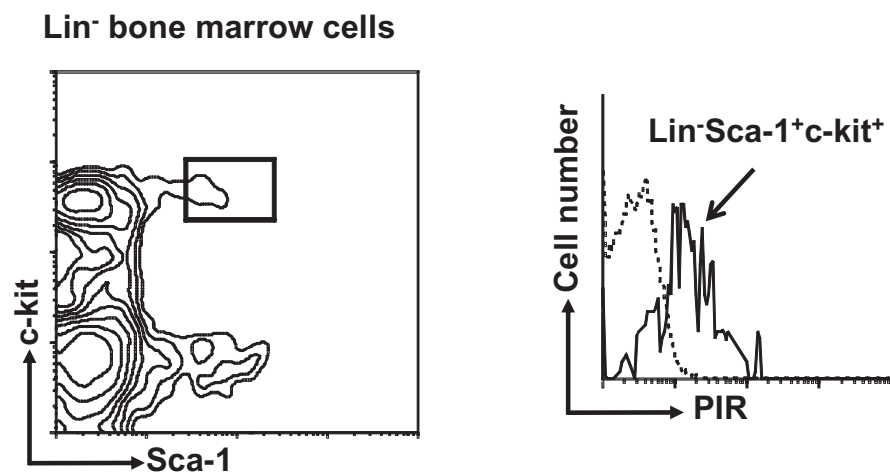


Fig. 2. Identification of PIR^+ progenitors in adult bone marrow. Adult (6 weeks) bone marrow cells were stained with PE-labeled mAbs specific for lineage markers (Lin : Ter119, B220, Mac-1, Gr1, CD4, CD8 and DX5) and anti-PE antibody-coupled magnetic beads before depletion of Lin^+ cells using a magnetic sorter. Enriched Lin^- cells were stained with a combination of FITC-anti-Sca-1, APC-anti-c-kit and PE-anti-PIR mAbs and analyzed by a FACSCalibur. Both c-kit and Sca-1 positive cells (*box*) were examined for PIR expression (*solid lines*). *Dotted lines* indicate the background staining with an isotype-matched control mAb. Note that hematopoietic stem cells express variable levels of PIR on their cell surface

inhibitory receptors (Lanier 2001; Ravetch and Lanier 2000). Like adult bone marrow, a significant fraction of the $c\text{-kit}^+/CD45^+/Lin^-$ hematopoietic progenitors in fetal liver at 11–15 days post coitus (dpc) were also found to express PIR proteins at variable levels (Masuda et al. 2005). A more detailed analysis of the progenitor subpopulations in fetal liver revealed that PIR is expressed by multipotent ($c\text{-kit}^+/Sca\text{-1}^{hi}/Lin^-$) and lymphoid ($c\text{-kit}^+/Sca\text{-1}^+/IL\text{-}7R\alpha^+/Lin^-$) progenitors, and not by myeloid ($c\text{-kit}^+/Fc\gamma RII/III^{hi}/Lin^-$) and erythroid ($c\text{-kit}^+/CD45^+/Lin^-$) progenitors (Masuda et al. 2005). Thus, PIR is selectively (or preferentially) expressed by early hematopoietic cells in adult bone marrow and fetal liver tissues.

3.3 PIR Expression by Thymocyte Progenitors

When adult thymocytes subdivided on the basis of CD4 and CD8 expression were examined for PIR expression, a small subpopulation of the CD4 and CD8 double-negative (DN) cells was found to express cell surface PIR at varying levels.

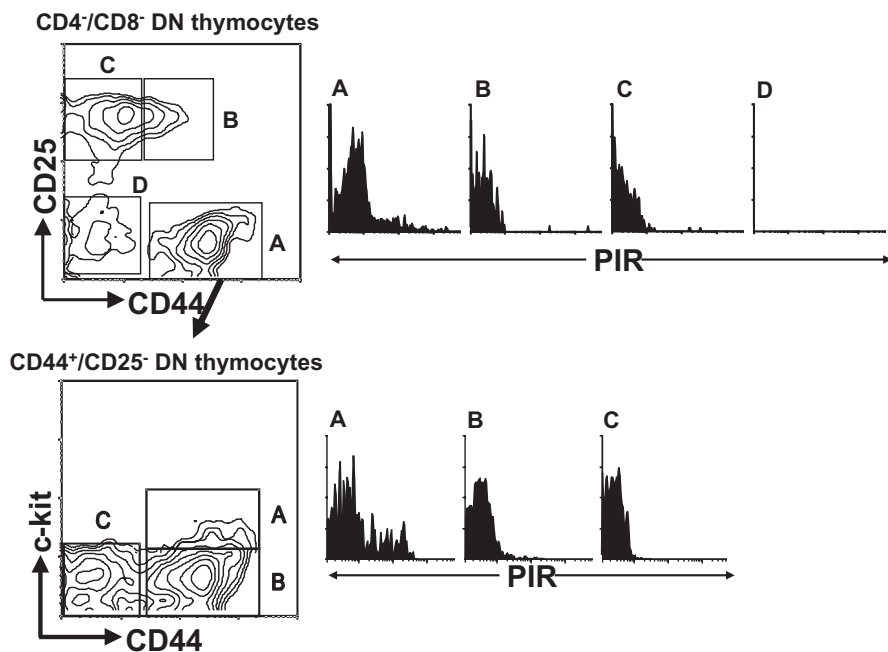


Fig. 3. Identification of PIR⁺ cells in thymus. Adult (6 weeks) thymocytes were stained with PE-labeled mAbs specific for CD4 and CD8 and anti-PE mAb-coupled magnetic beads before depletion of CD4⁺ and/or CD8⁺ cells by a magnetic sorter. Enriched CD4⁻/CD8⁻ DN thymocytes were stained with a combination of fluorescein isothiocyanate (FITC)-anti-CD44, APC-anti-c-kit, Cy5-anti-CD25, and PE-anti-PIR mAbs, and analyzed by a FACSCalibur. Cells in each box (in the left panel) are examined for PIR expression (solid profile) in the right panel. Note that small subpopulations of the CD44⁺/CD25⁻ cells (Fx A in top panel) and the CD44⁺/c-kit⁺ cells (Fx A in bottom panel) express PIR

The PIR⁺ cells were positive for c-kit and CD44 and negative for CD25 (c-kit⁺/CD44⁺/CD25⁻). Thus, they resided in the most immature thymocyte compartment, designated DN1, which includes newly arrived progenitor cells (Fig. 3). A similar predominance of cell surface PIR-B expression was observed for the neonatal DN1 thymocytes (not shown). Remarkably, the great majority of DN1 cells in fetal thymus at 12 dpc expressed PIR proteins at relatively high levels. The cell surface PIR levels on this subset of thymocytes decreased dramatically beginning around 13 dpc, prior to the expression of CD25, which coincided with the early progression phase of newly arrived T-cell progenitors (Masuda et al. 2005). Paired immunoglobulin-like receptor expression is thus down-regulated within the DN1 stage, a finding that has been confirmed using an in vitro culture system with stromal cells expressing the Notch ligands, wherein the PIR⁺/c-kit⁺/CD44⁺/CD25⁻ cells give rise to conventional DN1 cells. Immunohistochemical analysis of fetal and neonatal thymi revealed that PIR⁺ cells are selectively localized in the corticomedullary junction, the site of thymic entry for T-cell precursors. Paired immunoglobulin-like receptor is thus expressed by subpopulations of cells in the most immature compartment of thymocytes. These findings also raise the possibility that down-regulation of the surface PIR-B expression on newly arriving thymocyte progenitors is a prerequisite for their subsequent T-lineage differentiation.

3.4 Differentiation Potential of PIR⁺ and PIR⁻ Lymphoid Progenitors

To define the functional potential of PIR⁺ and PIR⁻ progenitor populations in hematopoietic tissues, we initially focused on the differentiation capability of PIR⁺ and PIR⁻ lymphoid (c-kit⁺/IL-7R⁺/Lin⁻) progenitors in fetal liver by using a high oxygen submersion fetal thymic organ culture for assaying multi-lineage progenitors (MLPs) (Masuda et al. 2005). This MLP assay has proven to be an excellent method for allowing a single progenitor cell to generate different progeny over a 10 day culture period with appropriate cytokine stimulation (Katsura 2002; Kawamoto et al. 1997, 2000). To our surprise, the PIR-positive population was greatly enriched for T-lineage progenitors as evidenced by the generation of progeny including CD4 and CD8 double-negative, double-positive, and single-positive $\alpha\beta$ T cells as well as $\gamma\delta$ T cells (Masuda et al. 2005; see Fig. 4). In a modified MLP assay system the PIR⁺ T-cell progenitors in fetal liver were also capable of giving rise to NK cells and DCs, thereby defining them as common progenitors for T cells, NK cells and DC (T/NK/DC progenitors). The PIR⁺ T/NK/DC progenitors appear to be selectively released from fetal liver into the circulation during 11–14 dpc, as PIR⁻/IL-7R⁺ progenitors are rarely observed in fetal blood. On their entry into the thymus, PIR expression is rapidly down-regulated prior to the onset of CD25 expression.

In contrast to the PIR⁺ progenitor population, the PIR-negative lymphoid progenitors (PIR⁻/IL-7R⁺/Lin⁻) contained B-lineage progenitors as evidenced by their ability to give rise to B220⁺/IgM⁻ pro-B/pre-B-cell as well as IgM⁺ B-cell progeny (Masuda et al. 2005). Notably, these PIR⁻ lymphoid progenitors can give rise to B cells within fetal thymic lobes even without adding exogenous cytokines. The

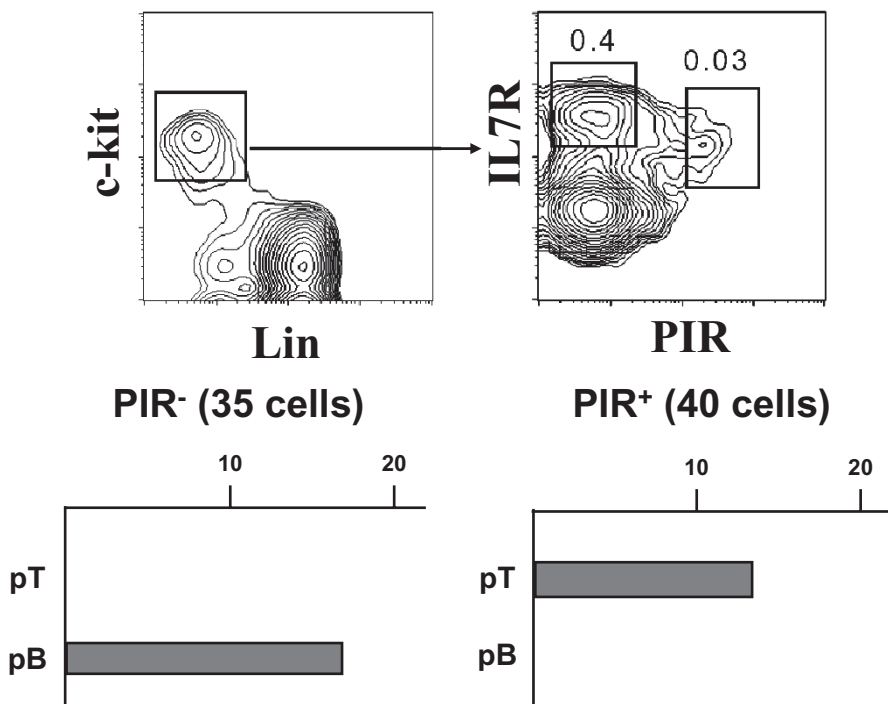


Fig. 4. Differentiation potential of PIR⁺ and PIR⁻ lymphoid progenitors. Fetal (15 dpc) liver cells were stained with FITC-mAbs specific for lineage markers (Lin), APC-anti-c-kit, Cy5-anti-IL-7R and PE-anti-PIR mAbs, and the c-kit⁺/Lin⁻ cells (box in the left panel) were examined for their expression of IL-7R and PIR (right panel). The IL-7R⁺/PIR⁻ cells and IL-7R⁺/PIR⁺ cells were sorted and a single cell was placed in fetal thymic organ cultures for assaying T-lineage or B-lineage committed progenitors (pT or pB). Note that among 35 isolated PIR⁻ lymphoid progenitors, 17 are pB and the remainder are untyped because no progeny were generated. In contrast, among 40 PIR⁺ lymphoid progenitors, 13 are pT and the remainder are untyped

commitment of these cells to the B-lineage differentiation therefore appears to be sustained even under conditions biased toward T-cell generation. The expression profiles of lineage-associated transcription factors correlate well with the differentiation capability of PIR⁺ and PIR⁻ lymphoid progenitors. While the expression levels of Ikaros, PU.1, Gata2 and Gata3 are comparable between the PIR⁺ and PIR⁻ population, transcription factors specific for T-lineage (Tcf-1) and B-lineage (EBF, Pax5, mb-1/Ig α , λ 5) are exclusively detected in PIR⁺ and PIR⁻ lymphoid progenitors, respectively (Masuda et al. 2005). Taken together, these findings indicate that PIR provides an excellent marker for pre-thymic progenitors with potential to give rise to T cells, NK cells and DCs in fetal liver and blood. We are currently conducting similar analyses for adult bone marrow in order to determine whether or not the PIR⁺ progenitors in the fetus and adults have the same hematopoietic potential. More importantly, these findings raise the question of PIR function during the development of hematopoietic cells.

3.5 PIR Expression by Dendritic Cell Precursors

Another unique population of cells expressing PIR was found among early hematopoietic cells when adult bone marrow cells were examined. Approximately two-thirds of the B220⁺/CD19⁻/DX5⁻ cells, which comprise ~0.5% of the nucleated bone marrow cells, were found to express high levels of PIR. The PIR^{hi}/B220⁺/CD19⁻/DX5⁻ cells also expressed CD11c, Ly6C, CD43, and variable levels of CD4 and major histocompatibility complex (MHC) class II (Fig. 5). RT-PCR analysis of the PIR⁺/B220⁺/CD19⁻ cells revealed the coordinate expression of PIR-A and PIR-B transcripts as well as FcR γ c and early B-lineage gene transcripts (e.g., B29/

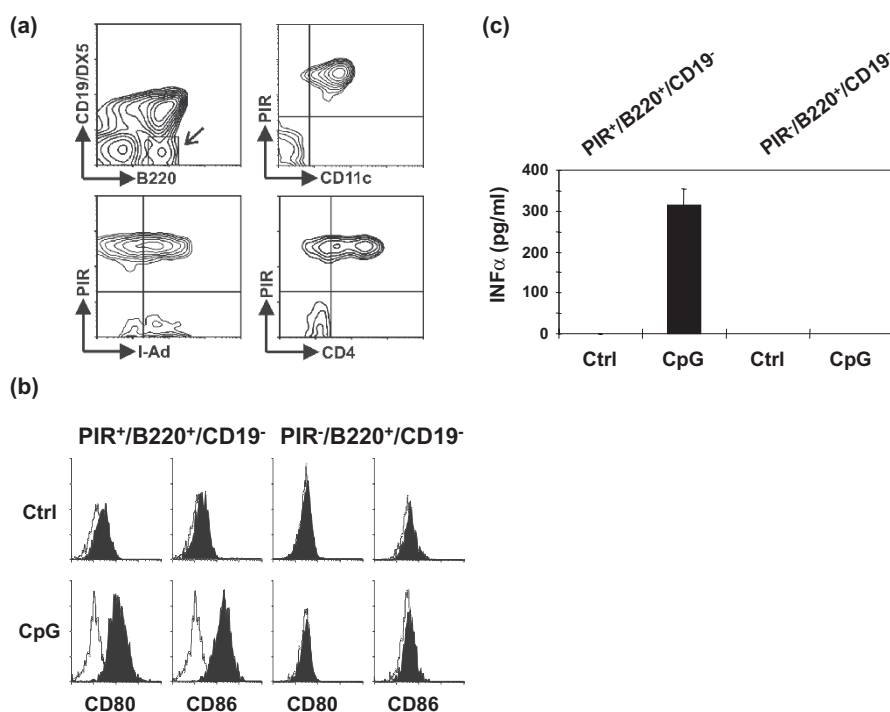


Fig. 5a–c. Paired immunoglobulin-like receptor expression by plasmacytoid DC precursors, pre-DC2. **(a)** Bone marrow nucleated cells were stained with a combination of FITC-anti-CD19, FITC-anti-DX5, Cy-anti-B220, PE-anti-PIR mAbs and APC-labeled mAb specific for CD11c, class II or CD4 before analyzing the B220⁺/CD19⁻/DX5⁻ cell population. Note that B220⁺/CD19⁻/PIR⁺ cells are positive for CD11c and express variable levels of class II and CD4. **(b)** The PIR⁺/B220⁺/CD19⁻ and PIR⁻/B220⁺/CD19⁻ subpopulations were sorted and cultured for 24 h in the presence or absence of CpG oligodeoxynucleotides. To compensate for DNase-mediated degradation, 10 μ l of 100 μ M CpG oligo were added at multiple time points during culture. The cultured cells were stained with biotin-labeled mAbs to CD80 or CD86, then with PE-streptavidin before flow cytometric analysis. **(c)** The interferon alpha (INF α) levels (pg/ml) in the culture supernatants were determined by an INF α enzyme linked immunosorbent assay

Ig β , germ-line μ , IL-7R α , bcl-2). Generation of the PIR⁺/B220⁺/CD19⁻ cells was unaffected in bone marrow samples from mice deficient for Ig μ heavy chain, recombination activating gene 1, IL-7R α or common cytokine receptor γ . When the B220⁺/CD19⁻ cells were divided into PIR⁺ and PIR⁻ sub-populations and cultured in the presence of CpG oligodeoxynucleotides to stimulate DC development, the PIR⁺ cells were induced to express the co-stimulatory molecules CD80 and CD86, while the PIR⁻ cells were unresponsive. The PIR⁺ population also produced interferon- α (IFN α), whereas the PIR⁻ population did not. Unlike the PIR⁻/B220⁺/CD19⁻ cells, the PIR⁺/B220⁺/CD19⁻ cells failed to proliferate in vitro under the B-lineage supportive conditions (Chen CC et al., unpublished). Collectively, these findings suggest that the PIR⁺/B220⁺/CD19⁻ cells population is enriched for DC precursors, most likely for pre-DC2 or plasmacytoid DC.

3.6 PIR Ligands

We have postulated that PIR may recognize multiple ligands based upon the sequence variability observed in the extracellular Ig-like domains of the PIR isoforms. While PIR-A and PIR-B have very similar extracellular regions, amino acid sequence diversity in the second amino terminal Ig-like domain of PIR-A is concentrated in regions predicted to be on the loops between β sheets by comparative modeling based on the structure of killer cell Ig-like receptor (KIR/CD158). At least four different patterns of diversity are recognized in such regions of PIR, thereby implying different recognition specificities.

Major histocompatibility complex class I or class I-like molecule specificity of PIRs has been suggested by several findings. Paired immunoglobulin-like receptor-B isolated from splenic B cells and macrophages were found to be constitutively tyrosine-phosphorylated, irrespective of cell activation status (e.g., small resting versus large activated B cells) and to be associated with SHP-1 tyrosine phosphatase (Ho et al. 1999). Recent studies of Pereira and Lowell indicated that PIR-B on resting, non-adherent bone marrow-derived monocytes was negligibly tyrosine-phosphorylated and that its phosphorylation was induced by adherence to plates (Pereira and Lowell 2003; Pereira et al. 2004). In *Lyn*^{-/-} mice, PIR-B tyrosine phosphorylation was greatly reduced, suggesting that *Lyn* is a major participant in the constitutive tyrosine phosphorylation of PIR-B (Ho et al. 1999). In contrast to the in vivo findings, tyrosine phosphorylation of PIR-B was not observed in most myeloid and B-cell lines, but could be induced by ligation of the PIR molecules, implying that the constitutive tyrosine phosphorylation of PIR-B is a consequence of interaction with self-ligands. Since some members of the human ILTs/LIRs (the closest relatives of mouse PIR) and KIR/CD158 have been shown to have binding specificity for different MHC class I alleles (Arm et al. 1999; Barten et al. 2001; Colonna et al. 1999; Cosman et al. 1999; Long 1999; Martin et al. 2002; Wagtmann et al. 1997), the PIR-B tyrosine phosphorylation status was examined in mice lacking β 2 microglobulin (β 2 m), the transporter associated with antigen processing

(TAP-1) or MHC class II. The level of PIR-B tyrosine phosphorylation was reduced by ~50% in $\beta 2m^{-/-}$ mice, but was not significantly altered in TAP-1 $^{-/-}$ or MHC class II $^{-/-}$ mice, suggesting that PIR-B may recognize an endogenous $\beta 2m$ -associated protein which does not require TAP-1 to traffic to the cell surface, for example MHC class I-like molecules (Ho et al. 1999). In support of this idea, the PIR-B tyrosine phosphorylation status was reduced by ~80% in CD1 $^{-/-}$ mice. However, we have not observed an *in vitro* interaction of recombinant soluble form of PIR-B/Fc γ chimeric protein, which is composed of the extracellular region of PIR-B and the Fc region of human IgG, with CD1 transfected cells (unpublished).

More compelling evidence of an interaction between PIR and MHC class I has come from surface plasmon resonance assays (Nakamura et al. 2004). Recombinant soluble PIR-B protein corresponding with its extracellular region (solPIR-B) was found to bind recombinant MHC class I monomers and tetramers (H-2L d , H-2D d , H-2K k , H-2K b , H-2K d) with an affinity (K_d) of ~300 nM and ~10 nM, respectively. These affinities are higher than those seen for the ILT/LIR interaction with the classical and non-classical MHC class I molecules (Chapman et al. 1999; Shiroishi et al. 2003). Interestingly, the solPIR-B also bound isolated $\beta 2m$ at a similar affinity range (~50 nM), suggesting that the PIR-B specificity is directed toward the $\beta 2m$ on MHC class I molecules (Nakamura et al. 2004). This could account for the apparently broad binding specificity of solPIR-B to MHC class I molecules. Consistent with this finding, incubation of MHC class I tetramers with splenic B cells and macrophages up-regulated the tyrosine phosphorylation status of PIR-B and FcR γ molecules by 10–80%. The binding of MHC class I tetramer to PIR-B expressed on normal splenic B cells and to PIR-A on PIR-B-deficient peritoneal macrophages was verified by confocal microscopic analysis using fluorochrome-labeled MHC class I tetramers and anti-PIR mAb, implying that both native PIR-B and PIR-A cell-surface molecules bind recombinant MHC class I tetramers (Chapman et al. 1999).

In HLA transgenic mouse models, mouse PIR-B has been shown to react with HLA-G, an MHC class I-like molecule expressed on fetal trophoblasts and thymic epithelial cells (Liang et al. 2002). This raises the interesting possibility that PIR-B may recognize the mouse HLA-G homolog, the Qa-2 antigen. Interestingly, the human PIR-B homologues, ILT2/LIR1 and ILT4/LIR2, were also found by surface plasmon resonance assays to bind HLA-G with a 3- to 4-fold higher affinity than classical MHC class I (Shiroishi et al. 2003). In collaborative studies conducted with Dr. Paul Bowness and his colleagues (Medical Research Council, Oxford, UK), the $\beta 2m$ -free heavy chain homodimers of HLA-B27, an allele strongly associated with ankylosing spondylitis (Bird et al. 2002), were found to react with the cell surface of transfectants stably expressing mouse PIR-B and PIR-A4 (Kollnberger et al. 2004). The latter finding was confirmed by an enzyme-linked immunosorbent assay in which the recombinant $\beta 2m$ -free HLA-B27 heavy chain homodimers (B27H $_2$) bound to the solPIR-B or solPIR-A3-coated wells in a dose dependent fashion, whereas the recombinant HLA-B27 heterodimers (B27/ $\beta 2m$) did not (unpublished). This suggests that unlike the interaction of PIR with mouse MHC class I, the $\beta 2m$ molecule is not required for the binding of PIR-B and

PIR-A3 to HLA-B27. Notably, it has been shown that β 2m-free HLA-B27 heavy chains bind ILT4/LIR2-transfected but not ILT2/LIR1-transfected cells (Allen et al. 2001). The fact that ILT2/LIR1 binding is more dependent on β 2m than ILT4/LIR2 could be explained by the finding that ILT2/LIR1 recognizes the side of MHC class I molecule with two contact surfaces, the non-polymorphic α 3 domain of heavy chain and the β 2m, in a 1:1 ILT2/LIR1-MHC class I stoichiometry (Chapman et al. 1999; Shiroishi et al. 2003; Willcox et al. 2003). Collectively, these findings suggest that, like ILT/LIR, PIR can recognize both classical and non-classical MHC class I molecules. It is also quite possible that PIR may recognize additional ligands including pathogen-derived ligands. In this regard, ILT2/LIR1 was originally identified as a receptor for UL18, an MHC class I homolog encoded by human cytomegalovirus (Cosman et al. 1997).

3.7 Paired Immunoglobulin-Like Receptor Function

The initial *in vitro* analysis of PIR function was conducted by using anti-PIR mAb as a surrogate ligand. While the anti-PIR mAbs do not discriminate PIR-B and PIR-A molecules, B cells and mast cells, unlike myeloid cells, express predominantly PIR-B on their cell surface. Paired immunoglobulin-like receptor co-ligation was found to inhibit B-cell receptor (BCR)-mediated Ca^{2+} mobilization and IgE-mediated mast cell activation (e.g., serotonin release), and this inhibition was observed only when the PIR-B and BCR or Fc ϵ RI were brought into physical proximity by ligation with a common secondary reagent (Bléry et al. 1998; Maeda et al. 1998a; Uehara et al. 2001; Yamashita et al. 1998b). It remains to be determined whether both types of receptors, PIR-B and BCR for B cells and PIR-B and Fc ϵ RI for mast cells, are brought together following ligation with their respective natural ligands. A different scenario was suggested for c-kit, a prototypic growth factor receptor tyrosine kinase controlling the development and differentiation of hematopoietic progenitor cells and mast cells. Simultaneous co-ligation of PIR-B and c-kit inhibited the c-kit ligand (stem cell growth factor)-induced Ca^{2+} mobilization and proliferative responses of mast cells, but this inhibition did not require bringing PIR-B and c-kit receptors into physical proximity with a secondary reagent (Chen et al. 2002). In the IL-3 dependent BaF/3 pro-B-cell line, IL-3 stimulation led to increased recruitment of SHP-1 tyrosine phosphatase to PIR-B, implying a functional link between PIR-B and cytokine receptor signaling (Wheadon et al. 2002). These findings suggest that the negative regulation of ITIM-bearing receptors like PIR-B may extend to non-ITAM-mediated cell activation, as observed for Fc γ RIIB and gp49 (Feldweg et al. 2003; Malbec et al. 1999). These findings also suggest that PIR-B may regulate a broad spectrum of cell activation responses (see other examples of leukocyte adhesion and chemotaxis below).

The role of PIR-B in granulocyte and macrophage functions has been studied by Lowell and colleagues (Pereira et al. 2004; Zhang et al. 2005). In their studies, PIR-B deficient granulocytes and macrophages were found to be hyper-responsive

to integrin cross-linkage. The PIR-B deficient phagocytes displayed enhanced adhesion, superoxide production and secondary granule release when plated on surfaces coated with integrin ligands. These findings are consistent with the biochemical evidence of enhanced phosphorylation of proteins initiated by integrin signaling in PIR-B deficient cells, thereby suggesting that PIR-B plays an essential role in attenuating the signaling cascade initiated by integrin ligation (Pereira et al. 2004).

PIR-B has also been suggested to be an important regulator of signaling through chemokine receptors, heterotrimeric G-protein-coupled receptors (GPCRs), in granulocytes and DCs (Zhang et al. 2005). As in B cells and macrophages, PIR-B on resting granulocytes was noted to be constitutively tyrosine phosphorylated by myeloid cell-specific Src family tyrosine kinases Fgr and Hck and was associated with SHP-1/SHP-2 phosphatases. This constitutive phosphatase association appeared to restrain chemokine-mediated granulocyte activation, since PIR-B deficient granulocytes and DCs were hyper-responsive to chemokine stimulation as determined by Ca^{2+} mobilization, Erk1/2 activation, actin polymerization and chemotactic responses (Zhang et al. 2005). Moreover, enhanced responses were observed with Fgr^{-/-}/Hck^{-/-} granulocytes and Fgr^{-/-} DCs. Notably, chemokine binding to GPCRs on normal granulocytes and DCs led to transient dephosphorylation of PIR-B (possibly by SHP-2), release of the SHP-1/SHP-2 phosphatases and reversal of tonic inhibition of signaling. This chemokine-induced dephosphorylation appeared to be selective for PIR-B, because another ITIM-bearing protein, signal regulatory phosphatase binding protein-1 α , was unaffected. Antibody-mediated cross-linkage of PIR-B (and PIR-A) on normal granulocytes was also shown to lead to transient dephosphorylation of PIR-B (Zhang et al. 2005). Thus, like Lyn in B cells (Chang et al. 1997; Katsuta et al. 1998; Wang et al. 1996), Src family tyrosine kinases Fgr and Hck function as negative regulators in chemokine signaling of granulocytes and DCs by maintaining the tonic phosphorylation of PIR-B. This analysis also raises the question of how chemokine signaling leads to selective PIR-B dephosphorylation and dissociation from SHP-1/SHP-2.

PIR-B-deficient mice have been found to have normal T and B-cell development, except for slightly higher levels of peritoneal B-1 cells (Ujike et al. 2002). As expected, however, PIR-B deficient B cells were hyper-responsive to BCR ligation and to T-cell independent antigens. Surprisingly, PIR-B^{-/-} mice had significantly augmented IgG1 and IgE responses to T-cell dependent antigens and produced more IL-4 and less IFN γ than wild-type control mice, suggesting an enhanced Th₂ response. The skewed Th₂ response in the PIR-B^{-/-} mice may be due to the immature phenotype of GM-CSF-induced, bone marrow-derived DCs. Interestingly, PIR-B deficient DCs had several constitutively tyrosine phosphorylated proteins and, upon GM-CSF stimulation, exhibited very transient tyrosine phosphorylation of the GM-CSF receptor common β chain when compared to wild-type control DCs, suggesting the PIR-B involvement in GM-CSF mediated signaling (Ujike et al. 2002). Thus, PIR-B may play an important regulatory role in B-cell responses and DC maturation, and indirectly may balance Th₁/Th₂ immune responses.

Another interesting feature of PIR-B^{-/-} mice is that they exhibit an exaggerated graft-versus-host (GVH) reaction (Nakamura et al. 2004). When sublethally irradiated PIR-B^{-/-} and control mice received allogeneic splenocytes, almost all PIR-B^{-/-} recipients died within 2 weeks, whereas approximately a half of the wild-type recipients survived over a 3-week period. The numbers of IFN γ -producing, donor-derived CD4⁺ T cells and CD8⁺ T cells were significantly increased in the PIR-B^{-/-} recipients. Recipient CD11c⁺ DCs in both types of hosts were activated and expressed high levels of MHC class I, co-stimulatory molecules as well as PIR. However, the DC population in PIR-B^{-/-} recipients was found to contain more IFN γ -producing cells than the DC population in wild-type recipients. This suggests that DCs are hyper-activated in PIR-B-deficient recipients, possibly due to the interaction between PIR-A on PIR-B^{-/-} DCs and allogeneic MHC class I on donor T cells. This abnormal interaction leads to increased production of IFN γ , a critical cytokine in lethal GVH disease, as well as to increased proliferation of donor cytotoxic T cells (Nakamura et al. 2004). Thus, PIR-A and PIR-B serve as counteracting receptors for allogeneic immune regulation.

It is interesting to compare results obtained in humans and rats with those mouse PIR-A/PIR-B studies. CD8⁺/CD28⁻ alloantigen-specific suppressor or regulatory T cells were found in humans to induce up-regulation of the ILT4/LIR2 and ILT3/LIR5 inhibitory receptors on antigen-presenting cells (APCs), thereby rendering them tolerogenic (Chang et al. 2002). The tolerogenic APCs displayed reduced expression of co-stimulatory molecules and induced antigen-specific unresponsiveness in CD4⁺ T helper cells. Notably, the cell surface levels of PIR in mice are also altered during cell activation. For example, IL-4, a multifunctional cytokine produced by Th₂ cells, was shown to reduce the expression of inhibitory receptors, including PIR-B on activated B cells, thereby releasing B cells from inhibitory receptor suppression (Rudge et al. 2001). In a rat heart transplant model, similar regulatory T cells (CD8⁺/FOXP3⁺) mediated tolerance to allogeneic heart transplants by inducing up-regulation of PIR-B in allogeneic DCs and heart endothelial cells and by rendering the grafts invulnerable to rejection (Liu et al. 2004). Collectively, these findings suggest that PIR has a regulatory function in the interaction between T cells and APCs, as a crucial priming step for T-cell mediated immune responses.

3.8 Conclusion

While PIR-A and PIR-B are among the earliest identified paired receptors with opposing signaling capabilities, more than 20 such related receptors have now been identified. The pairing of activation and inhibition is thought to be essential modulators for the initiation, amplification and termination of immune responses. Given the wide cellular distribution of PIR (B, monocyte/macrophages, DCs, granulocytes, mast cells, and megakaryocyte/platelets), it has been postulated that PIR-A and PIR-B play specific regulatory roles in host defense, including inflammatory,

coagulative, antigen-presenting, allergic, and humoral immune responses. In addition to their expression on these mature cell types, PIR is also expressed by: (i) early hematopoietic progenitors in both fetal liver and adult bone marrow, (ii) subpopulations of the most immature compartment of thymocytes, and (iii) subpopulations of B220⁺/CD19⁻ cells enriched for DC precursors, especially plasmacytoid DCs. Paired immunoglobulin-like receptor provides an excellent marker for prethymic progenitors in fetal liver and blood with potential to give rise to T cells, NK cells, and DCs. There is now compelling evidence that PIR recognizes classical and non-classical MHC class I molecules, analogous to the human PIR homologs ILT/LIR. Several remarkable functional alterations are observed in PIR-B^{-/-} mice, including (i) hyper-responsiveness of PIR-B-deficient B cells, (ii) enhanced Th₂ response to T-dependent antigens, (iii) exaggerated GVH disease, and (iv) hyper-responsiveness of granulocytes and macrophages to integrin ligation. Paired immunoglobulin-like receptor-B also proves to be an important regulator of signaling through chemokine receptors in granulocytes and DCs. The functional significance of the PIR proteins has been gradually unveiled since identification of the Pir genes in 1997, but we are still far away from seeing the complete picture of PIR biology.

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4

Self–nonself Recognition through B-Cell Antigen Receptor

Daisuke Kitamura

4.1 Introduction

Receptors on innate immune cells that recognize pathogens, such as Toll-like receptors, have been diversified and selected through evolution. In contrast, antigen receptors on B and T lymphocytes diversify enormously in each individual: genes for these receptors composed of multiple various parts are assembled randomly through gene recombination during development of these cells throughout the life of the individuals. While T-cell antigen receptor (TCR) recognizes antigenic peptides of proteins presented on the self MHC, B-cell antigen receptor (BCR) recognizes essentially any kinds of molecules. It is miraculous that only a few genes generate a repertoire of receptors that match essentially all of the molecular structures existing on earth including even newly generated chemical compounds, the mechanisms for which have mostly been clarified to date as briefly mentioned below. It is also amazing that each of the enormous number of receptors recognizes almost single specific structure. Another surprise is that such diverse receptors in an individual do not react with any structures contained in the self body (self-antigens) in principle, which is called self-tolerance.

B-cell antigen receptor and antibody, membrane-bound and secretory forms of Immunoglobulin (Ig), respectively, are produced from the same Ig genes through alternative splicing. The Ig genes are diversified during B-cell development through V(D)J recombination process in which D-J and then V-DJ recombination of heavy (H) chain gene locus successively take place in pro-B cells, and then V-J recombination of light (L) chain (κ or λ) genes in pre-B cells. Combination of multiple V, D, and J gene segments, and junctional nucleotide variations, makes enormous diversity among the assembled receptors. After successful recombination the of IgH gene, μ H chain product is assembled with invariant surrogate light chains,

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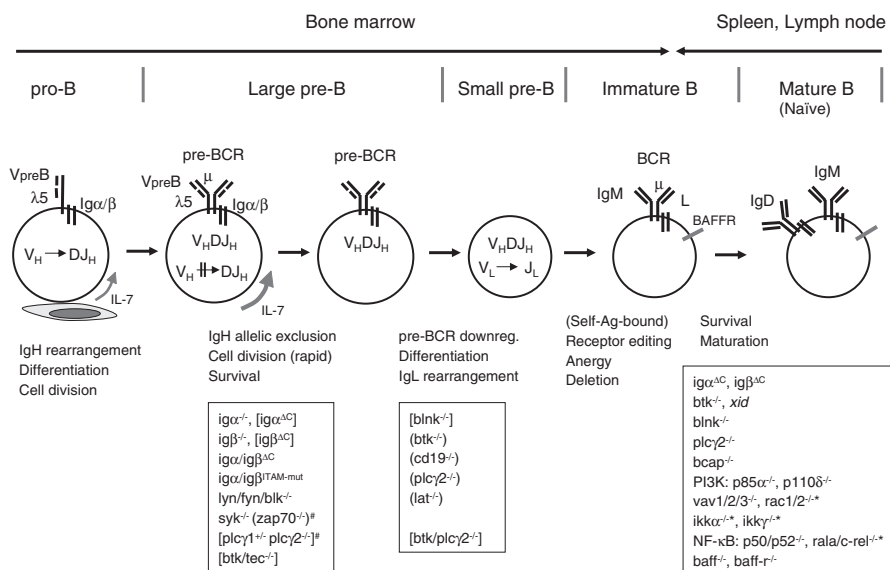


Fig. 1. Primary B-cell development regulated by pre-B/B-cell receptor signaling. Cellular events induced by pre-B-cell receptor (BCR)/BCR signals during the B-cell development are denoted below the symbolized cells of representative developmental stages. In boxes are listed the genotypes of the knockout mice showing defects (those with partial defects are in the *square brackets*) in the respective events. Gene symbols aligned with *slash* in between indicate double or triple gene knockout mice. In the *parentheses* are indicated the genotypes that significantly affect the phenotype only in combination with the adjoined genotype. *Hash mark*, defect of *IgH* allelic exclusion has been proven; *asterisk*, B-cell-specific conditional gene knockout mice or chimeric mice with the mutated hematopoietic cells have been analyzed

VpreB and $\lambda 5$, forms a pre-B-cell receptor complex (pre-BCR), and is expressed on the surface of the cells (Fig. 1). Signals through pre-BCR, synergistically with interleukin-7, induce proliferation of what are then called large pre-B cells, followed by differentiation into the next stage called small pre-B cells where L chain gene rearrangements take place. The pre-BCR signal also prohibits rearrangement of another *IgH* allele, thus contributing to H chain allelic exclusion, the phenomenon that a single B-cell expresses H chain from only one allele of the two. The principle that one lymphocyte holds one receptor specificity is the basis of the clonal selection theory of the lymphoid system [reviewed by Rajewsky (1996) and Karasuyama et al. (1996)].

After successful V-J recombination of L chain gene, L chain products and μH chain are combined into IgM that is the first BCR class to emerge on B cells. The BCR signals the decision of the fate of each newly generated B cell. Depending on the affinity and local density of the self antigen bound to BCR, self-reactive B cells are inactivated (anergy) or eliminated (deletion) from the primary repertoire, or the self-reactive BCR is replaced with a non-harmful one (receptor editing), during the immature B-cell stage. This negative selection of self-reactivity of B cells is the

basis for the self–nonself recognition (that is, tolerance for self) in the B-cell system and for avoidance of autoimmunity. After the negative selection, the remaining B cells emigrate into the peripheral lymphoid organs and further differentiate into so called mature B cells. The maturation and survival of B cells require antigen-independent signals from BCR. Upon encountering antigen through BCR and with T-cell help the mature B cells proliferate and undergo class switch recombination of H chain gene loci, then some of them differentiate into plasma cells that produce antibodies and others form germinal centers in which somatic hypermutation in the V region of H and L chains takes place. From the germinal centers develop memory B cells and long-lived plasma cells equipped with IgG or other classes of Ig containing the affinity-selected mutations in V regions. The random mutation of Ig genes should occasionally generate BCR that binds to self-antigen. However such self-binding B cells do not normally differentiate into memory or plasma cells, but are negatively selected by the mechanism that is largely unknown.

Both pre-BCR and BCR are bound with Ig α /Ig β transmembrane proteins that serve as an intracellular signal transduction subunit. Despite the invariability of this subunit, signals from these receptors induce diverse cellular responses depending on the developmental stages of the cells, or possibly on the nature of the antigen binding. As mentioned above, pre-BCR, not by binding to antigen but probably by self-ligation (Ohnishi and Melchers 2003), regulates V(D)J recombination, cell proliferation, and differentiation. Antigen-bound BCR on newly generated B cells signals anergy, deletion, or receptor editing, whereas that on mature B cells signals proliferation, positive or negative selection, and differentiation into memory or plasma cells. The fact that the same Ig α /Ig β signaling subunit induces such a diverse outcome suggests the presence of diversification and integration of the signaling pathways that eventually determine the cell response. This chapter gives an overview of our current knowledge on the molecular pathways of the pre-BCR and BCR signal transduction to understand the diversification and integration of signaling network, and to discuss from the signaling point of view on how B cells recognize self and nonself antigens.

4.2 Signal Transduction from BCR

4.2.1 Overview

B-cell antigen receptor signaling is primarily transduced from Ig α /Ig β heterodimer, invariant membrane proteins non-covalently associated with H chain of IgM or other Ig classes. Ig α and Ig β contain the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic regions that is crucial for the signal transduction (Flaswinkel et al. 1995). Both Ig α and Ig β are necessary for pre-BCR surface expression and thus for pro-B to pre-B-cell transition during the early B-cell development (Gong and Nussenzweig 1996; Pelanda et al. 2002). In mice with a targeted

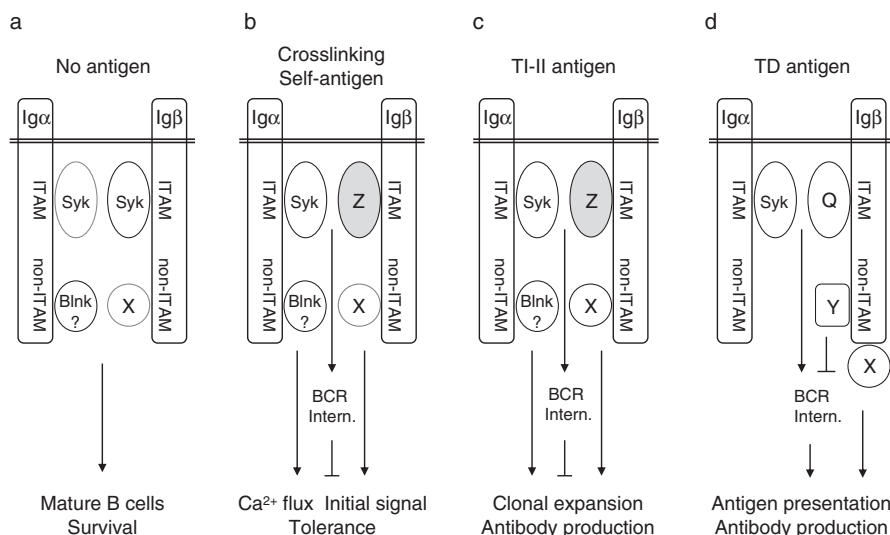


Fig. 2a–d. Differential function of ITAM and non-ITAM sequences in Igα and Igβ. *Each panel* indicates Igα and Igβ associated with one BCR (not shown). At least one ITAM of either Igα or Igβ likely functions through binding with Syk. There may be other ITAM-binding proteins (Z or Q). Although the essential non-ITAM sequences have not been identified, a BLNK-binding site in Igα may be the one. Putative Igβ non-ITAM-binding proteins are indicated as X or Y). **(a)** At least one ITAM of Igα or Igβ and non-ITAM part on the other (or both non-ITAM parts) are necessary for B-cell maturation and survival. **(b)** One ITAM mutation in either Igα or Igβ makes BCR-signal transduction stronger and induces more exaggerated tolerance, suggesting the presence of an inhibitory ITAM-binding protein (Z) and/or signal attenuation due to BCR-internalization promoted by the two ITAMs. ITAM and non-ITAM part in the same Igα (or Igβ) are enough for the initial signal and tolerance transduction. **(c)** One ITAM (particularly of Igβ) inhibits TI–II immune response as in **(b)** (Gazumyan et al. 2006). But non-ITAM parts of both Igα and Igβ are necessary for this response. **(d)** Both ITAMs contribute positively to BCR internalization and thus TD immune response. Non-ITAM part of Igβ may recruit a factor inhibiting BCR internalization (Y) as well as a factor activating B cells (X)

mutation that deletes a cytoplasmic domain of either Igα or Igβ (ΔC), this early development narrowly proceeds, but maintenance of peripheral B cells is impaired (Reichlin et al. 2001; Torres et al. 1996). Indeed, conditional deletion of Igα, of either whole molecule or a cytoplasmic domain, results in rapid loss of mature B cells (Kraus et al. 2004). In contrast, mutations of two tyrosine residues in an ITAM of either Igα or Igβ do not affect follicular B-cell development, suggesting the functional redundancy of the two ITAMs and a role for non-ITAM residues in B-cell maturation (Fig. 2a) However, at least one of these cytoplasmic domains and ITAMs therein is required for the pre-BCR-signaled B-cell development (Gazumyan et al. 2006; Kraus et al. 2001).

Two tyrosines in the ITAM are phosphorylated by cytoplasmic protein-tyrosine kinases (PTKs) of Src-family kinase (SFK) such as Lyn, Fyn, and Blk, and become docking sites for the two Src-homology (SH)2 domains of another PTK Syk. Recent

fluorescence resonance energy transfer analysis of living cells demonstrated that ITAM phosphorylation by SFK induces reversible conformational changes of cytoplasmic domains of IgH, Ig α and Ig β chains, which makes them open to Syk access (Tolar et al. 2005). Src-family kinase is potentiated by dephosphorylation of its C-terminal tyrosine, which is mediated by the transmembrane protein-tyrosine phosphatase CD45. Thus B cells from CD45-null mutant mice do not proliferate in response to BCR-ligation, although B-cell development is normal in these mice (Byth et al. 1996; Kishihara et al. 1993). Despite the central role of Lyn among the SFK for the initiator of BCR signaling, early B-cell development is unimpaired in Lyn-deficient mice, as in Fyn- or Blk-single knockout mice. However, Lyn/Fyn/Blk-triple knockout mice exhibit a developmental arrest at pro-B-cell stage, indicating the redundant function of the three kinases (Saijo et al. 2003). In the Lyn-single knockout mice mature B cells are moderately reduced in number but spontaneously activated and hyper-responsive to BCR stimulation in terms of Ca²⁺ flux, proliferation and antibody production, and the mice eventually develop autoimmune disease reminiscent of systemic lupus erythematosus (SLE). This is ascribed to a negative signaling role of Lyn: phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the cytoplasmic domains of inhibitory co-receptors such as CD22, CD72, and Fc γ RIIB, to which a protein-tyrosine phosphatase SHP-1 or an inositolide phosphatase SHIP is recruited (see Chapter 7).

Upon binding to the phospho-ITAM, Syk becomes active possibly through its conformational change and auto-phosphorylation. Like Ig α /Ig β , Syk is crucial for pre-BCR signaling because its ablation in mice results in a severe arrest of B-cell development at the pro-B-cell stage (Cheng et al. 1995; Turner et al. 1995), and a failure of H chain allelic exclusion (Schweighoffer et al. 2003). Yet another type of PTK, Btk, is also activated by Syk. The Btk gene is responsible for human X-linked agammaglobulinemia (XLA, also called Bruton's disease) and *xid* mice (spontaneous mutant mouse strain with B-cell deficiency), and Btk is essential for early B-cell development in humans. In mice, Btk is critical for B-cell maturation and activation: *xid* mice as well as Btk-knockout mice show the phenotype (referred to as *xid* phenotype hereafter) in which the number of mature B and peritoneal B1 cells are reduced, B-cell survival is shortened, serum IgM and IgG3 titer is low, BCR-mediated proliferation is abolished, and immune responses to thymus-independent type II (TI-II) antigens and primary, but not secondary, responses to thymus-dependent (TD) antigens are impaired (Khan et al. 1995; Kerner et al. 1995; Ridderstad et al. 1996). Activated PTKs phosphorylate and regulate the enzymatic activities of various signaling intermediates including phospholipase C γ 2 (PLC γ 2), phosphoinositide 3-kinase (PI3K), and Vav, which in turn transmit the signals into distinct pathways, in part, leading to the activation of nuclear transcription factors such as AP-1, NFAT, and NF- κ B (Kurosaki 2002; Reth and Wienands 1997). A cytoplasmic adaptor protein BLNK is phosphorylated by PTKs, recruits, and activates many of the signaling intermediates (Fig. 3).

CD19 is the coreceptor physically associated with BCR and also with complement receptor (CR) 2 and CD81, though it is unclear if these two complexes are

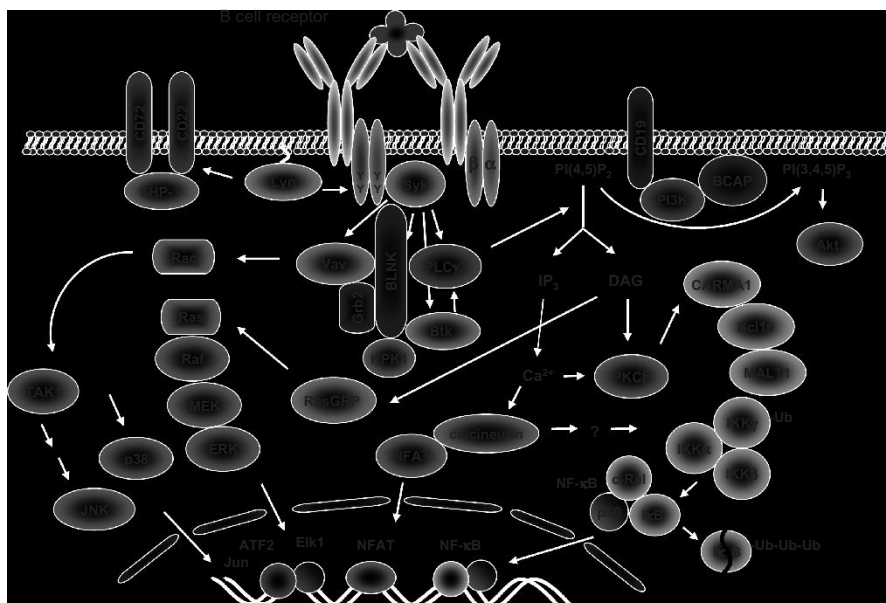


Fig. 3. Signal transduction pathways from B-cell receptor. Only representative pathways are shown. Note that all the depicted signaling events may not happen in the cells at the same time, but events may occur selectively depending on the developmental stages, activation or anergic states, nature of encountering antigen, etc. See text for more details. (See Color Plates)

independent of each other. Upon BCR ligation, tyrosine residues in the cytoplasmic region of CD19 are phosphorylated and bind phosphoinositide 3-kinase (PI3K) via its p85 α regulatory subunit. Class I PI3K phosphorylates phosphatidylinositol (PI)-4,5- P_2 in the plasma membrane and generates PI-3,4,5- P_3 , which recruits many enzymes required for B-cell survival and activation, such as phosphoinositide-dependent kinase 1 (PDK1), Akt (also known as PKB), Btk, Vav, and PLC γ 2, through their PH domains. It follows that PDK1 phosphorylates and activates Akt, which leads to cell survival. Thus mice deficient for a regulatory subunit, p85 α , or a catalytic subunit, p110 δ , of PI3K show the *xid* phenotype with a defect in marginal-zone (MZ) B-cell development and a marked defect in BCR-mediated Akt activation (Clayton et al. 2002; Fruman et al. 1999; Jou et al. 2002; Okkenhaug et al. 2002; Suzuki et al. 1999, 2003). On the other hand, there is a report showing that BCR-induced activation of Btk is independent of p85 α , and that Btk/p85 α double-knockout mice had more severe defects than either single-knockout mouse in B-cell maturation and BCR-induced proliferation (Suzuki et al. 2003). It should be noted that redundant regulatory and catalytic subunits of class I PI3K may be involved in CD19, BCR, and other receptors in B and other cells, making the interpretation of the results difficult.

4.2.2 *Adaptor Protein BLNK*

A B-cell-specific member of the SLP-76 family of adaptor proteins, BLNK (also known as SLP-65 or BASH), has been proven to be critical in the signal transduction of BCR (Fu et al. 1998; Goitsuka et al. 1998; Ishiai et al. 1999; Wienands et al. 1998). BLNK possesses multiple phosphotyrosine-based SH2-binding motifs, proline-based SH3-binding motifs, and a C-terminal SH2 domain. Upon BCR-ligation, BLNK is recruited to the BCR through its SH2 domain binding to a phosphorylated non-ITAM tyrosine in Ig α (Engels et al. 2001; Kabak et al. 2002). A leucine zipper motif in the N-terminal basic domain of BLNK has been shown to be necessary for its localization to the plasma membrane (Kohler et al. 2005). BLNK is primarily phosphorylated by Syk upon BCR stimulation and interacts with various signaling proteins. Simultaneous binding with Btk and PLC γ 2, through their SH2 domains, allows Btk-phosphorylation and activation of PLC γ 2 (Kurosaki and Tsukada 2000). In addition, BLNK binds another adaptor Grb2 and Vav, recruiting Vav to the plasma membrane and activating its GEF activity for a small GTPase Rac (Johmura et al. 2003). The SH2 domain of BLNK itself binds to Syk-phosphorylated HPK1, a serine/threonine kinase of the Ste20 family, and contributes to its activation that leads to NF- κ B activation (Sauer et al. 2001; Tsuji et al. 2001). BLNK has been shown to be necessary for BCR-mediated calcium ion (Ca²⁺) flux, the activation of mitogen-activated protein kinases (MAPKs) such as ERK, JNK, and p38, and transcription factors such as NFAT and NF- κ B in DT40 chicken B cells (Fu et al. 1998; Ishiai et al. 1999).

BLNK knockout mice exhibit a severe *xid* phenotype, additionally with an incomplete block at large pre-B-cell stage of early B-cell development (Hayashi et al. 2000; Jumaa et al. 1999; Pappu et al. 1999; Xu et al. 2000). B-cell antigen receptor-mediated induction of cyclin D2, cdk4, and Bcl-x is impaired and B cells fail to enter the cell cycle (Tan et al. 2001). These defects in cell cycle entry and survival are common to Btk- or PI3K-deficient mice (Glassford et al. 2003; Suzuki et al. 2003). The similar peripheral B-cell phenotype in BLNK-, Btk-, PLC γ 2-, and PI3K-deficient mice (Hashimoto et al. 2000; Wang et al. 2000) suggests their functional association and signifies the physical complex of these proteins, termed "BCR signalosome," in BCR signal transduction leading to survival, maturation, activation, and proliferation of peripheral B cells. In contrast to the result of DT40 cells, BCR-mediated activation of all MAPK is retained in primary B cells from BLNK-deficient mice (Tan et al. 2001).

BLNK is necessary, but not essential, for B-cell development since BLNK-deficient mice possess a small number of functional mature B cells that respond to T-cell-dependent antigen and conform memory B and long-lived plasma cells (Jumaa et al. 1999; Xu et al. 2000; Yamamoto et al. 2004). B-cell development is blocked nearly completely at the large pre-B-cell stage in the BLNK-deficient mice with combined deficiency of CD19, LAT, Btk, or PLC γ 2, suggesting that the presence of BLNK-independent pre-BCR signaling pathway (Hayashi et al. 2003;

Kersseboom et al. 2003; Su and Jumaa 2003; Xu et al. 2006). A similar functional redundancy in the promotion of pre-BCR-mediated development is reported for Btk and Tec, or PLC γ 2 and PLC γ 1 (Ellmeier et al. 2000; Wen et al. 2004). Alternatively, but not exclusively, BLNK might function to enhance the signal by locally and temporally concentrating signaling molecules such as Btk and PLC γ 2, since the early B-cell development and BCR signal transduction depend on the dosage of PLC γ 2 in the absence of BLNK (Xu et al. 2006), and also combined Btk/PLC γ 2 deficiencies cause the same developmental arrest at the large pre-B-cell stage as BLNK deficiency does (Xu et al. 2007). The local concentration of the signaling molecules might be negatively regulated by c-Cbl, a ubiquitin ligase targeting Ig α , Syk, and Lyn, as the early B-cell development is partially rescued in BLNK/c-Cbl-double knockout mice (Song et al. 2007).

4.2.3 Calcium Signaling

After BCR-ligation, PLC γ 2 is recruited to plasma membrane through its PH domain, and to BCR signalosome through binding to BLNK, as described above. PLC γ 2 is phosphorylated and activated by Btk, and then hydrolyzes phosphatidylinositol-4,5-bisphosphate [PI(4,5)P $_2$] in the plasma membrane into inositol 1,4,5-triphosphate (IP $_3$) and diacylglycerol (DAG). Binding of IP $_3$ to its receptors on the ER membrane, which functions as Ca $^{2+}$ channels, promotes flux of Ca $^{2+}$ from ER into the cytoplasm. Immediately follows the Ca $^{2+}$ influx through store-operated Ca $^{2+}$ channels or other cation channels on the plasma membrane, making a transient increase of intracellular Ca $^{2+}$ concentration. Accordingly, signalosome factors such as BLNK, Btk, and PLC γ 2 are necessary for the BCR-induced Ca $^{2+}$ flux response (Hashimoto et al. 2000; Kurosaki and Tsukada 2000; Wang et al. 2000). Interestingly, cytoplasmic tails or the tyrosines of ITAM in Ig α or Ig β are not essential and rather inhibitory for the Ca $^{2+}$ flux response and for tolerance induction, provided with one tail intact (Gazumyan et al. 2006; Kraus et al. 1999, 2001; Reichlin et al. 2001) (Fig. 2b) In contrast, a non-ITAM tyrosine, a binding site for BLNK, is necessary for full Ca $^{2+}$ flux response as well as BLNK phosphorylation and its downstream events induced by BCR crosslinking (Patterson et al. 2006).

The increase of Ca $^{2+}$ concentration induces activation of various calcium-dependent enzymes. One of such enzyme is Ca $^{2+}$ /calmodulin-dependent serine-threonine phosphatase calcineurin. Calcineurin is known to dephosphorylate and activate the cytoplasmic components of NFAT transcription factors (NFATc). B cells express NFATc1, 2, and 3 of the four family members. Dephosphorylated NFATc shuttles into the nucleus and transcriptionally activates the target genes. A relatively low level of Ca $^{2+}$ increase is enough to trigger the immediate NFATc translocation in B cells (Dolmetsch et al. 1997). Physiological role of the calcineurin/NFAT pathway in the B-cell system has been less studied compared with the T-cell system. Chimeric mice containing lymphocytes doubly deficient for NFATc1 and NFATc2 showed a hyperactivated B-cell phenotype, with markedly elevated

serum IgG1 and IgE and plasma cell expansion, despite T-cell dysfunction, suggesting B-cell intrinsic function of these NFATs to negatively regulate terminal differentiation (Peng et al. 2001). B-cell-specific knockout of calcineurin (by deleting the B1 subunit gene) has recently revealed that this molecule is not necessary for follicular B-cell development but for the BCR-mediated activation and proliferation of B cells. The B-cell specific calcineurin B1-deficient mice showed a complex phenotype: higher serum IgM, reduced number of B1 cells, enhanced T-cell-independent (TI) type 1 but normal TI type 2 responses, moderately reduced T-cell-dependent immune responses and plasma cell differentiation, and normal B-cell tolerance (anergy), thus calcineurin may also be involved in other receptors (Winslow et al. 2006). Together with a marked immunosuppressive effect of the calcineurin inhibitors such as FK506, these results suggest the presence of unidentified calcineurin targets other than NFAT that promotes immune responses.

4.2.4 Protein Kinase C

The protein kinase C (PKC) family includes classical PKC (cPKC: α , β , and γ), novel PKC (nPKC: δ , ϵ , η , and θ) and atypical PKC (aPKC: ζ and λ /I) isotypes. cPKC activation requires DAG (or its mimetic, phorbol esters such as PMA) and Ca^{2+} binding to its C1 and C2 domains, respectively, and nPKC requires DAG, whereas aPKC requires neither. Upon receptor stimulation, PKCs become phosphorylated, undergo conformational changes, and translocate to the plasma membrane to be activated. B-lineage cells express all PKC isoforms except PKC γ . Protein kinases C have been presumed to mediate key aspects of antigen receptor function, definite proofs for which have only recently been shown. PKC β -knockout mice exhibit the *xid* phenotype, except that the number of follicular mature B cells is not reduced (Leitges et al. 1996) and that BCR-mediated induction of cyclin D2 expression is not impaired (Su et al. 2002). On the other hand, PKC β is necessary for BCR-induced IKK-NF- κ B activation, Bcl-xL expression and survival (Su et al. 2002; Saijo et al. 2002). These facts suggest that the BCR signal pathway diverges downstream of the signalosome into PKC β -dependent and independent pathways.

In mice lacking PKC α , TCR-mediated T-cell proliferation is moderately impaired possibly due to low responsiveness to IL-2, but BCR-mediated B-cell proliferation is not. PKC α -deficient mice produced a reduced amount of antigen-specific IgG2a/2b antibodies upon immunization, suggesting a mild defect of Th1 cells (Pfeifhofer et al. 2006). PKC θ is known to be involved in TCR signal transduction, and the absence of PKC θ in mice leads to impaired T-cell activation, but B-cell function is normal (Sun et al. 2000).

Among other PKC isotypes, PKC ζ has been demonstrated to be involved in B-cell function in vivo. In PKC ζ -knockout mice, BCR-mediated survival, and proliferation in vitro are partially impaired and TD immune response in vivo is attenuated (Martin et al. 2002). It is indicated that PKC ζ upregulates the transcriptional activity of NF- κ B through direct phosphorylation of RelA (Leitges et al. 2001). In

contrast to the role for PKC θ in T cells, another nPKC member PKC δ has a negative regulatory role in B-cell activation. PKC δ -knockout mice have increased numbers of B cells in the periphery and increased serum antibodies, and develop systemic autoimmune diseases being accounted for by a breakdown of peripheral B-cell tolerance (Mecklenbrauker et al. 2002; Miyamoto et al. 2002). It has been suggested that suppression of pro-apoptotic PKC δ function is integrated in BAFF-receptor-mediated, but not BCR-mediated, signaling for B-cell survival (Mecklenbrauker et al. 2004; Nojima et al. 2006). Expression of PKC η is reported to be readily detectable in pro-B cells but to markedly decrease along with the developmental transition into pre-B cells (Morrow et al. 1999). Although PKC η -knockout mice have not been reported so far, we have recently reported that retroviral expression of PKC η , as well as BLNK, in the leukemic pre-B-cell line derived from a BLNK-knockout mouse causes differentiation toward B cells, represented by κ -gene rearrangement (Yamamoto et al. 2006).

It has been shown that protein kinase D1 (PKD1, also known as PKC μ), a serine-threonine kinase containing a C1 domain, is a common substrate of cPKC and nPKC isoforms, and is activated by the PKCs downstream of BCR and TCR (Spitaler and Cantrell 2004). It has been proposed that PKD1 negatively regulate BCR signal transduction through inhibition of Syk (Sidorenko et al. 1996). PKC ν (PKD3), another member of the PKD family, is abundant in B cells and activated upon BCR crosslinking in a BLNK/Btk/PLC γ 2-dependent manner, through phosphorylation by nPKC, but not by cPKC (Matthews et al. 2003). DT40 B cells express PKD1 and PKD3, and both are redundantly necessary for BCR-induced phosphorylation and nuclear export of class II histone deacetylases, and the target promoter activation, but not for phosphorylation of ERK and Akt (Matthews et al. 2006). Physiological role for PKDs in B-cell development and activation remains to be determined.

4.2.5 Mitogen-Activated Protein Kinase Pathway

Upon crosslinking of BCR, small membrane-bound GTPases such as Ras and Rac are activated and constitutively bound GDP is rapidly replaced with GTP by the enzymes generally called a guanyl nucleotide exchange factor (GEF). The GTP-bound form recruits several cytoplasmic enzymes to the plasma membrane, including the serine threonine kinase Raf1 in case of Ras, which stimulates Raf1-MEK-ERK cascade. However, the active state of the GTPases is only transient since the bound GTP is rapidly metabolized into GDT with the aid of GTPase-activating proteins (GAP). Therefore the GTPases are regarded as rapid molecular switches. Although their requirement in BCR signaling has started to be shown recently, their role for activators of mitogen-activated protein kinase (MAPK) pathway has been well established in other cell lineages.

In the BCR signal transduction, Ras is mainly activated by RasGRP3 with a minor contribution of RasGRP1, both being Ras GEF that is activated upon binding

with DAG (Coughlin et al. 2005; Oh-hora et al. 2003). It has recently been shown that PKC phosphorylates and activates RasGRP3, which is likely mediated through association of the two by binding to DAG at the plasma membrane (Aiba et al. 2004; Teixeira et al. 2003; Zheng et al. 2005). In B cells from RasGRP1/3 double null mutant mice, BCR ligation fails to induce activation of Ras and ERK as well as cell proliferation. Nevertheless, B-cell development is unaffected and T-dependent immune responses of only IgG1 and IgG2a isotypes are significantly impaired in the mutant mice (Coughlin et al. 2005). These results indicate that the Ras-ERK pathway of BCR signaling is essential for induction of cell proliferation but not for B-cell development and terminal differentiation. Ras-ERK pathway, possibly activated by other GEF such as Sos in cytokine-receptor signaling, may also play a role in B-cell development. For example, B cell-specific transgenic expression of a dominant-negative Ras mutant results in the developmental arrest at pro B-cell stage before the expression of pre-BCR (Iritani et al. 1997; Nagaoka et al. 2000). The same Ras mutant inhibits development or survival of a fraction of memory cells having high-affinity Ig mutations, and impairs differentiation of memory cells into antibody forming cells upon secondary antigen stimulation, which is rescued by overexpression of Bcl-2 (Takahashi et al. 2005). This suggests that Ras-mediated survival signal is critical for generation and response of memory B cells, although it is unknown for which receptor this signal is essential, BCR or cytokine-receptors. B cells express Raf-1 and B-Raf isoforms. It has been shown with DT40 cells that these two are necessary but mutually complementary in BCR-mediated ERK activation (Brummer et al. 2002). Contribution of MEK1 or MEK2 in BCR signaling is still unclear.

Rac is important for BCR-mediated reorganization of actin-cytoskeleton, and activation of JNK (SAPK) and p38. Rac is activated by Rac-GEF, Vav, in the BCR signal transduction. The B-cell adaptor molecule of 32 kDa (Bam32), containing SH2 and PH domains and acting downstream of PTK and PI3K, positively regulates Rac1 activation, actin remodeling and BCR internalization (Allam et al. 2004; Niiro et al. 2004). Various signaling cascades starting with GTP-bound Rac including PAK, MEKK, and SEK/MKKs that end with JNK/p38 activation have been reported in various receptor signaling, but little is known in BCR signaling. Loss of all Vav (Vav1–3) in mice results in a developmental arrest at immature B-cell stage, and at CD4/8 double-negative thymocytes as well. B-cell antigen receptor-ligation on the Vav-null B cells does not induce calcium flux and proliferation, but induces intact ERK1/2 activation. The numbers of immature and marginal zone B cells are moderately reduced. The Vav-null mice completely fail to mount both T-dependent and T-independent humoral responses, the latter being partly due to inability of MZ B-cell to differentiate into plasma cells (Fujikawa et al. 2003; Stephenson et al. 2006). Rac1/2-double knockout mice show the similar phenotype with more severe reduction of immature and MZ B cells (Walmsley et al. 2003). Recently, TAK1, a member of MAP3-kinase family, has been shown to be essential for BCR-mediated JNK, but not ERK or p38, activation (Sato et al. 2005; Shinohara et al. 2005). B-cell antigen receptor-induced strong calcium flux and calcineurin activation are also necessary for JNK activation (Dolmetsch et al. 1997; Healy et al.

1997). SEK1 (MKK4) is essential for the development of embryo, but B cells lacking SEK1 develop almost normally and produce antibody normally upon immunization in Rag-deficient recipient mice, and respond in vitro normally to BCR stimulation in terms of JNK activation and proliferation, indicating that SEK1 is dispensable for BCR signaling (Nishina et al. 1997; Swat et al. 1998).

4.2.6 Nuclear Factor (NF)- κ B Pathway

Transcription factor NF- κ B promotes transcription of various genes such as cytokines, cyclins, and anti-apoptotic genes. NF- κ B is a protein family consisting of homo- or heterodimer of the subunits NF- κ B1 (p50), NF- κ B2 (p52), c-Rel, RelA (p65) and RelB. p50 and p52 are generated by proteolysis of their precursors p105 and p100, respectively, and lack activation domain that is required for transcriptional activation of the target gene. p50 and p52 act as transcriptional repressors in the forms of homodimers, but form transcriptionally active complexes with Rel proteins. In most resting cells, NF- κ B is retained in the cytoplasm as an inactive complex with I κ B proteins. Upon antigen-receptor stimulation, I κ B kinase (IKK) complex consisting of two catalytic subunits, IKK α (IKK1) and IKK β (IKK2), and a regulatory subunit, IKK γ (NEMO), is activated and phosphorylates I κ B, which leads to polyubiquitination and proteasome-dependent degradation of I κ B, and to the following nuclear accumulation of active NF- κ B, mainly consisting of p50 and RelA or c-Rel. This signaling pathway is termed the “canonical” pathway (Hayden and Ghosh 2004). B-cell antigen receptor-ligation activates this pathway and expression of the target genes such as *bcl-2*, *bcl-x*, *A1*, *cdk4*, and *cyclinD2*, leading to proliferation of B cells. The BCR-induced B-cell proliferation and immune response are impaired in c-Rel-single knockout mice indicating a non-redundant role of c-Rel in the receptor-induced cell-cycle entry and survival (Grumont et al. 1998; Kontgen et al. 1995; Tumang et al. 1998).

In mature B cells, however, NF- κ B is constitutively active to some extent, and this activity is required for development and survival of mature B cells including marginal zone and peritoneal B1 cells, as revealed by p50/p52-double knockout mice, bone-marrow chimeras transferred with IKK α - or RelA/c-Rel-deficient fetal liver cells, or B-cell specific IKK β - or IKK γ -knockout mice (Franzoso et al. 1997; Grossmann et al. 2000; Kaisho et al. 2001; Pasparakis et al. 2002; Sasaki et al. 2006). The development and survival of mature B cells require “tonic” signal from unligated BCR (Kraus et al. 2004; Lam et al. 1997; Torres et al. 1996), as well as signal from BAFF receptor (BAFF-R) except for peritoneal B1 cells (Gross et al. 2001; Schiemann et al. 2001; Thompson et al. 2001). The BAFF-R signaling to NF- κ B activation utilizes both “canonical” and “alternative” pathways, the latter involving NIK, IKK α , RelB, and p52, but not IKK γ (Claudio et al. 2002; Pasparakis et al. 2002; Sasaki et al. 2006). The defect of B-cell development in the NF- κ B-deficient mice as mentioned above is, at least in part, ascribed to a defect in the canonical pathway from BAFF-R, since conditional expression of active IKK β

form recovers follicular mature and MZ B cells, possibly through PKC δ nuclear exclusion, in BAFF-R-deficient mice (Sasaki et al. 2006). RelB and p52 are essential for T-dependent humoral immune response, which is probably due to their critical role in CD40 signaling in B cells, and in TNF- and lymphotoxin-receptor signaling in splenic stromal cells (Franzoso et al. 1998; Weih et al. 2001). RelB in hematopoietic cells is necessary for MZ but not follicular B-cell development (Weih et al. 2001).

Btk, BLNK, PLC γ 2, PI3K p85 α , and PKC β have been shown to be necessary for BCR signal leading to NF- κ B activation (Bajpai et al. 2000; Hikida et al. 2003; Petro and Khan 2001; Petro et al. 2000; Saijo et al. 2002; Su et al. 2002; Suzuki et al. 2003; Tan et al. 2001). Interestingly, Src-family kinase, but not Syk, is required for Ig β -induced IKK-NF- κ B activation in pro-B cells (Saijo et al. 2003), though Syk is critical for pro-B to pre-B-cell development. In this regard, there has been no evidence that B-cell intrinsic NF- κ B activity is required for the early B-cell development. It has been shown that BCR-induced strong calcium flux and the following activation of calcineurin are necessary to trigger I κ B degradation and NF- κ B activation (Dolmetsch et al. 1997; Healy et al. 1997).

In peripheral B and T cells, three interacting proteins, CARMA1 (also known as CARD11), Bcl10, MALT1, are essential for IKK-NF- κ B activation, cell activation and proliferation in response to stimulation through BCR, CD40, and TCR, but not for development of mature T cells and B cells except B1 and marginal zone B cells (Egawa et al. 2003; Hara et al. 2003; Jun et al. 2003; Newton and Dixit 2003; Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003; Xue et al. 2003). Therefore, the CARMA1/Bcl10/MALT1 complex, as well as PKC β , are dispensable for the BCR “tonic” signal, but necessary for the “inductive” signal. It has been shown that BCR-induced PKC β -binding and phosphorylation of CARMA1 leads to NF- κ B activation (Sommer et al. 2005). Bcl10 and MALT1 have been shown to promote K63 polyubiquitination of IKK γ to activate IKK in other receptor signaling (Zhou et al. 2004). Thus, the CARMA1/Bcl10/MALT1 constitute an upstream part of the canonical pathway. Using DT40 cells, it has recently been shown that TAK1, a member of MAP3-kinase family, is essential for IKK and NF- κ B activation in response to BCR stimulation, and that PKC β -phosphorylated CARMA1 interacts with TAK1 and IKK, allowing TAK1-phosphorylation of IKK (Shinohara et al. 2005). On the other hand, BCR-mediated NF- κ B activation is intact in primary B cells from mice with B cell-specific TAK1-deficiency, implying context-dependent TAK1 requirement (Sato et al. 2005). Taken together, it is proposed that BCR signalosome leads to PKC β activation at plasma membrane, and that the CARMA1 modified by PKC β serves as a docking site for IKK $\alpha/\beta/\gamma$ complex where IKK γ -ubiquitination is mediated by Bcl10 and MALT1. It remains to be determined how IKK α/β is then activated.

It has been demonstrated that PI3K is required for BCR-mediated activation of NF- κ B, but not of Btk, implying a Btk-independent mechanism for NF- κ B activation mediated by PI3K (Suzuki et al. 2003). In this regard, in TCR-signal transduction, PI3K-downstream PDK1 is shown to recruit PKC θ , IKK complex, and CARMA1/Bcl10/MALT1 complex together, resulting in IKK activation (Lee et al.

2005). In addition, BCAP, a BCR-associated adaptor protein, is necessary for maintenance of c-Rel protein level, implying another mechanism regulating NF- κ B activation. Thus B cells from BCAP-knockout mice respond poorly to BCR cross-linking to express Bcl-xL, A1, Cyclin D2, and Cdk4, and to proliferate, and the mice exhibit the *xid* phenotype (Yamazaki et al. 2002; Yamazaki and Kurosaki 2003).

4.3 BCR Signaling Pathways for Immune Response

4.3.1 *Thymus-Independent Response*

Immune responses characterized by antibody production are classified as thymus-independent (TI) or thymus-dependent (TD) based on the requirement for T-cell help. Antigens that elicit TI responses are divided into type I and type II. The former (TI-I) antigens represented by lipopolysaccharide (LPS) are now known to stimulate polyclonal B cells through Toll-like receptors to proliferate and finally to differentiate into antibody-forming plasma cells, which is independent of BCR signaling. The latter (TI-II) antigens, represented by polysaccharide displaying repetitive epitopes, aggregate BCR and induce clonal expansion and differentiation of specific B cells into plasma cells in a short period (within a week). Typically, TI-II response is elicited by marginal zone B and B1 cells, which form short-lived plasma cells homing outside of the lymphoid follicles in the red pulp, and does not induce germinal centers (GCs) and affinity maturation of Ig (Fig. 4). In the TI-II response, B cells carrying BCRs with higher affinity to a given antigen respond more frequently to the antigen *in vivo* to proliferate and generate more plasma cells than those with lower-affinity BCRs, resulting in a selective production for high-affinity antibodies (Shih et al. 2002b). Recently it has been reported that TI-II response generates atypical memory B cells (Obukhanych and Nussenzweig 2006) and memory-type plasmablasts derived from B1b cells (Hsu et al. 2006).

4.3.2 *Thymus-Dependent Response*

In the primary response to TD antigens, antigen-specific B and T cells encounter and B cells are activated through antigen-bound BCR as well as by T-cell help through CD40 and cytokine receptors. After clonal expansion, some B cells form extrafollicular primary foci of plasmablasts secreting IgM, some undergo Ig class-switch and form the foci of IgG⁺ plasmablasts, and others aggressively proliferate in the B-cell follicles to form GCs (Fig. 4). Plasma cells from the foci are short-lived and produce antibodies for a couple of weeks after immunization. In GCs, proliferating B cells undergo Ig gene somatic hypermutation and selection for high-affinity BCR, and then the selected B cells differentiate into short-lived plasma,

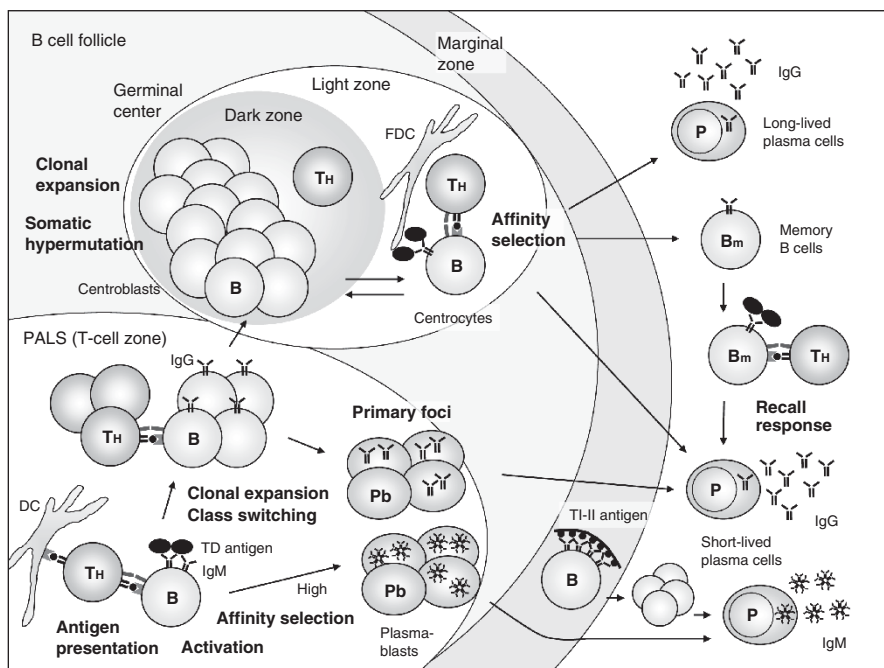


Fig. 4. Thymus-independent (TI)-II and thymus-dependent (TD) immune responses. See text for interpretation. Each event occurring in B cells during TD immune response is notated in bold. *B*, B cells; *T_H*, helper T cells; *Pb*, plasmablasts; *B_m*, memory B cells; *P*, plasma cells; *DC*, dendritic cells; *FDC*, follicular dendritic cells; *PALS*, periaarteriolar lymphoid sheath. (See Color Plates)

memory B or long-lived plasma cells. Memory B cells are maintained for a long time without persisting antigen (Maruyama et al. 2000) and respond promptly to a secondary challenge of antigen (so-called recall response) to produce a large amount of high affinity antibodies of typically IgG class.

A conserved transmembrane/cytoplasmic tail of IgG was shown to be responsible for the efficient TD IgG response including Ig affinity maturation, memory formation, and higher antibody production partly due to enhanced survival of plasmablasts after clonal expansion compared to those mediated by IgM (Kaisho et al. 1997; Martin and Goodnow 2002). Molecular mechanism accounting for the IgG tail function is still unclear, besides an augmented Ca^{2+} response, and involvement of CD22 is currently under debate (Horikawa et al. 2007; Wakabayashi et al. 2002; Waisman et al. 2007). In addition to the class-switching of BCR itself, T-cell-mediated activation may drastically alter the BCR-downstream signaling pathway, as it was reported that prior B-cell exposure to CD40-ligand results in BCR-mediated activation of NF- κ B and ERK pathways that are independent of Btk, PI3K or PLC γ 2 [Mizuno and Rothstein (2005) and references therein].

In TD response, B cells with initially higher-affinity receptors for antigen are more preferentially selected for expansion in germinal centers and later for memory

B cells than those with lower-affinity receptors in a competitive condition, although both respond almost equally in non-competitive condition (Dal Porto et al. 2002; Shih et al. 2002a). The former cells in germinal centers accumulate less somatic hypermutation in Ig V region (albeit no less in the intron) than the latter in non-competitive condition, possibly because the mutation in the high-affinity V gene would more likely result in the loss of affinity, rather than the gain (Shih et al. 2002a). However, B cells initially carrying BCR with affinity higher than a certain threshold expand early upon immunization and preferentially differentiate into extrafollicular short-lived plasma cells, and are less recruited into germinal center reaction and the following long-lived plasma or memory B-cell formation (O'Connor et al. 2006; Paus et al. 2006). It is still unknown how B cells that acquire high affinity during the germinal center reaction are selected for differentiation into memory B or long-lived plasma cells.

4.3.3 Immune Responses in Mutant Mice Deficient for BCR-Signaling Molecules

Mice devoid of a cytoplasmic tail portion of either Ig α or Ig β (Δ C), or one of BCR-proximal signaling molecules, such as Btk, PI3K (p85 α or p110 δ), PLC γ 2, PKC β , BLNK, or BCAP, are deficient for antibody production after immunization with TI-II antigens, indicating that the BCR signaling from both Ig α and Ig β through these signaling molecules is essential for clonal expansion and/or plasma-cell differentiation upon receptor aggregation (Hashimoto et al. 2000; Jumaa et al. 1999; Khan et al. 1995; Leitges et al. 1996; Okkenhaug et al. 2002; Reichlin et al. 2001; Ridderstad et al. 1996; Suzuki et al. 1999; Torres et al. 1996; Wang et al. 2000; Xu et al. 2000; Yamamoto et al. 2004; Yamazaki et al. 2002). Involvement of SFK and Syk in the immune response cannot be assessed because of their functional redundancy and indispensability in pre-B-cell development, respectively. CD45 is essential for T-cell development and function, and B-cell-intrinsic function of CD45 in immune responses has not been reported.

The primary IgG response to TD antigen is severely decreased but detectable despite the greatly reduced number of mature B cells in mice with Δ C mutation in either Ig α or Ig β , suggesting partial redundancy of the cytoplasmic tails in the TD response. Interestingly, in mice with replacement mutations at two tyrosine residues in ITAM of either Ig α or Ig β , follicular mature B cells develop normally and fully (even better) respond to TI-II antigen, in contrast to the Δ C mutants as mentioned above, suggesting redundant function of the two ITAMs as well as the importance of non-ITAM residues in Ig α /Ig β in the TI-II response (Gazumyan et al. 2006; Kraus et al. 2001) (Fig. 2c). In this regard, the mice with a mutation at a conserved non-ITAM tyrosine of Ig α , which is necessary for BLNK binding and phosphorylation, respond several fold less to TI antigen but normally to TD antigen, which is similar to the response of BLNK-knockout mice (Patterson et al. 2006; Yamamoto et al. 2004). On the contrary, TD IgG1 response is significantly reduced in either

ITAM mutant mice, indicating that both ITAMs of Ig α /Ig β are necessary for optimum TD response (Gazumyan et al. 2006; Kraus et al. 2001). In mice in which the cytoplasmic tail of Ig β has been replaced with that of Ig α (β_c - α_c mutation), follicular mature B cells develop almost normally and are long-lived, but respond barely to TI-II antigen and several-fold less to TD antigen. Taken together with the dispensability (actually inhibitory role) of Ig β ITAM for the TI-II response, it indicates that Ig β non-ITAM sequence is necessary for the TI-II response (Reichlin et al. 2004) (Fig. 2c). In B cells of the β_c - α_c mutant mice, constitutive as well as crosslinking-induced BCR internalization is accelerated, surface BCR level reduced, and BCR signal transduction weakened (Reichlin et al. 2004), which is an almost opposite phenotype of B cells of either ITAM mutant (Gazumyan et al. 2006; Kraus et al. 2001). This suggests that two ITAMs of Ig α /Ig β are necessary for the efficient BCR internalization, whereas the non-ITAM sequence in Ig β is involved in the negative regulation of the internalization (Fig. 2d).

The primary TD IgG response is delayed and reduced in Btk-, PKC β -, PI3K p110 δ -, or BLNK-deficient mice, with nearly absence of IgM production, which is not necessarily correlated with the number of mature B cells in each strain but may be due to less efficient activation and proliferation signaled through IgM BCR. It was reported to be normal in PLC γ 2- or PI3K p85 α -deficient mice, but kinetics data for them are missing. In addition the interpretation of the results requires some caution because the immune responses may be affected by the immunized antigens and conjugates or by genetic backgrounds of mouse strains. In contrast to the primary, the secondary IgG response to TD antigens is largely intact in these mutant mice (Hashimoto et al. 2000; Jumaa et al. 1999; Khan et al. 1995; Leitges et al. 1996; Okkenhaug et al. 2002; Ridderstad et al. 1996; Suzuki et al. 1999; Wang et al. 2000; Xu et al. 2000; Yamamoto et al. 2004). Finally, antibody affinity maturation, the recall response of memory B cells after a long-term interval, as well as long-term maintenance of specific antibodies, are intact in Btk- or BLNK-deficient mice (Ridderstad and Tarlinton 1997; Ridderstad et al. 1996; Yamamoto et al. 2004), while they remain unknown in other mutant mice.

In sharp contrast, CD19-deficient mice exhibited severely impaired TD primary and secondary responses including germinal center formation, antibody affinity maturation, memory B-cell formation and long-lasting antibody production, but rather enhanced TI-II response (Fehr et al. 1998; Rickert et al. 1995; Sato et al. 1995) (see also Chapter 7). The TD response is profoundly impaired, except for antibody affinity maturation, also in mice deficient for *Cr2* gene encoding CD21/CD35 complement receptors (Chen et al. 2000; Croix et al. 1996). These results are supported by the *in vitro* data showing that signaling through CD19/CD21 complex co-ligated with BCR by antigen-complement complex stabilizes the BCR and augments antigen processing/presentation (Cherukuri et al. 2001a,b), and *in vivo/vitro* data that CD21/CD35 signaling protects B cells from Fas-mediated apoptosis during primary TD-response (Barrington et al. 2005). Thus once the BCR class switches to IgG and T-cell help become available, BLNK/Btk signalosome is no more necessary, but the co-signaling from IgG and CD19 is still required for efficient induction of germinal center reaction and the following immune responses.

Among the downstream signaling pathways, CARMA1/Bcl10/MALT1 and cRel have been shown to be essential for B- and T-cell activation/proliferation through BCR, CD40, and TCR, and for both TI and TD immune responses (Hara et al. 2003; Jun et al. 2003; Kontgen et al. 1995; Newton and Dixit 2003; Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003). B-cell specific deletion of IKK β in mice exhibit reduced TI and TD immune responses, but it is unclear which defect is responsible for it, reduced follicular, MZ, and B1 cells, lowered proliferative signals from BCR, TLR, or CD40, or reduced survival (Li et al. 2003). Thus contribution of the NF- κ B pathway downstream of BCR for the TD immune response is still unclear. As mentioned above, Vav and Rac are also essential for both TI–II and TD responses, suggesting a signalosome-independent basic function of these molecules in BCR signal transduction. The significance of the Ras-MEK-ERK signaling pathway is currently unclear.

4.4 BCR Signaling Pathways for Self Tolerance

4.4.1 *Clonal Deletion*

Owing to the random nature of V(D)J recombination of Ig H and L genes and of H/L-chain pairing, many newly generated B cells express BCR that binds to self antigens. It has been proposed that such self-reactive B-cell clones are eliminated through BCR-signal-induced apoptosis in the bone marrow (clonal deletion), based on the in vitro model systems using ex vivo immature B cells or B lymphoma cell lines, and also in vivo model using transgenic mice expressing monoclonal BCR that reacts with membrane-bound (neo-) self-antigen or DNA (Fang et al. 1998; Lang et al. 1997; reviewed by Goodnow 1992; Nemazee et al. 1991). Although the mechanisms for the BCR-induced apoptosis of immature B cells have been extensively studied in the in vitro systems (reviewed by King and Monroe 2000), the specific mechanism that accounts for the BCR-mediated clonal deletion in vivo remains unclear. Detailed analysis of the anti-hen egg lysozyme (HEL) Ig/ membrane-bound-HEL (mHEL) transgenic mice revealed that the self-reactive immature B cells are not immediately deleted in the bone marrow but developmentally arrested at the immature B-cell stage, and ultimately die through apoptosis which can be rescued by artificial expression of bcl-2 (Hartley et al. 1993). This indicates that the signal from self-antigen bound IgM is likely to block maturation of self-reactive B cells but not to directly induce their apoptosis. Indeed the clonal deletion is a rare event in mice carrying self-reactive Ig transgenes targeted into the Ig gene loci so that they can be removed by de novo V(D)J rearrangements (receptor editing; see below). The arrested immature B cells expressing a low level of anti-self IgM in the mHEL/anti-HEL-Ig transgenic mice may represent the cells undergoing receptor editing. Accordingly BCR-ligation on immature B cells ex vivo induces Ig gene rearrangements but not apoptosis under appropriate culture conditions such as a use of feeder cells and/or monoclonal antibodies for the BCR

ligation (Hertz and Nemazee 1997; Melamed and Nemazee 1997; Sandel and Monroe 1999). Thus it is likely that the immature B cells, having failed to edit their self-reactive IgM into a non-harmful one within the permissive period for the rearrangement, die through apoptosis (Melamed et al. 1998), possibly by a temporally controlled program.

It was shown that soluble antigen induces apoptosis of germinal-center B cells *in vivo*, which was thought to reflect the elimination of self-reactive B cells newly generated by hypermutation of Ig genes during the immune response (Pulendran et al. 1995; Shokat and Goodnow 1995). Even the resting mature B cells are rapidly eliminated in the periphery after forced replacement of their BCR into a self-reactive one by Cre/loxP-mediated recombination (Lam and Rajewsky 1998). Such self-antigen-induced elimination of mature B cells may be responsible, at least partly, for the peripheral tolerance. However the BCR-signaling mechanism specific for such elimination is unknown.

4.4.2 Receptor Editing

Receptor editing is a process by which the specificity of BCR is altered by successive V gene (mainly of L chain) rearrangements when the BCR on newly generated B cells is bound by self-antigen with repeated epitopes such as membrane proteins or DNA. The “Ig knock-in” mice, in which Ig gene loci are replaced through gene-targeting technology with rearranged V genes of an autoantibody, have revealed that the receptor editing plays a major role in eliminating the self-reactivity in the primary B-cell pool. In such mice, secondary rearrangement of the Ig gene loci (primarily at the *Igκ*, then at the *Igλ*, and less frequently at the *IgH* loci) replaces the self-reactive V gene with new ones, which efficiently rescues originally self-reactive B cells from clonal deletion (reviewed by Nemazee 2006). Cellular deletion occurs only when further rearrangement is precluded by the lack of the leftover of unrearranged J segments or of RAG proteins (Halverson et al. 2004; Xu et al. 1998). In accord with these *in vivo* data, BCR engagement on bone marrow immature B cells *in vitro* has been shown to induce expression of RAGs (when it is suppressed by transgenic BCR expression) and secondary L chain gene rearrangements, but not apoptosis (Hertz and Nemazee 1997; Melamed et al. 1998; Sandel and Monroe 1999).

B-cell antigen receptor signaling pathway to receptor editing has not been extensively studied, partly because the study requires genetically modified mice at multiple loci including the Ig genes as aforementioned and a gene of interest. It has been reported that tyrosine kinase Syk and Btk, tyrosine phosphatase CD45 and co-receptor CD19, all of which are known to positively regulate BCR signal transduction, have been reported to be dispensable for receptor editing in anti-self MHC or anti-HEL Ig-transgenic mouse systems (Dingjan et al. 2001; Meade et al. 2004; Shvitiel et al. 2002a,b). However, the apparent receptor editing in Syk- and CD19-knockout mice could be ascribed to the lack of allelic exclusion of light

chains (Meade et al. 2004; Shvitz et al. 2002b). We have recently demonstrated that receptor editing in anti-DNA Ig knock-in mice is impaired in the absence of BLNK. Accordingly BLNK-deficient mice with normal *Ig* loci possess less Ig λ^+ B cells, have undergone less recombining sequence (RS) recombination, both of which are hallmarks of receptor editing, and respond more to DNA-immunization, than wild-type mice. Thus BLNK is necessary for the BCR signaling pathway leading to receptor editing that substantially contributes to the elimination of self-reactivity physiologically (Hayashi et al. 2004). It is currently unknown how BLNK transmits the signal for the receptor editing. Additional anti-self Ig knock-in mouse strains with genetic ablations of other BCR-signaling molecules would be necessary to solve this problem. Not only the membrane-bound but soluble self-antigens can induce the receptor editing to some extent (Hippen et al. 2005), indicating that the quantity of the BCR signaling plays a crucial role in determining the receptor editing. Signaling molecules such as BLNK may function to control the threshold of BCR signaling to induce the editing in an appropriate period of B-cell development. Bone-marrow microenvironment that has been shown to protect immature B cells from BCR-induced apoptosis and to allow receptor editing instead (Sandel and Monroe 1999) may also function to define a “time window” being permissive for the receptor editing during B-cell development.

4.4.3 *Anergy*

Self-reactive B-cell clones that have not been eliminated or edited in the bone marrow often become unresponsive to antigen, which is termed “anergy.” This was first demonstrated and has been best characterized by the double transgenic mice that express anti-HEL Ig (Ig^{HEL}) and soluble HEL antigen (sHEL) (Goodnow 1992). Since the membrane-bound HEL antigen causes receptor editing or clonal deletion in the same Ig-transgenic mice as described above, it is believed that B cells are anergized when BCR is bound to self-antigen with relatively low avidity such as sHEL. This is supported by the fact that increased BCR signaling quantity rendered by the mutation of protein tyrosine phosphatase SHP-1 or Lyn, which constitute negative regulatory pathways, results in clonal deletion, rather than anergy, in the sHEL/Ig^{HEL} mouse system (Cyster and Goodnow 1995; Cornall et al. 1998). In the same system, continued binding of self-antigen is required for the B cells to be kept in anergic state (Gauld et al. 2005; Goodnow 1992). The anergic B cells show several features that partly differ depending on the experimental systems: cell surface expression level of BCR (mIgM) is reduced (Goodnow et al. 1988) and that of CD5 is increased (Hippen et al. 2000), lifespan is shortened, and entry into the lymphoid follicles is prohibited (Cyster et al. 1994). Their BCR are desensitized and therefore the B cells do not proliferate in response to antigens even in the presence of cognate T-cell help but are instead eliminated by Fas-induced apoptosis (Ho et al. 1994; Rathmell et al. 1995), which depends on repression of B7.2 on anergic B cells (Rathmell et al. 1998). The shortened lifespan and the follicular

exclusion are evident when the anergic B cells and nonself-reactive B cells are coexist, which suggests that the anergic B cells fail to compete for a survival or chemotactic factor.

B-cell activation factor belonging to the tumor necrosis factor family (BAFF) is thought to be one of the survival factors that are critical to affect the tolerance, since overexpression of BAFF from transgene causes autoimmune disease featuring various autoantibodies in mice (Mackay et al. 1999). Although BAFF and its receptor (BAFF-R) are necessary for normal B-cell maturation and survival in the periphery (Mackay and Browning 2002), precise control of local concentration of BAFF appears to be important to maintain the follicular exclusion and the shortened lifespan of anergic B cells that are more dependent on BAFF (Lesley et al. 2004). BAFF negatively regulates the expression of pro-apoptotic protein Bim (Craxton et al. 2005) and the nuclear entry of death-promoting PKC δ (Mecklenbrauker et al. 2004). Loss of Bim or PKC δ has been shown to break B-cell anergy and to cause autoimmunity with autoantibodies in mice (Mecklenbrauker et al. 2002; Miyamoto et al. 2002; Oliver et al. 2006), indicating that Bim and PKC δ are crucial to shorten the life span of anergic B cells. In addition to the deregulated BAFF action, activation of anergic B cells through Toll-like receptors by pathogen-derived components such as unmethylated CpG-containing DNA or single-stranded RNA is proposed to break the tolerance and to induce autoimmune disease (Berland et al. 2006; Christensen et al. 2006; Leadbetter et al. 2002). Furthermore, overexpression of transgenic CD19 has been shown to break anergy in the sHEL/Ig^{HEL} system (Inaoki et al. 1997).

B-cell antigen receptor signaling pathway that induces and/or maintains anergy is poorly understood. B-cell development is blocked in Syk-deficient mice even in the presence of transgenic BCR expression, and therefore anergy induction cannot be tested (Cornall et al. 2000). Although Lyn-deficient mice develop autoimmune disease, they show normal tolerance induction in the sHEL/Ig^{HEL} system (Cornall et al. 1998). Recently it has been shown that the mice in which B cells are devoid of Cbl and Cbl-b, E3 ubiquitin ligases that target Syk and Ig α in B cells, often suffer from SLE-like autoimmune disease and in the sHEL/Ig^{HEL} system B-cell anergy is impaired: B cells are mature, express a high level of Ig^{HEL}, respond to BCR-ligation by CD86 upregulation and Ca²⁺ influx. However the Cbl/Cbl-b-deficient B cells are not hyper-responsive in vitro as well as in vivo in terms of activation, proliferation, and antibody production, although they show enhanced tyrosine phosphorylation of Syk and its substrate proteins, except for BLNK, and Ca²⁺ response upon BCR-ligation, and impaired BCR downmodulation (Kitaura et al. 2007). These results suggest that Ig α /Syk-ubiquitination by Cbl proteins and BCR downmodulation upon contact with self-antigen during the immature B-cell stage is necessary for the induction of B-cell anergy. PKC δ may also be necessary for the BCR signaling inducing anergy because PKC δ -deficient mice develop the similar autoimmune disease and in the sHEL/Ig^{HEL} system HEL-binding mature B cells and antibodies are not reduced, Ig^{HEL} is not downregulated, and BCR-induced CD86 upregulation, Ca²⁺ influx and proliferation are restored (Mecklenbrauker et al. 2002).

Using the sHEL/Ig^{HEL} system, it was shown that in anergic B cells basal calcium level is elevated because of repetitive calcium oscillations arising through continued BCR-stimulation by circulating self-antigen (sHEL), though BCR-ligation induces little initial calcium rise. This resulted in constitutive NFAT activation. ERK is also basically activated and fully activated by BCR ligation. Such activated phenotype of anergic B cells is dependent on the presence of CD45, suggesting the involvement of SFK-mediated signaling (Healy et al. 1997). Calcineurin-deficient B cells, however, have been shown to be normally tolerized in the same system, suggesting the basal NFAT activation is not necessary for the anergic state (Winslow et al. 2006). The anergic B cells do not respond by activation of JNK and NF- κ B upon BCR-ligation, which requires large transient calcium rise and calcineurin activity (Dolmetsch et al. 1997). Since both JNK and NF- κ B were fully activated in the anergic cells upon stimulation with phorbol ester plus calcium ionophore, the defect does not lie at CARMA1 or its downstream but probably at upstream of PKC β (Healy et al. 1997). This chronic ERK activation prevents anergic B cells from producing autoantibody in response to CpG DNA or LPS through TLRs (Rui et al. 2003, 2006). Similarly it was reported that sustained activation of the Raf-MEK-ERK signaling pathway induces cytokine nonresponsiveness in T cells (Chen et al. 1999). In B cells, the active ERK inhibits LPS-mediated plasma-cell differentiation, which is relieved by IL-2/IL-5, T-cell cytokines known to promote plasma-cell differentiation, through induction of ERK-phosphatase DUSP5 (Rui et al. 2006). It remains to be clarified which each alteration in signaling is responsible for, induction of anergy, maintenance or both.

4.5 Concluding Remarks: To Respond or Not to Respond, That is the Question

Taking together all the evidence described so far, mainly from *in vivo* experiments, I have drawn a picture illustrating the signaling pathways from BCR that induce different B-cell responses depending on the nature of antigens and on the developmental stages of the cells (Fig. 5). These pathways are deduced from many pieces of evidence and of many inferences therefrom, and thus the picture is far from complete, but might be useful to clarify the unsolved problems. The difficulty in completing the picture stems from an uncertainty of cell type affected by a gene knockout, and of receptors affected even in the case of B cell-specific gene knockout, given the target signaling molecule may function under multiple receptors and in multiple types of cells. A new technology that enables targeting a molecule in a specific receptor signaling should be desired. In addition, the problem of molecular redundancy (functional compensation of one gene mutation by other genes) needs to be solved.

Despite the very incomplete understanding for the BCR signaling pathways, it may be meaningful at this point to deduce the strategy of how the B-cell system manages to discriminate self from nonself. Unlike toll-like receptors that have

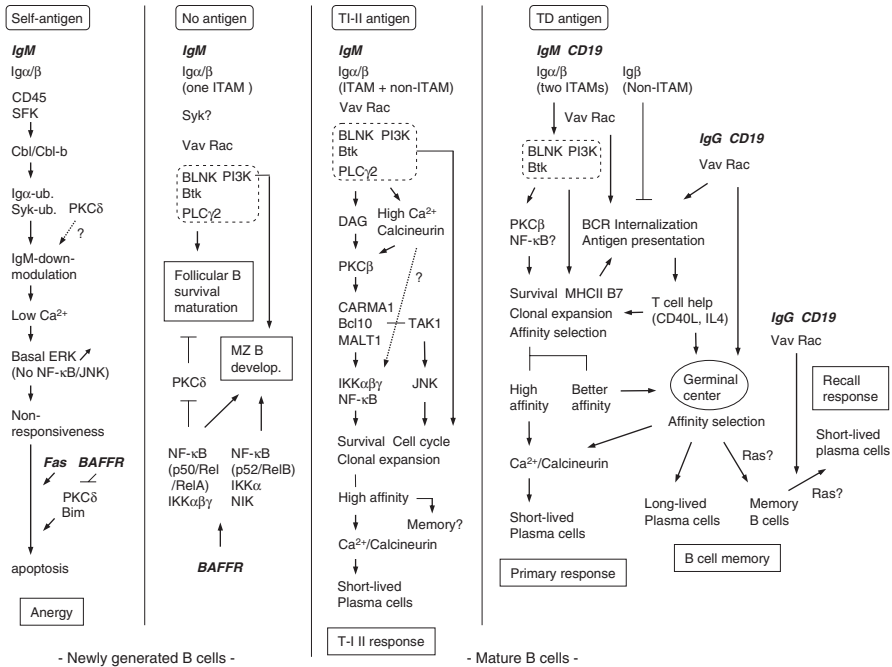


Fig. 5. B-cell receptor signaling pathways that induce B-cell anergy, maturation, TI–II, and TD immune responses. A hypothetical schema of B-cell receptor signaling pathways for each type of response (in boxes), which are deduced from many pieces of published data and inferences from them. Receptors inducing such responses are indicated in bold and italics. “BCR signalosome” is indicated by the dotted box. B1 cell development is not considered since it is substantially affected in a codominant fashion by the genetic background of 129 strains which many of the gene knock-out mice may have inherited (Corcoran and Metcalf 1999). See text for more details

evolved to recognize characteristic patterns of pathogens, BCR is primarily diversified in random fashion and has not evolved to recognize a particular structure. Therefore each B cell cannot discriminate self from nonself, but can respond to whatever the antigens in a manner depending on its developmental state and the valency of the antigens. Although it seems complicated how such a variety of B-cell responses are integrated into a whole B-cell system that performs humoral immune response to nonself and tolerance to self, I propose to take it rather simple as follows: the BCR signaling system may have evolved to let B cells respond to antigens as least toward antibody production. While a variety of inhibitory coreceptors, such as CD22, CD72, and FcγRIIB, obviously function to inhibit the activation signals from BCR, BCR signaling itself appears to be inhibitory. B-cell antigen receptor signal transduction machinery appears to be designed to transiently activate the cells but rapidly to endocytose any antigens that bind BCR, perhaps through Igα/Igβ ubiquitination by Cbl proteins, to ensure the cessation of the initial activation of the cells. In immature B cells that are not yet equipped for the antigen

presentation (e.g., low MHC class II expression), this leads to low BCR expression and an unresponsive state, which results in the anergy to self antigens. In mature B cells, this endocytosis proceeds to antigen presentation, and the cells eventually die unless they are rescued by cognate helper T cells (TD response). Thus the response of peripheral self-reactive B cells, which may have escaped the central tolerance or to have been newly generated through somatic hypermutation, is prohibited by the T-cell tolerance system. In addition, the response to the TD antigens is restricted by the need of BCR-colligation with CD19/CD21 via complement C3d that marks nonself antigens like pathogens. Such passive tactics of BCR signaling appear to be a basis for the B-cell system that responds only to nonself.

Only when the antigen is so highly repetitive and large, such as bacterial capsular polysaccharides or TI–II antigens, that BCR is not able to endocytose, is BCR aggregated on the cell surface and continues to signal. In mature B cells (mostly MZ and B1 B cells) this results in clonal expansion and antibody production that is not checked by T cells. But this will not cause autoimmunity to similar repetitive self antigens because the same signal induces receptor editing in immature B cells still expressing RAG proteins and apoptosis of RAG-negative immature B cells, thus eliminating B cells carrying BCRs that react with such self antigens. It has not been established why immature B cells are destined to die upon BCR aggregation, but it was recently proposed that low DAG production unbalanced with Ca^{2+} flux upon BCR ligation is responsible for induction of apoptosis rather than maturation in immature B cells (Hoek et al. 2006). The remarkable feature of the TI–II response that every one of the BCR signalosome molecules is indispensable for it, in contrast to their dispensability for TD response, implies that the BCR signaling machinery for the TI–II response is an exceptional version, which might be adapted to tight self-control as no T-cell control is available.

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5

How Do T Cells Discriminate Self from Nonself?

Catherine Mazza and Bernard Malissen

5.1 Introduction

There are two forms of immune responses, innate and adaptive. Individuals from species capable of innate immune responses possess limited repertoires of receptors dedicated to this task. Innate receptors recognize either stress-induced self-molecules (as exemplified by the NKG2D receptor, see Chapter 2), or bacterial, viral, or protozoan components that are difficult to mutate without an impact on pathogen replicative capacity (as exemplified by the Toll-like receptors, see Chapter 1). The specificity of these receptors is encoded in the germline, and although their expression may be restricted to a certain cell type, they are not clonally distributed (reviewed in Beutler 2003). Most individuals within a species capable of innate immune responses share very similar repertoires of microbial sensors (Pisitkun et al. 2006). Although the ligands of the Toll-like receptors were initially defined as “pathogen-associated molecular patterns,” it should be stressed that in the case of bacteria these ligands are not exclusively derived from pathogens. Therefore, sensors of innate immunity such as Toll-like receptors do not distinguish microbial commensals from pathogens.

Individuals from species capable of adaptive immune responses assemble large repertoires of T-cell antigen receptors (TCRs) and B-cell antigen receptors (BCRs) to anticipate encounter with any possible antigen. TCR and BCR diversity is generated somatically through site-specific DNA recombinations, and each receptor of a particular specificity is expressed in a clone of lymphocytes. The potential repertoire of $\alpha\beta$ TCRs that can be created by this process is exceedingly large and outnumber the number of T cells that are present in an individual at a given moment. The recognition by TCRs of antigenic peptides (p) bound to major histocompatibility complex (MHC)-encoded molecules is at the basis of adaptive

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immune responses. The fact that TCRs are blind to antigenic peptides that are not delivered to the cell surface through MHC molecules accounts for the MHC-restricted nature of antigen recognition by T cells. MHC class Ia and class II genes are highly polymorphic. This extraordinary diversity primarily involves the nucleotide sequences coding for the peptide-binding domain of the MHC molecules and is thought to reflect evolutionary pressure for recognition of a repertoire of antigenic peptides as broadly inclusive as possible. In contrast to the somatic site-specific DNA recombination that generates a vast TCR repertoire in each individual, the evolutionary forces operating on MHC molecules generate diversity at the population level. Accordingly, within a given species, the existence of multiple alleles for each MHC isoform increases the chance that at least a few individuals will express an allele capable of binding to at least one peptide derived from any pathogen encountered. MHC molecules do not discriminate self from non-self peptides. This ability fully relies on T cells and is in part acquired during T-cell differentiation in the thymus (central self-tolerance). Tolerance to self pMHC complexes that are not expressed in the thymus is further achieved after naïve T cells leave the thymus and circulate throughout secondary lymphoid organs (peripheral self-tolerance). Therefore, for T cells, the definition of the “immunologic self” is specified *de novo* in each individual and varies among individuals based on the inherited MHC class Ia and class II alleles, and on the repertoire of self-peptides capable of being presented by the products of these alleles (reviewed in Vivier and Malissen 2005). Considering, that some T cells normally reside in barrier organs that are laden with microbial symbionts (e.g., the gut), it is likely that a comprehensive definition of the “immunologic self” should also include the peptides derived from those obligate microbial symbionts or postulate tolerance rules proper to organ-specific immune responses (Raz 2007). Among the peptides that are sampled by MHC molecules and recognized by TCRs during immune responses to highly variable viruses (as exemplified by HIV), a few derive from conserved viral regions that, if mutated, had a substantial impact on viral replicative fitness. Because they are not prone to evade T-cell responses through escape mutations, these rare peptides might be used to induce highly focused immune responses resulting in a slower disease progression (Altfeld and Allen 2006).

The selection of the binding-specificity of TCRs and of innate sensors such as Toll-like receptors occurs at two different time scales since innate sensors are selected within a species over evolutionary time, whereas TCRs are selected during the life of an individual. Most receptors encountered in Biology are encoded in the germline and committed to bind a predetermined physiological ligand with invariant thermodynamic parameters. In the case of stochastically generated TCRs, there is no *a priori* physiological ligand, and a given pMHC complex is qualified as “the cognate ligand” only after it successfully activates a given T-cell clone. Because the manifestations of T-cell responses (proliferation, cytotoxicity, cytokine production, etc.) have distinct activation thresholds, quantitative differences in TCR ligand binding translates into qualitatively different signals and leads to responses that can range from maximal activation to desensitization (reviewed in Bongrand and Malissen 1998). Among the pMHC ligands that interact with a given TCR, those

denoted as agonists elicit the entire range of responses from expression of activation markers to stimulation of cell division and production of cytokines. Other pMHC ligands, termed partial agonists elicit some, but not all, of these responses. Finally, others are termed antagonists because they elicit no obvious response, except that they specifically block T-cell responses to agonist ligands. The ability of a given TCR to elicit a diverse range of biological responses according to its affinity for pMHC ligands plays a critical role in self–nonself discrimination. For instance, the developmental choices (deletion or differentiation into mature T cells) followed by immature T cells are primarily determined by the affinity of their TCRs for the self pMHC ligands expressed in the thymus (reviewed in von Boehmer et al. 2003). In this chapter, we provide an overview on the structure of the TCR complex, and on the way it recognizes foreign pMHC complexes and triggers T-cell activation. We also revisit the phenomenon of TCR binding degeneracy, a property with important biological consequences since there are far more potentially antigenic peptides in the environment of a mouse than it has T cells. Finally, we discuss whether the globally conserved mode of TCR docking on MHC molecules is due to coevolutionary pressure that shapes TCR and MHC gene products, or to the action of the CD4 and CD8 coreceptors.

5.2 Structure of the TCR Complex

5.2.1 Subunit Composition

The recognition of antigens by T cells and the ensuing transduction of intracellular signals are accomplished by a multisubunit transmembrane complex denoted as the TCR–CD3 complex. Mature T cells can be divided into two lineages on the basis of their antigen-binding TCR module. In adult mice, most T cells express TCR heterodimers consisting of covalently associated α and β chains, whereas a minor population expresses an alternative TCR isoform consisting of covalently associated γ and δ chains. The TCR α , β , γ , and δ chains each comprise an amino-terminal, clonally variable (V) region, and a carboxy-terminal constant (C) region. Their type I, single-pass transmembrane segment is followed by a very short cytoplasmic tail. Peptide loops homologous to immunoglobulin (Ig) complementarity-determining regions (CDRs) protrude at the membrane-distal end of TCR V domains. They exhibit interclonal sequence variation and constitute the binding site for pMHC ligands (see Fig. 1).

Transport of TCR heterodimers to the cell surface is dependent on their prior assembly with the invariant CD3 polypeptides. As shown in Fig. 2, the CD3 γ , CD3 δ , and CD3 ϵ subunits are expressed as noncovalently associated CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ pairs, whereas CD3 ζ polypeptides combine to form disulfide linked CD3 $\zeta\zeta$ homodimers. The stoichiometry of the TCR–CD3 complex has been the object of intense debate (Hayes and Love 2006). The $\alpha\beta$ TCR–CD3 complex likely contains a single $\alpha\beta$ TCR heterodimer that assembles with single CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, and

Fig. 1. Structure of a TCR-peptide-major histocompatibility complex class I (pMHC) complex (LC13, PDB accession number: 1mi5). The TCR is on the top, the α chain is colored *light blue*, and the β chain is colored *light pink*. The MHC molecule is on the bottom, colored *yellow* with the noncovalently associated β 2-microglobulin in *magenta*. The antigenic peptide is in *pink*. The complementarity-determining region (CDR) loops are colored in *green* (CDR3 α and β), *blue* (CDR1 α and β), and *red* (CDR α and β). (See Color Plates)



CD3 $\zeta\zeta$ pairs (Fig. 2). Aside from their role in allowing expression of the TCR at the cell surface, CD3 subunits are also responsible for coupling the antigen-binding TCR heterodimers to intracellular signaling pathways. None of the CD3 subunits possesses a cytoplasmic domain endowed with recognizable enzymatic activity. However, each contains one or several copies of a conserved sequence that is referred to as an immunoreceptor tyrosine-based activation motif (ITAM). ITAMs are also found in the transducing subunits of the BCR, the receptors for the Fc domain of IgE and IgG, activating natural killer (NK) cell receptors, and glycoprotein VI (GPVI), a receptor supporting platelet adhesion to collagen. The probable evolutionary relationships existing between the ITAM-containing signaling subunits associated with immunoreceptors is mirrored by their capacity to activate similar signaling pathways (Wegener et al. 1992). In the case of the

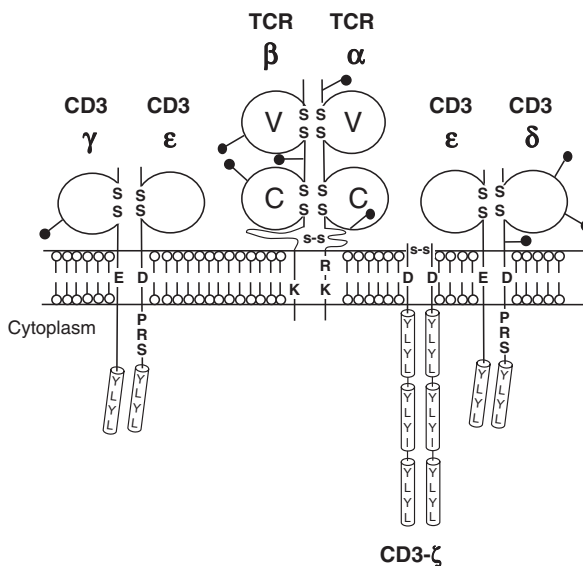


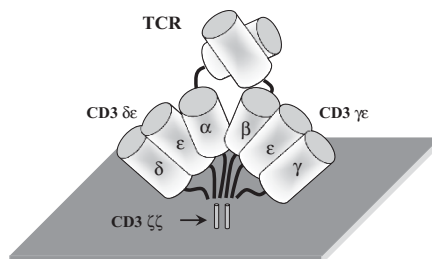
Fig. 2. Putative subunit composition of $\alpha\beta$ T-cell receptor (*TCR*) complexes. Each of the three basic residues (*K* and *R* in the single-letter amino acid code) in the transmembrane segment of the *TCR* α and β polypeptides serves as critical contact for one of the three pairs of acidic residues (*D/D* in the case of *CD3* $\zeta\zeta$ and *D/E* in the case of *CD3* $\gamma\epsilon$ and *CD3* $\delta\epsilon$) found in the transmembrane segments of the *CD3* signaling dimers. The immunoreceptor tyrosine-based activation motifs (ITAMs) found in each of the *CD3* subunits are shown as cylinders containing tyrosine-based docking motifs (*YLYL*). Sites with N-linked carbohydrates are indicated by *black dots*. The *semi-circles* depict sequence segments that fold as C or V immunoglobulin domain. The *CD3* ζ chain forms a disulfide-linked homodimer, and has an extracellular portion only nine amino acids long. *PRS* corresponds to the proline-rich sequence found in the cytosolic juxtamembrane segment of *CD3* ϵ . *S-S* corresponds to an intrachain or interchain disulfide bond

TCR, the ITAMs found in the *CD3* subunits are phosphorylated by the Src-family protein tyrosine kinases (PTK) *Lck* and *Fyn*. Phosphorylated *CD3* ITAMs subsequently recruit *ZAP-70*, a cytosolic tyrosine kinase belonging to the *Syk*-family. As a result, *ZAP-70* becomes activated and phosphorylates a number of downstream molecules, among which the adaptor molecule *Linker for Activation of T cells (LAT)* plays a cardinal role in that it coordinates the assembly of one of the signalosomes linking the *TCR-CD3* complex to a wealth of signaling pathways (reviewed in Malissen et al. 2005).

5.2.2 Quaternary Structure

The four pairs of polypeptides ($\alpha\beta$, *CD3* $\gamma\epsilon$, *CD3* $\delta\epsilon$, and *CD3* $\zeta\zeta$) that compose $\alpha\beta$ *TCR-CD3* complexes are brought together via non-covalent interactions involving residues found in their helical transmembrane segments (Call et al. 2002). The

Fig. 3. A model depicting the association of the CD3 and $\alpha\beta$ TCR ectodomains



TCR α chain transmembrane segment contains both a lysine and an arginine residue, whereas that of the TCR β chain contains a single lysine residue. Each of the basic residues found in the TCR transmembrane segments interacts with a pair of acidic residues donated by the transmembrane segments of a CD3 dimer to constitute a tight three-helix association. The formation of three distinct three-helix associations (TCR α -CD3 $\delta\epsilon$, TCR α -CD3 $\zeta\zeta$, and TCR β -CD3 $\gamma\epsilon$) constitutes the driving force for TCR-CD3 complex assembly and contributes to specifying its composition (Call et al. 2002).

Analysis of the extracellular domains of CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ dimers showed that the stalks that connect them to the transmembrane segments are shorter than those found in the TCR $\alpha\beta$ heterodimer (Sun et al. 2004). When considered together with the tight association existing between the transmembrane segments of the TCR and of the CD3 dimers, this suggests that the extracellular domains of the CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ heterodimers lie just below the TCR C domains. As depicted in Fig. 3, the TCR $\alpha\beta$ heterodimer with a vertical dimension of 80 Å likely projects from the cell membrane, and is flanked on either side by the shorter (40 Å) CD3 heterodimers (Sun et al. 2004). Epitope mapping analysis and structural modeling further suggested that the CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ dimers map to the TCR β - and TCR α -side, respectively, and that the glycosylated CD3 γ and CD3 δ subunits lie away from the TCR heterodimer (Fig. 2). When the widths of the CD3 $\delta\epsilon$ and CD3 $\gamma\epsilon$ dimers, 50 Å and 55 Å, respectively, are added to that of the $\alpha\beta$ TCR heterodimer (58 Å), the full TCR-CD3 complex spans approximately 160 Å (excluding glycans).

As stated by Garcia and colleagues (Garcia and Adams 2005), “the extracellular contacts between the TCR and the CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ dimers may simply be imposed by the transmembrane interactions, like balloons that bump into each other because their strings are held together in a bunch, with no specificity whatsoever.” However, existing data suggest that specific extracellular interactions occur between the $\alpha\beta$ TCR and the CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ dimers. $\gamma\delta$ TCR heterodimers have an overall shape distinct from $\alpha\beta$ TCR heterodimers, and their C domains present unique molecular surfaces for the docking of CD3 subunits (Allison et al. 2001). As a consequence, in the mouse, CD3 $\delta\epsilon$ dimers fail to associate with $\gamma\delta$ TCR heterodimers, and two CD3 $\gamma\epsilon$ dimers are incorporated per $\gamma\delta$ TCR complex (Hayes and Love 2002, 2006). The absence of the CD3 $\delta\epsilon$ dimer does not impede, however,

signaling through $\gamma\delta$ TCRs. Following cross-linking with anti-CD3 antibodies, $\gamma\delta$ TCR complexes induce phosphorylation of appropriate substrates, mobilization of Ca^{2+} , and activation of MAP-Kinases with a greater amplitude than $\alpha\beta$ TCR complexes. It is thus likely that the CD3 $\delta\epsilon$ pair additionally found in $\alpha\beta$ TCRs constitutes a device needed for coupling $\alpha\beta$ TCRs to the CD8 or CD4 coreceptors rather than to a unique signaling cassette (Doucey et al. 2003a). Another example of the influence exerted by the ligand-binding module on the subunit composition of the TCR–CD3 complex can be found in developing T cells. In contrast to mature T cells, immature T cells lack TCR α chain and express a substitute known as the pre-TCR α chain (pT α). pT α associates with TCR β and gives rise to pre-TCR complexes which are composed of CD3 $\gamma\epsilon$ and CD3 $\zeta\zeta$ dimers, and lack or are loosely associated with CD3 $\delta\epsilon$ dimers (von Boehmer 2005). Therefore, the nature of the ligand-binding module found in TCR complexes ($\alpha\beta$ TCR, $\gamma\delta$ TCR, and pre-TCR) clearly influences the spectrum of associated CD3 transducing subunits.

ITAM-containing signaling subunits that are evolutionary related to CD3 subunits have been also co-opted by other immunoreceptors such as Fc receptors and activating NK cell receptors (Malissen 2003a). The latter contain ligand-binding polypeptides with extracellular domains that belong to the Ig or to the C-type lectin families. The amino terminus is located in the extracellular space for Ig family polypeptides and in the cytoplasm for C-type lectin polypeptides. Despite this distinct topology, the transmembrane segment of the ligand-binding polypeptides of both families of activating NK cell receptors contain a basic residue (lysine or arginine) that associates according to a three-helix mode with signaling homodimers (DAP-10, DAP-12, etc.) that contain an aspartic acid in their transmembrane segments. Interestingly, activating NK receptors have inhibitory counterparts that lack a transmembrane lysine, and contain immunoreceptor tyrosine-based inhibitory (ITIM) in their longer cytoplasmic tail. Provided that inhibitory NK receptors are evolutionary more ancient than activating NK receptors, it has been suggested that, following gene duplication, one of the ancestral daughter gene gained an activating function through introduction of a positive charge into its transmembrane segment, and lost its inhibitory function by introduction of a stop codon prior to the sequence coding for the ITIM (Abi-Rached and Parham 2005). The acquisition of such “activating” functionality occurred independently in NK cell receptors belonging to the Ig or C-type lectin families, and might constitute an example of convergent evolution. Therefore, the noncovalent assembly mechanism that holds together the ligand-binding and transducing subunits and is based on membrane-embedded polar interactions constitutes a “signature” of activating immunoreceptors (Feng et al. 2005). During evolution, it likely permitted the “cheap” capture of some predating signaling pathways and also allowed some combinatorial diversification. For instance, the mouse NKG2D receptor can alternatively associate with either DAP-10 or DAP-12 (Diefenbach et al. 2002), and the $\alpha\beta$ TCR complexes expressed on CD8 $\alpha\alpha^+$ -intestinal intraepithelial lymphocytes with CD3 ζ or Fc ϵ RI γ dimers (Guy-Grand et al. 1994).

5.3 How Does the $\alpha\beta$ TCR Convey Signals Across the Membrane?

How information is passed from the $\alpha\beta$ TCR antigen-binding site to the CD3 ITAMs, a process termed TCR triggering, remains highly controversial (Malissen 2003a). None of the existing models satisfactorily accounts for the sensitivity of TCR recognition and the fact that the TCR embodies not only a switch that turns on signals in response to pMHC ligand, but also a timer that determines how long the signal will stay on. This “timer” dimension was originally revealed by the correlation existing between the bioactivity of a given pMHC ligand and the lifetime ($t_{1/2}$) of its association with the TCR. A recent study showed, however, that if changes in heat capacity of the TCR–pMHC interaction (ΔC_p) were combined with the $t_{1/2}$ of the interaction, a better correlation was found with pMHC bioactivity (Krogsgaard et al. 2003). In contrast to the situation observed for the $\alpha\beta$ TCR, for most germline-encoded receptors encountered in biology, the “timer” dimension is only revealed through the synthesis of weak agonist analogs of an agonist physiological ligand.

Apart from a model developed by Gil and colleagues (Gil et al. 2002, 2005) that suggests that the unmasking of a proline-rich sequence (PRS) found in the CD3 ϵ cytoplasmic tail is the seminal event in TCR triggering, most other models posit that tyrosine phosphorylation of CD3 ITAMs constitutes the initial trigger. All these models of TCR triggering can be organized into three categories. The first one postulates the occurrence of pMHC-induced changes in the quaternary structure of the TCR–CD3 complex, whereas the second one considers that the whole TCR–CD3 complex behaves as a rigid body and stresses the importance of ligand-induced homo-oligomerization or hetero-oligomerization with the CD4 or CD8 coreceptors. The third model considers that the seminal event in TCR triggering is the exclusion of the protein tyrosine phosphatases (PTPases) from the close-contact zones that form between a T-cell and an antigen-presenting cell (APC). Before discussing each of these models, it should be emphasized that in the absence of structural data on the organization of the extracellular domains of the TCR–CD3 complex under pMHC-liganded and -unliganded conditions, these models remain largely speculative (Mitra et al. 2004).

5.3.1 *Models Implying Architectural Changes in TCR–CD3 Quaternary Structure*

The elegance of the concept of allosteric control where binding of a ligand at one site affects a distant functional site through a conformational change has stimulated efforts to document whether allosteric changes occur during TCR triggering. Some studies suggested that after TCR engagement, a conformational change in the quaternary structure of the TCR–CD3 complex demasks the evolutionary conserved

PRS sequence found in the cytosolic juxtamembrane segment of CD3 ϵ , and permits the docking of the Nck adaptor through its first Src-homology (SH) 3 domain (Gil et al. 2002, 2005). Ligand-induced PRS exposure appears to occur prior to and independent of ITAM phosphorylation. Considering that overexpression of a dominant negative form of Nck inhibits early T-cell activation events, it has been suggested that PRS exposure constitutes a crucial “trigger” in the causality chain initiated by TCR engagement. Note that among ITAM-containing subunits, the PRS is unique to the CD3 ϵ polypeptide. Therefore, considering that $\alpha\beta$ and $\gamma\delta$ TCRs are the only immunoreceptors that contain CD3 ϵ polypeptides, they may function via a mode distinct from that of all other ITAM-associated immunoreceptors.

In mutant T cells expressing a CD3 ϵ polypeptide where all the proline residues of the PRS sequence have been converted into alanine, the interaction between CD3 ϵ and Nck was abolished, confirming that it depends on the CD3 ϵ PRS. This interaction was, however, dispensable for T-cell development and function (Szymczak et al. 2005). The demonstration that the Nck–PRS interaction is biologically irrelevant does not invalidate the hypothesis that the TCR–CD3 complex undergoes conformational change after pMHC binding. Exposure of the PRS may be only one among several changes that occur in the architecture of the TCR–CD3 complex as a result of TCR engagement. For instance, it has been shown that ligation of the TCR leads to exposure of the amino terminus of CD3 ζ , which is ordinarily buried within the complex, and to the dissociation of CD3 ζ from the remainder of the TCR–CD3 complex (La Gruta et al. 2004). Note that the strong association observed between the two transmembrane helices of the CD3 $\zeta\zeta$ transmembrane likely precludes them from undergoing rotational motion (Call et al. 2006). In contrast to PRS unmasking, the architectural changes involving CD3 ζ are dependent on Src family PTKs and thus presumably occur after or concurrently with kinase activation. The biological relevance of the changes postulated to occur in the TCR–CD3 quaternary structure remains however to be validated.

The view that conformational changes in the TCR–CD3 complex mediate critical “data transfer” from the external environment to the intracellular compartment is inconsistent with comparison of crystal structures of soluble $\alpha\beta$ TCRs in their liganded and unliganded states. Although these structural data contribute only part of the information required to improve our understanding of TCR triggering in the context of the cell surface, they clearly show that TCR engagement does not demask a “trigger” in the TCR ectodomain resembling the dimerization motif found in the epidermal growth factor receptor (EGFR) ectodomain (Garrett et al. 2002; Ogiso et al. 2002). Moreover, no positional shift occurs at the membrane-proximal ends of the TCR following binding to pMHC, although conformational changes in the TCR antigen-binding site can be considerable (reviewed in Rudolph et al. 2006). Likewise, the TCR structure is essentially the same whether bound to agonist, superagonist, or antagonist pMHC ligands. However, one exception reported by Kjer-Nielsen is the subtle difference in the conformation of the A-B loop of the α domain in the crystal structure of the human TCR LC13 in isolation and in complex with its cognate pMHC ligand (Kjer-Nielsen et al. 2003). Considering that the α domain shows both some flexibility and an unusual Ig fold due to the absence of

a typical outer β -sheet, it has been speculated that it can switch to a distinct conformation upon pMHC binding. This switch may affect the organization of CD3 components and trigger the activation cascade. Interestingly, the structure of the TCR C γ and C δ domains is distinct from that of C α (Allison et al. 2001). Both the C γ and C δ domains are composed of a “classical” Ig domain with an outer β -sheet made of three strands. This suggests that in the case C α plays an important function in data transfer during TCR triggering, the corresponding mechanism does not extend to $\gamma\delta$ TCR–CD3 complex.

The view that TCR–CD3 quaternary changes are implicated in critical “data transfer” is also inconsistent with the observation that the ectodomains of the TCR–CD3 complex are dispensable for T-cell activation. For instance, following their artificial attachment to the inner leaflet of the membrane and their fusion to the FK506-binding protein 12 (FKBP12), monomeric CD3 ζ chains can be oligomerized by the small synthetic molecule FKBP12, and trigger activation events that faithfully mimic those induced by antibody-mediated cross-linking of the TCR–CD3 ectodomains (Spencer et al. 1993). Moreover, in many engineered TCRs, extracellular domains and ITAM-containing intracellular domains have been mismatched via different intervening transmembrane segments. Given that the majority of these hybrid receptors were capable of activating T cells, little selectivity and structural constraint seems required at the level of the segments connecting extracellular and intracellular domains (Choudhuri et al. 2005b). These rather loose constraints should be compared to the tight constraints needed for the activation of seven-transmembrane-domain receptors, a class of receptors where the transmembrane segments act as a switch that transmit ligand-induced conformational changes across the membrane to large heterotrimeric G proteins.

5.3.2 *Models Not Relying on TCR–CD3 Quaternary Changes*

The fact that ITAM-containing transducing subunits can indiscriminately cooperate with ligand binding units belonging to the Ig superfamily or to the C-type lectin family raises the question of how ligand binding to such diverse extracellular domains can be communicated to the common architecture of the cytoplasmic domains of the transducing subunits. It has been suggested that all the ITAM-associated immunoreceptors use a common strategy for conveying signals through the membrane that consists in changing the local, steady-state balance of intracellular PTKs and PTPases through ligand-induced oligomerization (reviewed in Davis and van der Merwe 2006; Malissen 2003a; Strong and McFarland 2004). Once phosphorylated by the Src family PTK Lyn, the ITAM-associated with the transducing subunits of the receptor for the Fc domain of IgE (Fc ϵ RI) recruit Syk, a PTK that constitutes the founding member of the Syk family. Using the Fc ϵ RI receptor, a more tractable receptor than the TCR, it has been possible to demonstrate that when the relative Lyn/Fc ϵ RI molecular ratio is low, the Lyn-mediated phosphorylation of the Fc ϵ RI ITAMs is absolutely dependent on prior ligand-induced

receptor oligomerization. In contrast, at a high Lyn/Fc ϵ RI ratio, the rate of spontaneous ITAM phosphorylation is faster than the rate of spontaneous ITAM dephosphorylation. Accumulation of tyrosine-phosphorylated Fc ϵ RI subunits, even in the absence of ligand-induced oligomerization, is the result (Scharenberg et al. 1995; Vonakis et al. 2001). Therefore, for at least some ITAM-based receptors, changing the intracellular balance of PTKs and PTPs can replace external stimuli and convert a ligand-activated switch into a constitutively active device. The ability to manipulate the ground-state PTK/PTP ratio in a ligand-dependent and focal manner also lies at the heart of the three models of TCR triggering to be described next.

5.3.2.1 “pMHC-Mediated Heterodimerization” Model

The TCR binds to the top of the MHC peptide-binding groove, whereas the CD4 and CD8 molecules bind to an invariant surface found at the bases of the MHC class II (MHCII) and MHC class I (MHCI) peptide-binding domain, respectively. Because the two surfaces recognized by the TCR and the CD4/CD8 molecules are non-overlapping, it is thus sterically possible for a single pMHC/II complex to be bound simultaneously by a specific TCR and a CD4/CD8 molecule (Chang et al. 2005). Early work suggested that this simultaneous engagement is required for optimal TCR triggering, and led CD4 and CD8 to be denoted as “coreceptors”. The divalent nature of a pMHC monomer is the basis of the “pMHC-mediated heterodimerization” model of TCR triggering (Malissen 1996). According to this model, pMHC-dependent aggregation of a monomeric TCR–CD3 complex and of a Lck-associated coreceptor molecule suffices to modify in the TCR vicinity the steady state PTK/PTP balance in favor of PTK, and to trigger T-cell activation. This model applies equally well to CD8-dependent, MHCI-restricted TCRs as to CD4-dependent, MHCII-restricted TCRs. We will discuss it below for the former case.

The activation of ZAP-70, the “effector” PTK in charge of propagating TCR-elicited signals requires a “priming module” made of Lck and of ITAM-containing CD3 subunits (reviewed in Malissen 2003a). This module is primarily intended to create high affinity, phosphotyrosine-based docking sites for the tandem SH2 domains found in ZAP-70, and to destabilize the inhibited conformation assumed by ZAP-70 under resting conditions (Brdicka et al. 2005). The “priming” Lck kinase is anchored in the membrane via lipid modification, and also associates to the cytoplasmic tail of CD8 through a zinc chelate complex. Thereby, CD8 constitutes a specialized device that can bring, via translational diffusion, Lck into contact with a pMHCI-occupied TCR–CD3 complex and increase its local concentration. Following CD8–MHCI association, the probability of an encounter between a CD8-associated Lck and the CD3 cytoplasmic tails of pMHC-engaged TCR–CD3 complex increases. This results in strong phosphorylation of the CD3 ITAMs and subsequent recruitment and activation of ZAP-70. Although TCR–CD8–pMHCI ternary complexes have a greater stability (longer half-life) than TCR–pMHCI binary complexes (Luescher et al. 1995), the primary function of CD8 is probably not to reinforce the adhesiveness between T cells and antigen-presenting cells, but

rather to increase the time an Lck kinase will reside in contiguity with CD3 ITAMs and ZAP-70.

What physiological benefit might result from the fragmentation of the TCR into receptor and coreceptor subcomplexes recognizing two distinct sites on the same pMHC ligand? It has been hypothesized that this structural fragmentation lies at the heart of T-cell physiology in that it permits a kinetic discrimination between short- and longer-lived TCR–pMHC interactions (Germain and Stefanova 1999). Provided that in the resting state most coreceptors are physically separated from the pool of TCR–CD3 complexes, some time must elapse between the encounter of a pMHC-occupied TCR–CD3 complex and of a coreceptor (Yachi et al. 2005). This time delay in coreceptor recruitment depends primarily on the surface density of TCRs and of coreceptors, and thus fixes a kinetic threshold to TCR triggering. A long lived TCR–pMHC complex would then have a high probability of recruiting a coreceptor and of leading to TCR triggering, whereas signaling proficient TCR–CD8–pMHC ternary complex would have a low probability of assembling if a low affinity pMHC ligand dissociates almost immediately from the TCR. Consistent with the above views, it should be mentioned that the contribution of CD8 to TCR triggering becomes particularly important when TCR–pMHC interactions exhibit K_D values above $3\mu\text{M}$. Therefore, since the majority of TCR–pMHC interactions occur in the micromolar range, they likely require CD8 to result in TCR triggering (Holler and Kranz 2003).

According to the “pMHC-mediated heterodimerization” model, the CD4 and CD8 coreceptors constitute “kinetic gatekeepers” allowing the length of occupancy of the TCR binding site to be measured. Therefore, they constitute “intrinsic components” of the TCR–CD3 complex rather than “plain” co-inhibitors and co-stimulators. The function of the latter is more likely to be to relay information on the cellular environment in which antigen recognition occurs and to act as “context” detectors capable of tuning activation thresholds. In support of the “pMHC-mediated heterodimerization” model, monomers of pMHC have been shown capable of activating adherent T cells (Delon et al. 1998). This observation is, however, controversial and other studies have suggested that soluble pMHC ligands need to be at least dimeric to trigger T-cell activation (Stone and Stern 2006). It has been suggested that these inconsistencies in the nature of the basic signaling unit that results in TCR triggering reflect differences in the state of activation of the T cells under study. For instance, in the case of adherent T cells, coreceptor-associated Lck kinases might be in a “high” state of activation, whereas when T cells are kept in solution, coreceptor-associated Lck kinases might be in a “low” state of activation (Doucey et al. 2003b; Randriamampita et al. 2003). In the latter instance, dimerization of TCR–CD3–CD8 complexes would result in two Lck kinases being brought together, and thereby convert them through trans-autophosphorylation into a “high” state of activation (Malissen 2003a). Importantly, however, the “pMHC-mediated heterodimerization” model fails to explain the fact that $\gamma\delta$ T-cell activation does not rely on the presence of the CD4/CD8 coreceptors. Elucidating the nature and the valency of the ligands recognized by $\gamma\delta$ T cells will certainly help our understanding of the mechanisms leading to coreceptor-independent $\gamma\delta$ TCR firing, and will probably also shed light

on the fact that for some $\alpha\beta$ TCRs expressed on T-cell clones and hybridomas, coreceptors are clearly dispensable when agonist ligands are expressed at supra-physiological concentrations.

5.3.2.2 “Pseudodimer” Model

Because the intracellular machinery that generates pMHC ligands does not sort out foreign peptides from self peptides, the surface of a cell that is being scanned by TCRs is a mosaic of self- and foreign-pMHC ligands. In contrast to other models of TCR triggering that focus on foreign, agonist pMHC ligands, the “pseudodimer” model confers an important role to MHCII molecules bearing peptides derived from self-proteins, and that are referred to here as endogenous pMHCII complexes (Krogsgaard and Davis 2005; Krogsgaard et al. 2005). The “pseudodimer” model was originally developed for CD4⁺ T cells. It stems from the observation that endogenous pMHCII complexes accumulate in immunological synapses when agonist pMHCII complexes are also present to drive activation. Although they are unable to induce TCR triggering on their own, endogenous pMHCII complexes are capable of facilitating TCR triggering by very low densities of agonist pMHCII complexes. Based on the assumption that in the resting state some CD4 coreceptors are physically associated with TCR–CD3 complexes and that, in contrast to CD8, CD4 could not bind the same pMHCII as the TCR, a “pseudodimer” model was proposed in which the basic signaling unit involves two TCRs, one CD4 molecule, one MHCII molecule bound to an agonist peptide, and a second MHCII molecule bound to a self-peptide. According to that model, a cooperative effect results from the complexation of one TCR binding to an agonist pMHCII ligand and another binding to an endogenous pMHCII, with CD4 acting to link the two TCR–CD3 complexes. The “pseudodimer” model appears only applicable to some CD8⁺ T-cell clones (Cebecauer et al. 2005; Sporri and Reis e Sousa 2002).

Considering that the affinity of CD4 for MHCII is much lower than the affinity of CD8 for MHCI, Choudhuri and colleagues (Choudhuri et al. 2005a) suggested that the “pseudodimer” and “pMHC-mediated heterodimerization” models can be integrated into a single one. They proposed that the affinity of the CD8–MHCI interaction is sufficient to recruit CD8 to pMHCI-engaged TCR–CD3 monomeric complex and initiate triggering. In contrast, the CD4–MHCII interaction is too weak to drive the assembly of TCR–CD3–CD4 complexes, and an additional weak interaction between a CD4-preassociated TCR–CD3 complex and a MHCII molecule bearing an endogenous peptide is simultaneously required to stabilize CD4–MHCII interaction and initiate triggering. Note that according to the “pseudodimer” model, the Lck molecule associated to CD4 can phosphorylate CD3 subunits belonging to either of the two TCR–CD3 complexes present in the “pseudodimer”.

It has been previously suspected that a weak reactivity toward self pMHC complexes expressed on the APCs found in secondary lymphoid organs permits the survival of naïve T cells in the periphery and keeps them in a state of heightened antigen sensitivity (Stefanova et al. 2002). The observation that suboptimal TCR

signals might prime naïve T cells and allow them to respond strongly to a subsequent agonistic challenge is reminiscent of the situation documented for the IFN- α/β signaling pathway (Taniguchi and Takaoka 2001). Interestingly, the “pseudodimer” model extends the role attributed to self pMHC complexes by demonstrating that they also assist the TCR during agonist stimulation, and thus constitute bona fide “accessory self-peptides”. Therefore, the thymus is not the only anatomical site where the TCR makes biologically relevant, low-affinity contacts with self pMHC complexes. Therefore, the purpose of TCR $\alpha\beta$ positive selection is probably to select a peripheral TCR repertoire that can use self pMHC complexes to achieve maximal sensitivity toward the same self MHC molecules bound to foreign peptides.

5.3.2.3 “Kinetic-Segregation” Model

The cumulative length of the ectodomains of a pair of TCR–pMHC molecules is relatively short, spanning approximately 14 nm, whereas receptor PTPases known to inhibit TCR triggering, including CD45 and CD148, have much larger ectodomains. This observation suggested that the passive, size-based exclusion of the PTPases from the close-contact zones that form between a T-cell and an APC probably increases the half-lives of phosphorylated species in that region and thus favors tyrosine phosphorylation of TCR–CD3 complexes (Springer 1990). By holding the T-cell and APC membranes the appropriate distance apart, small adhesion molecules such as CD2 likely facilitate size-based exclusion of bulky PTPases and the encounter between the TCR and its pMHC ligand. It should be stressed that prior to the encounter of an APC, intermolecular collisions are likely to occur between Lck and CD3 subunits in the plane of the T-cell membrane. These transient interactions probably result in some constitutive phosphorylation of the CD3 subunits of the TCR complex. However, due to the action of PTPases, the net level of CD3 phosphorylation is kept too low for activation, although it may be sufficient to heighten T-cell sensitivity and promote T-cell survival (Stefanova et al. 2002). Size-based exclusion of PTPases constitutes the basis of the model referred to as the “kinetic-segregation model” (Choudhuri et al. 2005b). Importantly, this model allows for weak, ligand-independent TCR triggering in the PTPase-free, close-contact zones. The presence of agonist pMHC ligands in the close-contact zone essentially prevents ligand-engaged TCRs to diffuse outside of the close-contact zone and thus increases the half-life of the phosphorylated CD3 ITAMs they associate with. This increase is commensurable to the half-life of the TCR–pMHC interaction. It is important to note that the “kinetic-segregation model” avoids an absolute requirement for coreceptor by postulating that free Lck molecules associated with the membrane inner leaflet suffice to phosphorylate the CD3 subunits present in the PTPase-free close-contact zones. According to this model, the function of coreceptors is thus limited to signal amplification by recruiting additional Lck in the vicinity of ligand-engaged TCRs and by stabilizing their interaction with their substrates (CD3 ITAMs and ZAP-70) (Davis and van der Merwe 2006).

5.3.2.4 Other TCR Triggering Models

Provided that the CD3 subunits are tightly associated to the TCR, the whole TCR–CD3 complex would likely behave as a rigid body, and it has been suggested that pMHC binding pushes the whole TCR–CD3 complex into the cell membrane. This “piston-like” movement could expose CD3 ITAMs to the constitutive action of Lck and trigger the signaling cascade (reviewed in Krogsgaard and Davis 2005). Finally, it should be mentioned that TCR–CD3 complexes have been postulated to exist in the membrane as higher-order structures (TCR oligomers) even in the absence of cognate pMHC ligands (Schamel et al. 2005). Binding of pMHC to these postulated oligomers could alter their distance or the relative orientation of their individual components and result in TCR triggering.

5.4 TCR Assembly During Intrathymic Development

The α and β chains of the TCR control both the physiology of mature T cells and the unfolding of the intrathymic T-cell development program. During the latter process, TCR chains constitute key components of molecular sensors that counteract the stochastic nature of V(D)J recombinations and prevent the possible emergence of cells bearing strongly autoreactive TCRs (von Boehmer et al. 2003). Genetic studies have greatly contributed to the definition of the two consecutive developmental checkpoints that are controlled by these sensors and at which T cells progressing along the $\alpha\beta$ -lineage undergo programmed cell death if they fail to rearrange TCR genes productively or if they express a TCR with inappropriate specificities.

5.4.1 TCR β selection

Transition through the earliest developmental checkpoint requires the operation of a sensor known as the pre-TCR. Because TCR β gene rearrangements precede rearrangements at the TCR α locus, the pre-TCR complex lacks a TCR α chain. It is composed instead of a pT α TCR β heterodimer that is non-covalently associated to CD3 $\gamma\epsilon$ and CD3 $\zeta\zeta$ dimers (reviewed in Malissen et al. 1999). At this stage of development, the pT α and CD3 components of the pre-TCR are already available and it is the TCR β polypeptide that constitutes the rate-limiting factor in the assembly of the pre-TCR complex. As stressed by Harald von Boehmer, the invariant pT α subunit of the pre-TCR is more than just a structural substitute for the missing TCR α chain (von Boehmer 2005). It contains a unique proline-rich motif in its cytoplasmic tail that appears important for proper pre-TCR signaling. It has been further suggested that once assembled, the pre-TCR signals without binding to exogenous ligands. This cell-autonomous and ligand-independent mode of action

is likely due to the fact that, once incorporated in the pre-TCR complex, pT α ectodomains have the ability to spontaneously form oligomers (Yamasaki et al. 2006). The phenotypic transition induced by the pre-TCR is generally referred to as β -selection. There is no selection for particular V β gene products at this check-point; it ensures only that double-negative (DN) CD4⁻CD8⁻ cells with productive TCR β gene rearrangements become double-positive (DP) CD4⁺CD8⁺ cells (Wilson et al. 2001). The pre-TCR also contributes to establish clonal expression of TCR $\alpha\beta$ heterodimers in that it prohibits further TCR β chain rearrangements on the second TCR β allele through the process of allelic exclusion.

5.4.2 TCR $\alpha\beta$ selection

Signals emanating from the pre-TCR also trigger the induction of a high rate of TCR α rearrangements among DP cells. Following productive TCR α rearrangements and substitution of pT α by TCR α , DP cells express at their surface clonally distributed mature $\alpha\beta$ TCR–CD3 complexes. Based on the specificity of their TCR for self-pMHC ligands, a small percentage (3 to 5%) of DP cells are rescued from programmed cell death and induced to differentiate into CD4⁺CD8⁻ and CD4⁻CD8⁺ single positive (SP) cells. Such second developmental checkpoint that marks the boundary between the DP and the SP stage of thymic differentiation is referred to as TCR $\alpha\beta$ selection, and is dependent on TCR binding specificity (see below). It is ablated by a number of genetic defects affecting (1) the assembly and proper cellular display of self pMHC ligands at the surface of thymic stromal cells (2) the synthesis of TCR–CD3 complexes (3) the CD4 and CD8 coreceptors, and (4) components of the signaling cassette activated by $\alpha\beta$ TCRs (reviewed in (Malissen et al. 1999)). SP cells correspond to the end products of the intrathymic $\alpha\beta$ T-cell differentiation sequence, and gradually exit from the thymus to reach peripheral secondary lymphoid organs.

Because the site-specific DNA recombination reactions that affect the TCR genes result in the random reassortment of a limited set of germ-line encoded CDR1 and CDR2 loops and of a large set of somatically encoded CDR3 loops, the population of DP T lymphocytes present in the thymus shows a diverse repertoire of clonally distributed $\alpha\beta$ TCRs. To function appropriately, TCRs have to match the combination of self-MHC gene products that are coincidentally inherited and available from conception. Consequently, after the generation of an MHC allele-neutral TCR repertoire in DP thymocytes, the phase of epigenetic molecular matching known as positive TCR $\alpha\beta$ selection is intended to favor the development of those clones expressing TCRs capable of weakly interacting with self-pMHC. Most DP thymocytes express TCRs with insufficient self-reactivity to mediate positive TCR $\alpha\beta$ selection and die in situ through programmed cell death. A subset of DP thymocytes bearing TCRs with excess self-reactivity is also eliminated by apoptosis. This last mechanism of clonal deletion is known as negative TCR $\alpha\beta$ selection and results in central tolerance. Therefore, whereas negative thymic selection prevents

the emergence of strongly autoreactive T-cell clones, positive selection prevents the overloading of the periphery with T cells expressing TCR unable to fit structurally and cooperate with the inherited MHC alleles. It should be stressed that the nature and complexity of the self-peptides involved in positive selection and their degree of structural relatedness to the antigenic peptides that are encountered in the periphery remains controversial (Hogquist et al. 2005). The antigenic specificity of a given T-cell clone is fixed by the irreversible shut-down of the V(D)J recombinase machinery that is associated with positive TCR $\alpha\beta$ selection. Moreover, in contrast to Ig genes, TCR genes are not subjected to somatic hypermutation in the periphery.

5.4.3 Secondary TCR α Rearrangements and Editing

The structure of TCR α locus is well suited to secondary rearrangements, providing a given DP cell with the possibility to express consecutively several TCRs and increasing its probability of being positively selected and thus of escaping “death by neglect”. It is not clear, however, whether there is enough time between consecutive rounds of TCR α rearrangements to test each novel TCR α chain for binding to self pMHC ligands (Davodeau et al. 2001). It has also been suggested that DP cells can escape death by negative selection using secondary rearrangements as well (Hogquist et al. 2005). The physiological role of this phenomenon, referred to as receptor editing, remains however to be determined.

5.5 How TCRs Bind pMHC

5.5.1 General Features

The first TCR–pMHCI crystal structures have permitted to define a few “general” features of TCR–pMHCI interactions (Bankovich and Garcia 2003; Housset and Malissen 2003; Krogsgaard and Davis 2005; Rudolph and Wilson 2002; Rudolph et al. 2006). For instance, the docking of the TCR on the pMHC is such that the V α and V β domains are closest to the amino- and carboxy-terminal residues of the antigenic peptide, respectively. Moreover, the extremely variable CDR3s are generally located over the center of the pMHCI surface and make contacts with the antigenic peptide as well as with the MHC α helices, whereas the less variable CDR1–CDR2 loops contact the termini of the bound peptide and residues of the MHC α helices (CDR1s), or exclusively the central part of the MHC α -helices (CDR2s). This “diagonal” TCR docking topology allows the relatively flat TCR binding surface to “slot” between the two high “peaks” that are found near the amino termini of the two MHC α helical regions, and thus maximizes the readout of the few peptide residues that are exposed to the TCR.

MHCII molecules bind processed peptides as long as 20 amino acids such that the peptide termini extend out of the open ends of the MHC groove (Batalia and Collins 1997). The peptide backbone in MHCII molecules is bound in an extended polyproline type II conformation and adopts a rather deep course in the binding groove. This contrasts with the situation observed in MHCI alleles where conserved hydrogen bonds fix the position of the amino and carboxy termini of the bound peptides, forcing the central part of peptides longer than eight amino acids to bulge out of the groove and become more accessible for TCR inspection. The structure of several TCR–pMHCII complexes has been recently determined (Hahn et al. 2005; Hennecke and Wiley 2002; Li et al. 2005a; Maynard et al. 2005; Reinherz et al. 1999). As previously observed for MHCI-restricted TCRs, the orientation of the TCR over the pMHCII surface maximizes contacts with the bound peptide by avoidance of the high points of the MHCII helices and of the ridge created by the amino terminus of the bound peptide. Analysis of the collection of available TCR–pMHCI and TCR–pMHCII complexes reveals the extent of variation afforded around the standard “diagonal” docking mode, and shows that there is no docking mode proper to MHC class I or class II molecules. A single TCR, denoted Ob.1A12 and originating from a patient with multiple sclerosis, showed a totally unusual binding topology (Hahn et al. 2005). Ob.1A12 recognizes with low affinity an immunodominant peptide of myelin basic protein (MBP residues 85–99) bound to HLA-DR2. Rather than being centered over the pMHCII surface it is instead positioned over the P2 peptide residue. This binding mode centers Ob.1A12 over the high point of the DR α helix and on the ridge created by the N-terminal extension of the peptide, indicating that these topological features do not preclude TCR binding as previously postulated. Moreover, the two CDR3 loops of Ob.1A12 create a dome-shaped cavity that is large enough to accommodate both an MHC residue and the P2 side chain. This feature is unprecedented, as the corresponding pocket found in some other TCRs accommodates a single peptide side chain but no MHC residue.

5.5.2 Flexibility of the TCR Antigen-Binding Site

Several TCRs have been crystallized in their unliganded and liganded states. Comparisons of these structures showed important changes that are restricted to the antigen-binding site, and that unevenly affected the various CDRs. For instance in the case of the KB5-C20 and LC13 TCRs, large-scale conformational reorganization of CDR3 loops have been observed upon binding to the pMHC surface, whereas CDR1 and CDR2 loops were the object of “en bloc” movements or of moderate conformational changes (Kjer-Nielsen et al. 2003; Reiser et al. 2003). In contrast, the 1G4 TCR showed very little conformational change upon recognition of HLA-A2 loaded with a peptide denoted ESO 9C and derived from a tumor-specific antigen (Chen et al. 2005). The ESO 9C-HLA-A2 ligand displays a prominent methionine-tryptophan “peg” in the central portion of the ESO 9C peptide,

which forms a large protrusion from the surface of the pMHC. The cavity formed between the CDR1 α , CDR3 α , and CDR3 β loops, and used by 1G4 to accommodate the methionine-tryptophan peg was preformed in the unliganded state. Therefore, in contrast to the conformational flexibility observed during the formation of TCR–pMHC complexes involving the KB5-C20 and LC13 TCRs, the 1G4 TCR showed that a well optimized pMHC-binding surface can be achieved through relatively small structural changes.

Thermodynamic parameters have been measured for a number of TCR–pMHC interactions establishing a recurrent thermodynamic profile whereby TCR ligation is governed by favorable enthalpic forces and unfavorable entropy (Boniface et al. 1999). The entropic penalty probably results from the stabilization of flexible CDR3 loops upon docking to the pMHC, whereas the favorable enthalpic forces result from the extensive network of bonds formed upon ligation. However, this thermodynamic signature may only apply to a subset of TCRs. For instance, it has been shown that the interaction of the LC13 TCR with the FLR–HLA-B8 ligand is entropically and enthalpically driven (Ely et al. 2006). Structural analyses of the LC13–FLR–HLA-B8 complex revealed that the CDR3 loops are well ordered in the unliganded state and undergo a conformational isomerization to adopt the liganded state, with the simultaneous expulsion of water molecules from the TCR–pMHC interface. The thermodynamics of LC13–FLR–HLA-B8 ligation is not the only exception. A similar thermodynamic profile is also found in the case of the formation of other TCR–pMHC complexes (Davis-Harrison et al. 2005; Mazza et al. 2007).

5.5.3 TCR Flexibility Facilitates TCR Binding Degeneracy

Binding specificity and degeneracy are imprecise but widely used concepts in immunology. Degeneracy (also called polyspecificity or molecular promiscuity) can be defined as the ability of a given receptor to interact with structurally distinct ligands. Conversely, specificity (also called monospecificity or molecular monogamy) can be defined as the ability of a given receptor to interact with one or a few ligands that are closely related in structure. Note that drug action is based on binding degeneracy since most drugs bind to sites that evolved to interact with physiological ligands. Degeneracy in protein-mediated recognition can be achieved through structural flexibility of the binding site, allowing the receptor to adapt to distinct ligands through conformational changes. Alternatively, studies of the interaction between the human growth hormone and the growth hormone receptor and of the NKG2D receptor with MHCI-like ligands have shown that an identical and rather rigid surface can be used to contact chemically different ligand surfaces (McFarland et al. 2003). It has also been shown that a given antibody can recognize several 14-mer peptides unrelated in sequences via differential usage of residues located in the binding site, the constellation of interacting residues being unique for each peptide. Finally, binding degeneracy can also be achieved via the

recognition of sequence-independent features. For instance in the case of the MHC peptide-binding groove, interactions with the peptide main chain provides a generic and largely sequence-independent set of interactions.

A given TCR can recognize other pMHC ligands in addition to its cognate pMHC ligand. When the cross-recognized pMHC ligand fortuitously displays the same constellation of TCR contact residues, a phenomenon referred to as molecular mimicry, cross-recognition may be achieved without flexibility in the TCR antigen-binding site (Lang et al. 2002). In contrast, the BM3.3 TCR provided the first example of bona fide TCR degeneracy, in that the flexibility of the TCR antigen-binding site is exploited to facilitate its adaptation to distinct pMHC surfaces (Reiser et al. 2003). Comparative analysis of the BM3.3 TCR in complex with two distinct peptides, pBM1 and VSV8, bound to H-2K^b, showed that BM3.3 recognition focused on peptide position 6 (P6) of pBM1 and of VSV8. P6 is the only position contacted by the TCR that shows a homologous asparagine to glutamine replacement in the cross-recognized VSV8 peptide. All the other positions contacted by the TCR in VSV8 corresponded to non-conservative replacements, and affected TCR binding in a positive (replacement at P4) or a negative (replacement at P7) way. The structurally different replacement observed at P4 of VSV8 was exploited through CDR3 α flexibility, which enables the residues at the apex of the CDR3 α loop to be used fully and to compensate in part for the loosened interactions between the CDR3 β loop and the residue found at P7 of VSV8. Altogether, these results show how structurally different replacements at P4 and P7 were accommodated through CDR3 α loop flexibility, and demonstrate that the BM3.3 TCR adopts unique structural solution to adapt to each peptide. Therefore, TCR cross-reactivity does not always require a high degree of pMHC structural mimicry, and TCR degeneracy is primarily permitted by the malleability of the TCR antigen-binding site.

5.5.4 Affinity, Degeneracy, and Bioactivity

It has been originally suggested that the absence of mature T cells with high affinity TCRs towards foreign pMHC ligands is due to the fact that those TCRs are detrimental to T-cell physiology (as postulated by the “serial engagement model” of T-cell activation [Gonzalez et al. 2005]). Conversely, high-affinity TCRs have been postulated to have a higher probability of cross-reacting with several self-pMHC complexes, leading to intrathymic deletion of the corresponding T cells (Donermeyer et al. 2006; Huseby et al. 2006). However, in the case of antibodies, no obvious link has been found between affinity maturation during secondary B-cell responses and the magnitude of crossreactivity (James and Tawfik 2003). In some instances, affinity maturation of antibodies might even result in the generation of more rigid antigen-binding sites with a narrower specificity. Moreover, in the case of TCRs engineered in vitro to yield variants capable of binding the cognate pMHC ligand with higher affinity, the resulting increase in affinity (up to 26 pM) was not

systematically associated with a broadening of specificity (Holler et al. 2003; Laugel et al. 2005; Li et al. 2005b). Therefore, TCR affinity and cross-reactivity appear to constitute two independent variables, and the absence of mature T cells with high-affinity TCRs is likely due to the fact that long TCR–pMHC bond lifetimes are detrimental to T-cell activation when pMHC ligands are present in limited amounts (Gonzalez et al. 2005).

5.5.5 Raison d'Être of TCR Degeneracy

Although the notion of degeneracy tends to be linked with deterioration in quality, it has been argued that TCR degeneracy has been selected to allow TCRs to recognize a universe of structurally diverse peptide antigens much larger in number than its own combinatorial diversity and than the number of T cells contained at a given moment in each individual (Mason 1998). Landsteiner and Pauling similarly hypothesized in the 1930s, that provided that a given antibody exists as an ensemble of isomers, each with a different antigen-binding site structure capable of accommodating a different ligand, then functional diversity could go far beyond primary sequence diversity. T-cell antigen receptor degeneracy is also at play during intra-thymic T-cell positive selection and antigenic responses in the periphery. In these two instances, the cross-recognized ligands consist of structurally distinct peptides presented by the same self-MHC molecule. T-cell antigen receptor degeneracy is also directly involved in heterologous immunity (Selin et al. 2004), and allows adaptive immunity to cope with antigenic variation during antiviral immunity (Goulder and Watkins 2004). Considering that much of the burden of infectious diseases today is caused by antigenically variable pathogens that can escape from adaptive immunity induced by prior infection or vaccination, TCR, as well as antibody, degeneracy is of immense importance for vaccine design. Finally, considering that the TCR can translate small quantitative differences in ligand binding into qualitatively different signals, TCR degeneracy does permit the occurrence of T-cell responses that range from maximal activation to desensitization. In the absence of TCR-binding degeneracy, no variation in TCR–pMHC bond lifetimes would be allowed for a given TCR, and its signaling properties would be limited to a mere on-off switch.

5.5.6 Alloreactivity and Xenoreactivity

Effector T cells preferentially interact with target cells bearing MHC alleles that the T cells had experienced in the thymic environment during development, and physiological cross-recognized ligands consist primarily of distinct peptides presented by the same self-MHC molecule. However under experimental or clinical conditions, TCRs can even react against MHC molecules not seen during thymic selection. For instance, many TCRs selected to respond to foreign peptides bound

to a self-MHC molecule display a concomitant cross-reactivity for intraspecies allelic variants of self-MHC molecules that can differ by up to 20 amino acids. This property, termed *alloreactivity*, causes graft rejection and graft-*versus*-host disease. Most of these polymorphic residues line the peptide-binding groove where they determine peptide-binding specificity, while a few of them are located on the top of the α -helices that form the groove and are thus available for TCR contact. Based on crystal structures of TCR-pMHC allocomplexes, it appears that during *allorecognition*, TCRs neither avoid contacting the bound peptide, nor focus on the polymorphic residues that are exposed on the top of the allo-MHC α helices (Housset and Malissen 2003). Although structures corresponding to the same TCR in complex with a self- and an allo-MHC are not available yet, a parsimonious interpretation of the available data suggests that during *allorecognition*, TCRs exploit the similarities rather than the differences between the top of the helices of self- and allo-MHC molecules, and that the high precursor frequency of *alloreactive* T cells is accounted for by the fact that allo-MHC molecules display a totally new constellation of endogenous peptides against which the repertoire of mature T cells has not been negatively selected in the thymus (Housset and Malissen 2003).

Some TCRs can even find compatible landmarks on MHC molecules belonging to other species. Such cross-reactivity for MHC across species is termed *xenoreactivity*. For instance, the mouse TCR repertoire is capable of recognizing transgenic human HLA molecules, although with a greatly reduced efficiency (Firat et al. 2002). This trans-species cross-reactivity is likely due to the shared amino acids that occur at positions through the entire spectrum of mammalian MHCI molecules. By elucidating the structure of a mouse TCR bound to a human MHCI molecule Buslepp and colleagues (Buslepp et al. 2003) provided the first hints to the structural basis of TCR *xenoreactivity*. They showed that *xenoreactive* recognition follows the same conserved docking orientation as self-pMHC and allo-pMHC recognition. Moreover, the bound peptide is engaged as a specificity element, and the TCR does not appear to focus on the species-specific residues that are exposed on the outer surface of xeno-MHC helices. Therefore, TCRs might exploit the similarities rather than the differences between the top of the helices of self- and xeno-MHC molecules, and avoid a global repositioning on binding to a xeno-pMHC surface. T-cell antigen receptor trans-species cross-reactivity appears thus permitted by the shared amino acids that fortuitously occur at TCR contact positions through the entire spectrum of mammalian MHC molecules (Malissen 2003b). A direct consequence of these observations is that in a clinical allotransplantation set-up, selecting donor-recipient combinations that maximize the differences in the set of residues that are sensed by the TCRs should dampen the strength of T-cell *allosponses* (Housset and Malissen 2003). Conversely, MHC alleles differing by only a minimal mismatch might induce *allosponses* with as great or even with a greater magnitude than observed across more disparate MHC allelic differences. Consistent with these views, Macdonald and colleagues (Macdonald et al. 2003) have recently demonstrated that powerful *alloreactivity* occurs across a “minimal” mismatch that involves one residue on the $\alpha 2$ helix that is inaccessible to direct TCR recognition and only alters the selection of the peptides.

5.5.7 Revisiting the Extent of TCR Degeneracy and the Specificity of Cross-Reactivity

Recent data suggest that TCRs are probably much less degenerate than assumed on the basis of theoretical considerations (Mason 1998), or on the utilization of combinatorial peptide libraries (Maynard et al. 2005). For instance, in the case of the BM3.3 TCR system, the upward pointing peptide residue P6 serves as a focal point for the BM3.3 TCR (Reiser et al. 2000; Reiser et al. 2003). P6 tolerates very few if any substitution and thus constitutes a “primary TCR contact residue” according to the nomenclature introduced by Allen and colleagues (Shih and Allen 2004). Residue P4 and P7 constitute “secondary TCR contact residues” and some mutations in these residues are less detrimental to the recognition process. The existence of “primary TCR contact residues” explains how some T cells can discriminate between peptide structures that differ by a single oxygen atom in a peptide side chain (for instance as the result of a phenylalanine to tyrosine substitution), and achieve specific recognition under conditions where there is generally a 10^3 – 10^4 excess of peptides that derive from self-proteins and may differ from the foreign peptide by only a single amino acid. Conversely, the malleability of the CDR3 α loop allows the BM3.3 TCR to cope with some non-conservative substitutions in the P4 and P7 secondary TCR contact residues, and account to its degeneracy. Importantly, each alternative docking solution achieved by BM3.3 involves a highly specific and fortuitous bond network (Reiser et al. 2003). As vividly stated by Garcia and colleagues, “CDR3 can assume different conformations to recognize a limited number of alternative peptides with a high degree of specificity, but they are not easily accommodating limp noodles” (Garcia and Adams 2005). Therefore, the BM3.3 TCR illustrates how a single TCR antigen-binding site can display both high specificity and degeneracy, two structural properties that appear a priori conflicting. It also demonstrates how the tight constraints imposed by a primary TCR contact residue and the limited number of permissible changes allowed in the secondary TCR contact residues significantly restrict the number of potential ligands that are recognizable as agonist by a given TCR.

5.5.8 Receptor Versus Cellular Specificity

Receptor specificity and cross-reactivity constitute empirical and assay-dependent properties. Accordingly, the sensitivity of some in vitro assays is sometimes much too high and reveals cross-reactions that are not biologically relevant (Zinkernagel 2002). For instance, the observation that cytotoxic T cells stained with pMHCI multimeric probes does not always mean that they are capable of productive responses in vivo. Importantly, the range of cross-reactivity exhibited by a given TCR is not a property intrinsic to the TCR. It also depends on the developmental stage of the T-cell clone that expresses it, and on the architecture of the signaling

network that acts downstream of the TCR. For instance, it has been shown that the protein tyrosine phosphatase SHP-1 can increase the specificity of TCR signaling by preventing spurious T-cell activation with large quantities of low-affinity pMHC ligands, while permitting sensitive responses towards small quantities of more strongly binding pMHC complexes (Altan-Bonnet and Germain 2005; Stefanova et al. 2003). Importantly, the point where ligand discrimination is set is not “hard-wired” into the TCR but can be modulated by changing the concentration of key components of the feedback loops that control the TCR signaling cassette. A state of TCR-signaling hypersensitivity can also be induced during lymphopenia (Goldrath and Bevan 1999), following adhesion-induced T-cell priming (Doucey et al. 2003a), or in the presence of the CD4 or CD8 coreceptors (Donermeyer et al. 2006; Holler and Kranz 2003; Renard et al. 1996), resulting in broadened TCR specificity.

T-cell antigen receptor (or more appropriately T cell) specificity is thus a property of the whole T cell that can be either sharpened by negative feedback loops that blunt signals emanating from weak pMHC ligands, or conversely relaxed by enhancing the output of the TCR signaling cassette (Altan-Bonnet and Germain 2005; Lin et al. 1997). Therefore, factors not directly related to the affinity of the TCR–pMHC interaction can determine the outcome of a T-cell response. This is due to the fact that the TCR is only one part of a complex cellular sensor, and that the state of activation of APCs and even the quality of the neighboring T cells can dramatically influence the response of a given T-cell clone. For instance, a study by Kedl and colleagues (Kedl et al. 2003) showed that high-affinity T cells can induce a selective loss of peptide-MHC complexes from the surface of antigen-presenting cells (APCs). By reducing the antigenic determinants on APCs, high-affinity T cells likely outcompete low-affinity ones. Along the same line, regulatory T cells can “deactivate,” in a direct or indirect (via APCs) manner, signaling proficient T cells (Fontenot and Rudensky 2005). Therefore, regardless of the fact that functional specificity originates from the V domains of TCRs, operational end points for assessing immunological specificity must be higher-level cellular functions or even organismal functions. As stated by R. Zinkernagel, observing whether a mouse ends up “legs up or legs down” following a viral challenge remains the ultimate cut-off for adaptive immunity.

5.6 What Causes the Restriction in Orientation Imposed on TCR–pMHC Interactions?

All but one of the TCRs analyzed to date bind to pMHC with roughly similar diagonal geometry. However, the shapes and chemical properties of the interacting surfaces found in these complexes are so diverse that no fixed contact exists between conserved TCR residues and a conserved area of the MHC α -helices (Gagnon et al. 2005; Garcia and Adams 2005; Housset and Malissen 2003; Rudolph et al. 2006). This absence of recognizable landmarks or pivot points within the

TCR–MHC interface suggested that the molecular cues responsible for enforcing the globally conserved mode of TCR docking on MHC molecules are encoded outside of the TCR/MHC pair, and might, for instance, be sought in the action of the CD4 and CD8 coreceptors. It has been argued that the conserved orientation of the TCR over the pMHC corresponds to the need to interact maximally with the peptide. However, these considerations would also be compatible with TCR engaging the pMHC with a 180° rotation relative to the observed orientation. Therefore, prior to the phase of intrathymic TCR $\alpha\beta$ selection, the repertoire of assembled TCR binding sites might contain TCRs capable of engaging pMHC with a 180° rotation relative to the “canonical” orientation. However, considering that CD8 likely interacts specifically with the CD3 $\delta\epsilon$ dimer of the TCR–CD3 complex (Doucey et al. 2003a; Yachi et al. 2005), it is plausible that in the case of a TCR–pMHCI docking geometry rotated by 180°, the Lck kinase associated with the CD8 coreceptor will be prevented from phosphorylating the CD3 ITAMs and ZAP-70, thereby impeding selection of the clones bearing those TCRs. Therefore, the need for coreceptor function during TCR $\alpha\beta$ selection may eliminate all the DP cells that are present in the pre-selected TCR repertoire and that express TCR antigen-binding site unable to engage the pMHC surface according to a fixed “signaling-proficient” geometry (Buslepp et al. 2003). The non-canonical docking observed for the coreceptor-dependent Ob.1A12 TCR (see section 5.5.1) further questioned the view that MHC restriction is encoded in the CDR1–CDR2 loops of the TCR V domains.

Although TCR–pMHC crystal structures have not revealed a straightforward “TCR–MHC recognition code,” loose guidance cues in charge of steering TCR–pMHC interactions can still be encoded within the germline encoded CDR1–CDR2 loops of the TCR V domains and in the MHC α helices themselves. A wide range of TCR docking modes can be deduced from all the TCR–pMHC structures solved so far (Rudolph et al. 2006). In some TCR–pMHCI complexes, the CDR2 loops of both V α and V β domains contact the central part of the MHC $\alpha 2$ and $\alpha 1$ helices, respectively. However, in other TCR–pMHC complexes, the two MHC helices are not contacted in a concerted fashion. For instance, the occurrence of contacts between the CDR2 β loop and the MHC $\alpha 1$ helix is clearly optional and contingent on the size of the CDR3 β loop (Ding et al. 1998). Conversely, in the JM22-MP(58–66)-HLA-A2 complex, the JM22 TCR footprint is translated such that the CDR2 α loop makes minimal contact with the MHC $\alpha 2$ helix (Stewart-Jones et al. 2003). Considering that the range of footprints adopted by the CDR1–CDR2 loops encompasses several turns of the MHC α helices and that the detailed atomic features of these interactions are very different, it has been suggested that the apex of the CDR1 and CDR2 loops and the top of the MHC α helix function as a kind of molecular “Velcro” offering multiple “continuous” docking possibilities (Housset and Malissen 2003). Along the same line, Huseby and colleagues have suggested that the CDR1–CDR2 loops primarily contact the main chain atoms of the MHC α helices, and have thus an intrinsic predisposition to adapt to their top regardless of their amino acid side chain content (Huseby et al. 2005). This postulated propensity may account for the fact that several mouse TCRs are capable of

recognizing both pMHC I and pMHC II ligands (see for instance Ge et al. 2006). Whether these unique TCRs use a largely sequence-independent set of interactions with the MHC surface as postulated by Huseby and colleagues (Huseby et al. 2005), or exploit the existence of fortuitous, side-chain encoded sequence similarities on the top of MHC I and MHC II helices remains, however, to be demonstrated.

Recent data, however, are not consistent with the view that the top of the MHC α helices constitutes “sticky” matrices promoting TCR binding in different registers. For instance, comparison of two TCRs (172.10 and scD10) that use the same V β gene segment (V β 8.2), showed that a set of interactions dependent on amino acid side chains might play a key role in steering TCR–pMHC interactions. Comparison of the 172.10-MBP-1-11-I-A^u and scD10-CA-I-A^k complexes showed that the V β 8.2 CDR1–CDR2 loops establish the same multi-point contact patch with the I-A^u and I-A^k MHC α 1 helix (Maynard et al. 2005; Reinherz et al. 1999). This superimposable region comprises five shared TCR residues interacting with five shared MHC residues, forming five hydrogen bonds and ten van der Waals contacts. The correspondence of the CDR1–CDR2 contacts with the MHC α 1 helix in the two structures is highly suggestive of a conserved anchor point. In contrast, comparison of the KB5-C20-pKB1-H-2K^b and BM3.3-pBM1-H-2K^b complexes revealed that the CDR1–CDR2 loops found in the V β 2 domain used by both TCRs adopt different binding registers when docking to the same MHC α helix (Reiser et al. 2002). Therefore, the CDR1–CDR2 loops of a given TCR V domain might alternatively adopt a small set of discrete docking geometries on a given MHC allele (Maynard et al. 2005). The “docking subsite” to be used by a given V would then depend primarily on the V partner it associates with. In support of this view, it should be noted that in each of the KB5-C20-pKB1-H-2K^b and BM3.3-pBM1-H-2K^b complexes, the V β 2 domain was associated with a distinct V α , whereas the V α found in 172.10 and D10 TCRs belonged to the same V α subfamily. It is thus possible that the unique combination of CDR1–CDR2 loops found in a given V α V β pair fixes the overall geometry of the TCR–pMHC interaction, the associated CDR3 loops being able to adapt to this imposed geometry for some pMHC ligands.

The present day TCR V gene segments have evolved through several rounds of duplication. It is thus possible that the position and nature of the primordial set of CDR1–CDR2 residues involved in contacting a fixed site on MHC helices and in steering the binding orientation of a primordial TCR–MHC pair have drifted during the process of V gene diversification, making their recognition difficult. Accordingly, the globally conserved diagonal mode of interaction adopted by all the present day TCR–pMHC pairs could result from a founder-like effect, and constitute variations on the binding geometry adopted by an ancestral TCR–pMHC pair (Housset and Malissen 2003).

The HLA-B*3508 allele binds a 13-amino acid peptide (LPEP) derived from the BZLF1 antigen of Epstein–Barr virus. Considering that the MHC class I antigen-processing pathway and antigen-presentation pathway is biased toward 8- to 10-amino acid peptides, LPEP constitutes thus an unusually long peptide (Burrows et al. 2006). When bound to HLA-B*3508, LPEP adopts a bulged conformation in its center while maintaining conserved networks of hydrogen bonds at its amino

and carboxy termini. In addition, LPEP assumes a rigid conformation and has thus the potential to block sterically TCR engagement. Indeed, determination of the structure of LPEP–HLA-B*3508 in complex with the SB27 TCR showed that the bulged peptide prevented a full engagement of the TCR with the HLA molecule. The interface with the TCR was dominated by peptide-mediated interactions rather than by MHC-mediated contacts; in other TCR–pMHC complexes, up to two third of atomic contacts are with the MHC itself (Tynan et al. 2005). However, the SB27 TCR was still MHC-restricted in that it contacted both residues 65 and 69 of the $\alpha 1$ helix and residues 150–158 of the $\alpha 2$ helix. Further structural studies of TCRs specific for other longer MHCI-bound peptides should confirm the need for minimal TCR–MHC contacts, or conversely reveal the occurrence of MHC-restricted T-cell recognition without significant TCR–MHC contacts, thereby indicating that MHC restriction is a consequence of extrinsic forces such as coreceptor interactions (Burrows et al. 2006).

It has been suggested that a large part of the binding energy of TCRs for pMHC ligands stems from direct contacts between the TCR and the MHC, the CDR3-peptide interaction modulating a preexisting affinity of the TCR CDR1–CDR2 loops for the MHC and raising the binding energy above the threshold required for productive engagement (Baker et al. 2001; Wu et al. 2002). In contrast to that view, a recent study using the LC13–FLR–HLA-B8 system showed that the interaction between the CDR1–CDR2 loops and the MHC helices contributed very little energy to the contact interface, whereas almost all of the binding energetics derived from peptide-CDR3 contacts (Borg et al. 2005). Likewise, in the case of intrathymic positive selection, the underlying energetic principles may vary according to the TCR and to the MHC class and allele under study.

Given the difficulty, so far, in seeing a TCR–MHC recognition code from crystal structures, Garcia and colleagues have recently stressed the limits of interpretations that are solely based on TCR–pMHC crystals (Garcia and Adams 2005). They suggested that the “TCR–MHC recognition code” may become only manifest at the level of the initial TCR–pMHC encounter complex. When the putative encounter complex relaxes into the most energetically stable final complex, the original contacts adopted by the CDR1–CDR2 and “signing” MHC restriction may undergo some reorganization that will depend on CDR3-peptide interactions and will thus differ for each CDR3 combination. In support of these views, recent studies of the kinetics and thermodynamics of TCR–pMHC binding suggest that conformational changes at the TCR–pMHC interface occur after an initial permissive encounter (Lee et al. 2004; Miley et al. 2004).

5.7 Rationalizing the Purpose of TCR $\alpha\beta$ Positive Selection

The proportion of *de novo* assembled TCRs capable of interacting with self-pMHC complexes is difficult to estimate due to the possible existence of sequential TCR α locus rearrangements (see section 5.4.3), and to the efficient removal of apoptotic

thymocytes. It has been, however, suggested that prior to TCR $\alpha\beta$ selection, up to 30% of de novo assembled TCRs can interact with the self-pMHC complexes encountered in the thymus (Merkenschlager et al. 1997; Zerrahn et al. 1997). This is considered a much higher frequency than would be expected from the generation of receptors through the random rearrangement of gene segments that do not have an inherent predisposition for interactions with MHC, like those involved in the assembly of Ig V regions. Note that although antibodies with TCR-like specificity (i.e. recognizing the MHC-bound peptide as a specificity element) can be selected (Hulsmeyer et al. 2005), it is generally assumed that, among a repertoire of random antibodies, the great majority will fail to bind to a given pMHC ligand in a peptide-specific manner. This assumption is based on the fact that antibodies are likely to treat pMHC ligands in an “opportunistic mode”, binding any epitope provided that some energetic prerequisite are met. Therefore, were antibodies V regions ever to be used in lieu of TCR V regions, most of the T-cell clones expressing them would be excluded from TCR $\alpha\beta$ selection. Altogether, these considerations suggest that evolution has shaped the germline encoded CDR1–CDR2 loops, so that they have an intrinsic ability to bind MHC α helices, allowing TCR to focus only on MHC and not on any other cell surface proteins. As previously discussed, the postulated interactions between CDR1–CDR2 and MHC are probably weak and can be modulated, in a positive or negative fashion, by the associated CDR3s and the side chains of the bound peptide (Wang and Reinherz 2002). Note that the idea that receptors expressed on T cells, might be intrinsically biased to bind MHC proteins was first proposed more than 30 years ago, at a time when the structure of the TCR was still elusive (Jerne 1971).

After the generation of an MHC allele-neutral TCR repertoire in DP thymocytes, the phase of molecular matching known as positive selection favors those TCRs capable of coping with a composite surface made of self-peptide side chains and of MHC determinants that are both conserved and allele specific. Provided that there is no global repositioning of the TCR V domains on the MHC surface when docking on the self peptide-self MHC ligands responsible for their intrathymic selection and on the foreign peptide-self MHC ligands that are encountered in the periphery, it can be inferred from the crystal structure of TCR bound to foreign peptide-self MHC ligands that during positive selection the TCRs are not particularly “obsessed” with the recognition of the few allele-specific residues that are found on the top of the MHC α helices (Malissen 2003b). In MHC Ia molecules, the residues of the $\alpha 1\alpha 2$ platform that display the greatest sequence variability point toward the peptide-binding groove resulting in allelic specificity for peptide binding whereas residues on the top of the α helices are more conserved. It has however been argued that these few allele-specific residues constitute a nuisance in that they affect the binding of some V α or V β domains and preclude the selection of the corresponding DP cells (Schumacher and Ploegh 1994). When discussing this kind of steric conflict, Ron Germain (Germain 1990) suggested that it would have been less “wasteful” to conserve those regions of the MHC molecule able to contact the TCR, altering only those that confer peptide-binding specificity. This strategy would maximize the usable fraction of TCRs among those generated by random V(D)J gene segment rearrangements and chain assortment, as no receptor would be excluded from selection because of a failure to interact with the MHC

molecules available in the thymus. However, the selective pressure driving MHC polymorphism, likely due to the necessity to diversify the repertoire of sampled peptides, clearly “spilled over” to residues accessible to the TCR, making necessary a phase of epigenetic molecular match.

In contrast to the models that consider the polymorphic residues found on the top of the MHC helices and available to TCR contacts as the nuisance that accounts for most of DP cell loss, we have argued that during TCR $\alpha\beta$ selection there are probably fewer constraints linked to matching the few allele-specific residues found on the top of the MHC helices than there are in adapting to the generic features that are imposed on the bound peptides by the architecture of a given MHC peptide-binding groove, and to the diversity of peptide side chains that point toward the TCR (Housset and Malissen 2003). For instance, TCR read-out of peptides that follow a flat and deep course within the MHCI groove, as exemplified by octapeptides bound to H-2K^b and H-2K^k, generates structural constraints distinct from the readout of H-2D^b- and H-2L^d-bound peptides that protrude out of the C-terminal part of the peptide binding-groove (reviewed in Kellenberger et al. 2005). However, despite the malleability of the TCR antigen-binding site and the occurrence of limited conformational adjustments in the pMHC surface, it is likely that the great majority (~70%) of the V α V β combinations expressed at the surface of the pre-selected DP cells remains “neglected” due to steric conflict with the residues of the self-peptides accessible to TCR contact. Therefore, it is possible that while most germline-encoded CDR1–CDR2 loops are capable of productive interactions with any given MHC allele, the repertoire of somatically generated CDR3 loops has difficulty to match the generic features that the MHC peptide-binding grooves impose on the bound peptides, and coincidentally on the peptide residues accessible to TCR contact. The fact that most MHCII-bound peptides adopt a rather deep and flat course in the binding groove might facilitate the interaction of MHCII molecules with the TCR CDR1–CDR2 loops, and make MHCII molecules more “accommodating” than pMHCI molecules to the binding of de novo assembled TCRs.

5.8 Recessive and Dominant Tolerance

The potential to generate random repertoire of $\alpha\beta$ TCRs has led to the evolution of complex quality-control mechanisms that eliminate or suppress T-cell clones that strongly react with self-pMHC molecules. It has been suggested that innate-like T lymphocytes (NKT cells and some subsets of $\gamma\delta$ T cells) are autoreactive by design (Bendelac et al. 2001). However, it should be stressed that conventional $\alpha\beta$ T cells are “trained” on self pMHC molecules and selected to have a low-affinity for such self pMHC complexes. Therefore, conventional $\alpha\beta$ T cells are also autoreactive on design and operate on the edge of autoimmunity (Aguado et al. 2002; Lin et al. 1997). For conventional $\alpha\beta$ T cells, the immunologic self corresponds to the pMHC molecules that are seen during intrathymic development and on the surface of peripheral, resting dendritic cells (DCs). In the thymus, the pool of MHC-bound self-peptides available for repertoire selection comprises

intrathymically expressed ubiquitous antigens and antigens specific to various types of thymic APCs: cortical and medullary thymic epithelial cells (cTECs and mTECs), thymic DCs, macrophages, and thymic B cells. Promiscuous gene expression by mTECs has recently extended the scope of central tolerance to self-constituents expressed by many peripheral tissues, including some that are only encountered during adulthood (Derbinski et al. 2005). This fascinating mechanism is controlled by the *Aire* gene product, and provides a way for developing T cells to anticipate the variety of self-proteins they will encounter while patrolling the body at later time points (Mathis and Benoist 2004). Furthermore, self-antigens can gain access to the thymus either via the blood circulation or by association with immigrating cells (Bonasio et al. 2006). Therefore, in contrast to the cortex that is a compartment genuinely unique to the thymus, there is some functional similarities between the medulla and peripheral secondary lymphoid organs.

Because not all self-antigens are expressed in the thymus, additional tolerance mechanisms exist in the periphery (Goodnow et al. 2005; Klein et al. 2000). In contrast to central tolerance that acts on self-reactive T lymphocytes before they become immunocompetent, peripheral tolerance acts on mature T cells. Peripheral presentation of self-determinants by DCs in the absence of tissue-damage, inflammation, or adjuvants does not prime naive T cells but rather results in abortive immune responses. This last mechanism of peripheral tolerance together with central tolerance are referred to as “recessive tolerance” because deletion of an individual autoreactive T-cell clone according to one of these processes does not affect other self-reactive T-cell clones. In contrast, the recently recognized regulatory T cells (Treg cells) are capable of acting in a dominant, trans-acting way to control self-reactive T cells and terminate conventional immune responses (Coutinho 2005). Treg cells are generated in the thymus upon high-affinity recognition of self-pMHCII ligands, and develop, under the influence of the Foxp3 transcription factor, along a unique differentiative pathway geared to anti-inflammatory and antiproliferative functions (Fontenot and Rudensky 2005). Treg can also be generated in the periphery subsequent to immune stimulation. Therefore, dominant tolerance constitutes an additional mechanism to compensate for incomplete representation of self in the thymus.

5.9 Evolutionary Perspectives

It has been argued that innate cell recognition strategies are more difficult to decipher than those used by B and T cells, where antigen receptors dominate the differentiation, activation, and effector function of these lymphocytes (Lanier 2005). For instance, rather than being regulated by a single receptor, NK cell activation results from the integration of signals emanating from multiple activating and inhibitory receptors that often display overlapping binding specificity. In contrast to the situation observed in T cells and B cells, Syk and ZAP70 are not required for NK cell development, and many NK effector functions are intact in mice that

are genetically deficient in both of these kinases. Therefore, unlike B and T lymphocytes, a single signaling pathway does not dominate the differentiation and effector function of NK cells (Chiesa et al. 2006). However, it should be stressed that the BCR and $\alpha\beta$ TCR signaling pathways are not autonomous and that their output can be tuned by a number of positive (CD28, CD19) or negative (CD5, CD22, Fc γ RIIB) regulators. Upon activation, some T cells are also capable of expressing inhibitory or activating receptors identical to the ones used by NK cells, and de novo expression of these inhibitory receptors is capable of aborting potentially productive TCR–pMHC interactions. Likewise, the function of the semi-invariant TCRs found on natural killer T (NKT) cells and on some subsets of $\gamma\delta$ T cells is tuned by inhibitory and activating receptors (Bendelac et al. 2001). As documented for the NKG2D activating receptors, it is likely that these inhibitory and activating receptors recognize self molecules that function as generic flags of cell damage or of other nonphysiological processes (Gasser et al. 2005). Therefore, the strategies used to activate both adaptive and innate immune effectors are rather similar and result from the integration of positive and negative signals emanating from a multitude of receptors (Vivier and Malissen 2005).

Natural killer T cells and $\gamma\delta$ T cells resemble NK cells in that they are “ready-to-go” cells. In contrast to naïve, conventional $\alpha\beta$ T cells that undergo a phase of antigen-driven clonal expansion that is associated with the acquisition of diverse functions (T helper type 1, 2, and 17 in the case of CD4 T cells), the effector functions of NK, NKT, and $\gamma\delta$ T cells are triggered without prior proliferation and chromatin remodeling of genes coding for effector molecules (granzyme, perforin, and cytokines) (Lanier 2005). The fact that NK cell receptors are germline-encoded and present on a large proportion of NK cells makes them well suited for such explosive-like defense mechanisms. Likewise, to behave as “ready-to-go” effector cells and thus avoid a lengthy, antigen-driven phase of clonal expansion and differentiation into effector cells, NKT cells and some subsets of $\gamma\delta$ T cells require mechanisms of TCR repertoire selection that ensure that a given TCR will be distributed “ab initio” on a relatively large cell subpopulation. The “simplest” solution to this problem is found in the $\gamma\delta$ T cells present at body surfaces. The programmed usage of certain V and J gene segments containing short homology repeats near their coding ends in association with an absence of terminal deoxynucleotidyl transferase activity allows them to express TCRs containing a single or a rather limited number of canonical V(D)J junctions. NKT cells also express quasi invariant TCR α chains, however in contrast to the strategy used by $\gamma\delta$ T cells, their limited TCR repertoire results from stringent cellular selection mechanisms acting on a population of intrathymic progenitors expressing randomly assembled TCRs. It is thus paradoxical to see that somatic, site-specific DNA recombination can be used to generate TCRs that have almost no diversity and recognize self molecules that function as generic flags of cell infection or other cell dysregulations. Finally, it should be noted that there is probably a link between the existence of somatically assembled and clonally distributed antigen receptors and the appearance of a pool of long-lived naïve circulatory precursors that can undergo antigen-driven clonal expansion and give rise to both effectors and memory T cells (Pancer and Cooper

2006). This unique feature necessitates processes that control independently the numbers of naïve and memory T cells, safeguarding both a diverse repertoire of naïve T cells for the control of newly emerging pathogens as well as a reservoir of memory T cells to rapidly eliminate pathogens that have been already encountered. In contrast, following encounter with their stimulatory ligands, innate-like cells are poised to become effectors without proceeding through a phase of proliferation. However, these contrasted views need probably to be tempered since both hapten-specific NK cells have been observed in a model of contact hypersensitivity, and an NK cell subset bearing an activating receptor specific for cells infected with mouse cytomegalovirus has been found to undergo a phase of “antigen-driven” proliferation akin to clonal expansion of conventional $\alpha\beta$ T cells (Yokoyama 2006).

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6

Fc Receptors

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6.1 Introduction

An immune response is characterized by a delicate balance; strong enough to eliminate foreign pathogens but at the same time well controlled and highly specific to prevent destruction of self-tissues. Antibodies are essential to defend the body against invading microorganisms and antibodies bound to their respective antigen in the form of immune complexes (IC) have long been recognized to be potent inflammatory stimuli. The existence of cellular receptors for antibodies was anticipated as early as 1960 (Boyden and Sorkin 1960). Starting with the molecular cloning of cellular receptors for IgG and IgE, subsequently Fc receptors (FcR) for all antibody isotypes (IgM, IgA, IgD, IgE, and IgG) have been identified (Ravetch 2003). Fc receptors are widely expressed throughout the hematopoietic system and are essential regulators of immune cell activation. By recognizing the Fc portion of antibodies in immune complexes, Fc receptors link ancestral pathways of innate immunity to the specificity of the adaptive immune system. Their cellular expression pattern on myeloid effector cells, mast cells and B cells predicted their involvement in different types of inflammatory responses, allergy and B-cell regulation. Indeed, effector mechanisms shown to be triggered by FcR crosslinking include antibody dependent cellular cytotoxicity (ADCC), phagocytosis, release of inflammatory mediators and antigen presentation (Ravetch 2003). Moreover, the potent immunoregulatory functions of ICs (consisting of IgG or IgM antibodies), ranging from a strong enhancement to complete suppression of antibody responses in

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addition to their more overt role as effector molecules for the elimination of foreign antigens, can now be ascribed to specific FcRs (Heyman 2000).

At the same time, however, inflammatory processes need to be tightly regulated to prevent destruction of self-tissues. In particular, low-affinity self-reactive antibodies (autoantibodies) found in healthy individuals need to be rendered harmless (Wardemann et al. 2003). Research over the last couple of years has identified multiple checkpoints that function to ensure orderly progression through an immune response (Goodnow et al. 2005; Grimaldi et al. 2005). The basis for these checkpoints is the establishment of discrete thresholds that define narrow windows of response; these thresholds are usually generated by the coexpression of activating and inhibitory molecules on the same immune effector cells. FcRs are a prime example for such regulation as activating FcRs are usually coexpressed with inhibitory counterparts, thereby setting thresholds for antibody mediated immune effector cell activation and release of inflammatory mediators. On B cells the inhibitory Fc γ -receptor IIB (Fc γ RIIB) regulates activating signals delivered by the B-cell receptor, ensuring that only B cells with a high-affinity B-cell receptor specific for foreign antigens can become activated. Similarly, inhibitory FcR expression on dendritic cells (DC) might prevent spontaneous DC activation and expansion of autoreactive T cells.

The aim of this chapter is to explain that this complexity exists to distinguish between self and nonself, thus avoiding autotoxicity and uncontrolled inflammation. We will focus mainly on the murine IgG and IgE FcRs, for which substantial data concerning their regulation and role in different physiological and pathological conditions *in vivo* are available. For a more in depth view about recent developments in Fc α -receptor biology the reader is directed to several excellent recent reviews covering that topic (Monteiro and van de Winkel 2003; Otten and van Egmond 2004). We will start with a brief description of basic Fc receptor biology, followed by an overview over activating and inhibitory FcR signaling pathways and examples where thresholds set by these contrasting signals are essential to maintain a balanced immune response and where disturbance of these thresholds leads to an uncontrolled inflammatory response and ultimately to destruction of self-tissues.

6.2 Fc Receptors—Basic Facts

In general Fc receptors can be divided into two classes: the activating and the inhibitory FcRs (Table 1). Whereas the activating receptors cannot signal autonomously and have to associate with additional adaptor molecules to be functional, the inhibitory receptor is a single chain molecule that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytosolic tail. A notable exception to this rule is human Fc γ RIIA that can transmit activating signals by itself. Therefore, a functional FcR consists of a ligand binding α -domain associated with signaling adaptor molecules containing immunoreceptor tyrosine-based activation motifs

Table 1. Human and mouse Fc receptors and their ligands

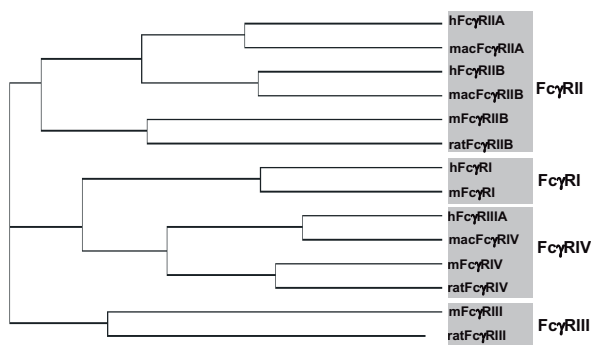
Protein family	FcαRI (CD89)		FcεRI (CD23)		FcγRI (CD64)		FcγRIIA (CD32)		FcγRIIB (CD32)		FcγRIIA (CD16)		FcγRIIB (CD16)	
	IgG superfamily	IgG superfamily	IgG superfamily	C-type lectin	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily	IgG superfamily
Ligand	IgA ₁ = IgA ₂	IgE	IgE	IgE	IgG3,1 > 4 >> 2	IgG3 > 1 >> 2,4*	IgG3 > 1 >> 2,4*	IgG3 > 1 > 4 > 2	IgG3 > 1 > 4 > 2	IgG1,3 >> 2,4*	IgG1,3 >> 2,4*	IgG1,3 >> 2,4*	IgG1,3 >> 2,4*	IgG1,3 >> 2,4*
Affinity (M ⁻¹)	10 ⁷	10 ¹⁰	10 ⁶ –10 ⁷	10 ⁶ –10 ⁷	10 ⁸ –10 ⁹	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	10 ⁶	10 ⁶	10 ⁶	10 ⁶	10 ⁶
Signaling (subunit)	Activating (γ-chain)	Activating (γ- and β-chain)	Activating	Activating	Activating (γ-chain)	Activating	Activating	Inhibitory	Inhibitory	Activating (γ- or ζ-chain)	Activating	Activating	None	(gpi-anchor)
Expression	Macrophage Neutrophils	Mast cells Basophils	B cells Follicular dendritic cells	B cells Follicular dendritic cells	Macrophages Neutrophils	Macrophages Neutrophils	Macrophages Neutrophils	Macrophages Neutrophils	Macrophages Neutrophils	Macrophages Mast cells	Macrophages Mast cells	Macrophages Mast cells	Neutrophils	Neutrophils
	Eosinophils Interstitial dendritic cells	Eosinophils Platelets			Eosinophils Dendritic cells	Eosinophils	Eosinophils	Eosinophils	Eosinophils	Basophils NK cells	Basophils NK cells	Basophils NK cells		
	Kupffer cells	Dendritic cells				Platelets Dendritic cells	Platelets Dendritic cells	Dendritic cells FDC	Dendritic cells FDC	Dendritic cells	Dendritic cells	Dendritic cells		
Mouse receptor	Igα/μ receptor	FcεRI	FcεRII	FcεRI	FcγRI	FcγRIII (extracellular)	FcγRIII	FcγRIIB	FcγRIIB	FcγRIV	FcγRIV	FcγRIV	Not identified	Not identified
Ligand	IgA, IgM	IgE	IgE	IgE	IgG2a	IgG1, 2a, 2b	IgG1, 2a, 2b	IgG1, 2a, 2b	IgG1, 2a, 2b	IgG2a, 2b	IgG2a, 2b	IgG2a, 2b	n.a.	n.a.
Affinity (M ⁻¹)	10 ⁸ /10 ⁹	10 ¹⁰	10 ⁶ –10 ⁷	10 ⁶ –10 ⁷	10 ⁸	10 ⁵ –10 ⁶	10 ⁵ –10 ⁶	10 ⁶	10 ⁶	10 ⁷	10 ⁷	10 ⁷	n.a.	n.a.
References	Monteiro and van de Winkel, 2003	MacGlashan, 2005	Gould et al., 2003	Hulet and Hogarth, 1994; Ravetch and Kinet, 1991										

* Indicates that allelic variants exist that show different affinities for antibody isotypes (see Dijkstra et al., 2001) n.a., not available

(ITAM). Depending on the cell type the associated signaling adaptor molecules vary. Whereas in the majority of cells, such as monocytes, macrophages, neutrophils, and dendritic cells FcRs are associated with the common gamma chain (γ -chain), in human natural killer (NK) cells FcRs are found in combination with the zeta chain (ζ -chain) (Table 1). Both γ - and ζ -chain are present as dimers linked by disulphide bonds. In the case of the Fc ϵ RI and human Fc γ RIIIA, additional β -chains have been identified in the receptor complex in certain cell types. In addition to the signaling function, these molecules are important for cell surface expression of the respective α -chains. For example, animals that don't express the γ -chain lack cell surface expression of all activating Fc γ -receptors and several other non-FcR-related proteins such as PIR-A and NK cell cytotoxicity receptors (Moretta et al. 2001; Ravetch 2003). As expected, γ -chain knockout animals were demonstrated to have significant defects in antibody-dependent effector cell responses such as phagocytosis of ICs, ADCC and inflammatory responses (Clynes and Ravetch 1995; Park et al. 1998; Sylvestre and Ravetch 1994; Takai et al. 1994; Zhang et al. 2004).

Regarding FcR genetics humans have 8 genes that encode Fc γ -receptors (Fc γ RIA/IB/IC, Fc γ RIIA/B/C, and Fc γ RIIIA/B) located on chromosome 1. In contrast to the duplication and diversification processes that have led to presence of multiple genes in the human genome (Qiu et al. 1990), the majority of other species including the mouse have four different classes of IgG Fc receptors that correspond to their human counterparts: Fc γ RI (CD64), Fc γ RII (CD32), Fc γ RIII (CD16) and Fc γ RIV. Fc γ RIV is a recently identified receptor, conserved in all mammalian species with intermediate affinity (10^{-7} M) and restricted subclass specificity (Davis et al. 2002; Mechetina et al. 2003; Nimmerjahn et al. 2005) and is most closely related to human Fc γ RIIIA (Fig. 1). Fc γ RI displays high affinity for the antibody constant region (10^8 – 10^9 M $^{-1}$), Fc γ RII and Fc γ RIII have a low affinity for the Fc-portion ($\sim 10^6$ M $^{-1}$) (Hulett and Hogarth 1994; Ravetch and Kinet 1991). The low-affinity Fc receptor genes are clustered in close proximity to each other in syntenic regions on chromosome 1 in humans, chimpanzees and mice. In contrast, the high-affinity Fc γ RI is located on chromosome 3 in mice and chromosome 1 in humans and chimpanzees. Mirroring this complexity of Fc γ -receptors is the existence of several IgG isotypes that show differential binding to Fc γ Rs (Table 1). In the mouse,

Fig. 1. Fc γ R families. Cladogram showing the alignment of selective Fc γ -receptors in humans (*h*), macaques (*mac*), mice (*m*), and rats



the high-affinity Fc γ RI exclusively binds IgG2a, the medium affinity Fc γ RIV binds IgG2a and IgG2b and the low-affinity receptors Fc γ RIIB and III bind IgG1, IgG2a and IgG2b. Moreover, it has only recently been appreciated that the IgG Fc receptors show significant differences in their affinity for individual antibody isotypes rendering certain isotypes more strictly regulated than others (Nimmerjahn et al. 2005). This represents a second layer of complexity and is of major importance for understanding Fc receptor dependent antibody mediated effector functions *in vivo* and for the design of antibody-based therapies. This important point will be discussed in greater detail later.

Although several proteins have been suggested to bind IgA, the best described receptor for IgA to date is CD89 (Fc α RI). It is a gamma chain dependent receptor that binds IgA with intermediate affinity and is located on chromosome 19 in humans and chimpanzees; no homologous protein has been found in mice. However, a protein that can bind IgA as well as IgM has been identified in mice and called Fc α / μ -receptor (Shibuya et al. 2000). Like its human homologue, it binds IgA with intermediate affinity and is expressed on the majority of murine B lymphocytes, myeloid and also nonhematopoietic cells like mesangial cells (reviewed in Meteiro and van de Winkel 2003; Otten and van Egmond 2004). In contrast to the prevailing view that IgA antibodies are generally anti-inflammatory due to their capacity to block entry of pathogens at mucosal surfaces, recent evidence suggests that serum IgA can trigger inflammatory reactions as well (Otten and van Egmond 2004).

In contrast, both mice and humans contain two different IgE receptors. Fc ϵ RI has a high affinity for IgE (10^{10} M^{-1}), is located on chromosome 1 in mice and humans and is expressed on allergic effector cells including basophils and mast cells. Its expression has been detected on other cell types, for example on human antigen presenting cells like dendritic cells and macrophages. In contrast to the tetrameric form of the receptor (α -, β - and γ -chain dimer) on allergic effector cells, the receptor complex on other cells lacks the β -chain (MacGlashan, 2005; Novak et al. 2001). It has been suggested that this additional molecule is important for proper assembly of Fc ϵ RI and functions as an amplifier of γ -chain mediated signaling (Dombrowicz et al. 1998; Donnadieu et al. 2003). The low-affinity IgE receptor (Fc ϵ RII; CD23) is a structurally unrelated protein that forms trimers and contains a C-type-lectin domain. It is located on chromosome 8 in mice, chromosome 19 in humans and chromosome 20 in chimpanzees. It is important for controlling IgE production and transport and has been implicated in the enhancement as well as the downregulation of IgE responses following immunization with IgE immune complexes (Heyman 2002).

In addition to the classical Fc receptors a new family of FcR-like or homologous proteins has been identified recently. These proteins have significant sequence homology to classical FcRs in their extracellular domains and contain ITAM or ITIM motifs in their cytosolic portions. Several of these molecules, e.g. FcRX and FcRH1–5 are expressed on human and mouse B cells. Despite significant sequence homology to classical Fc receptors, attempts to demonstrate antibody-binding activity for these molecules have been unsuccessful, so Fc γ RIIB remains the only antibody binding Fc receptor on B cells. Nevertheless, these Fc receptor related

molecules show a restricted expression pattern during different stages of B-cell development and might become important new markers for defining B-cell developmental stages or malignancies (reviewed in Davis et al. 2002; Davis et al. 2005).

6.3 Fc Receptor Signaling

In the following sections we will summarize the current models of how activating and inhibitory signals are triggered by crosslinking of cell surface Fc receptors by immune complexes. We will start with the signaling cascades triggered by activating Fc receptors followed by inhibitory signaling pathways and their counter-regulation of activating signals. The outcome of these opposing signaling events (cell activation or inhibition) is determined by several factors including the relative affinity of the antibody isotypes incorporated in immune-complexes, the expression level of activating and inhibitory Fc receptors and the cytokine environment which can influence expression levels of Fc receptors.

6.3.1 Signaling Pathways of Activating Fc Receptors

The first step in triggering signaling pathways by activating Fc receptors is the aggregation or cross linking of these receptors by immune complexes (Fig. 2). The affinity of the majority of activating Fc receptors for monomeric antibodies is not sufficient for stable binding and induction of signaling. High-affinity receptors, like Fc γ RI and Fc ϵ RI, can associate with monomeric IgG or IgE antibodies, but activating signals are only triggered upon replacement of bound IgG by immune complexes (in the case of Fc γ RI) or upon allergen binding and concomitant crosslinking of cell surface bound IgE (Galli et al. 2005; Gould et al. 2003). Ligands that bind with low affinity cannot trigger sustained receptor aggregation and might even behave as antagonistic ligands (Torigoe et al. 1998).

The overall signaling cascades triggered by the different activating Fc receptors and other activating receptors like the B-cell or T-cell receptor are very similar. As shown for Fc α RI aggregation by ICs induces a re-location into cell membrane sub-domains called lipid rafts that are enriched in signaling molecules such as Src-protein kinases (Lang et al. 1999). Tyrosine residues in the ITAM motif of the γ -chain then become phosphorylated by Src kinases creating SH2-domain docking sites for the subsequent recruitment of Syk kinases. Depending on cell type and the receptor in question, different members of the Src-kinase family are involved in phosphorylation of the γ -chain. Whereas Lyn is crucial for the Fc ϵ RI pathway in mast cells, Lck is associated with Fc γ RIIIA in natural killer (NK) cells. In macrophages, both of these kinases and additionally Hck have been suggested to be important for γ -chain phosphorylation after crosslinking of Fc γ RI or Fc γ RIIA. This

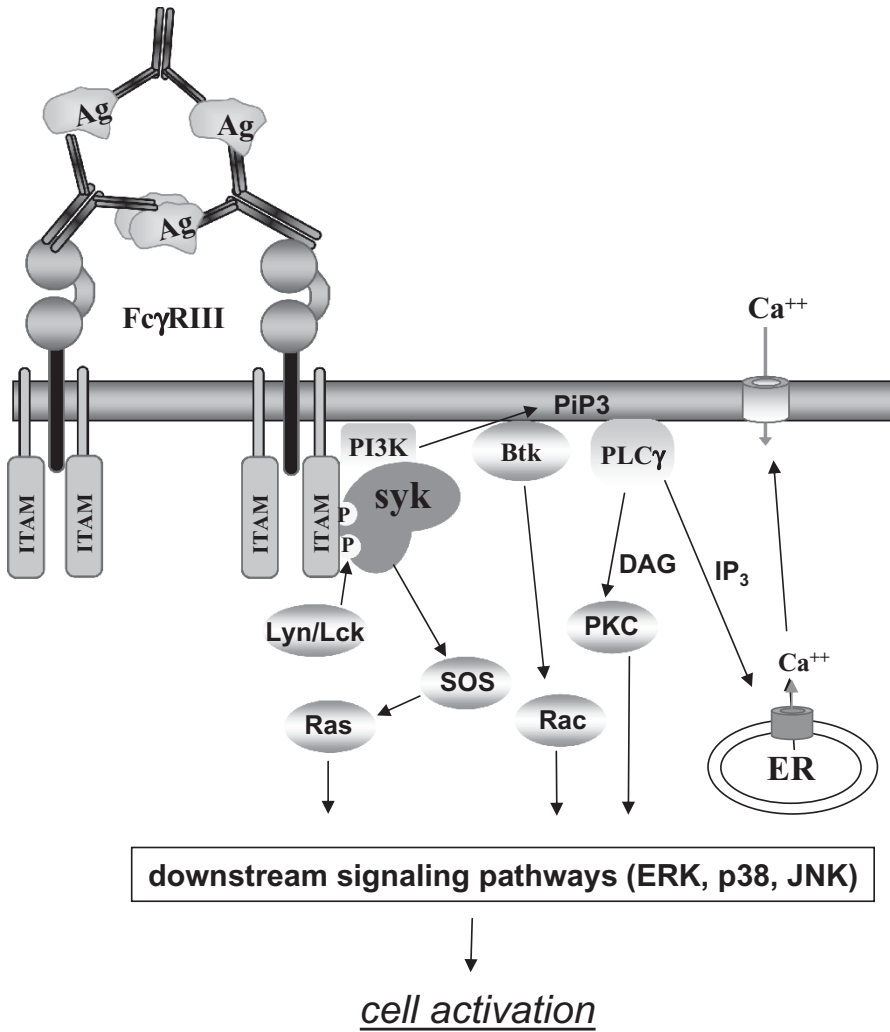


Fig. 2. Signaling pathways triggered by activating Fc receptors. After crosslinking of activating Fc receptors, the receptor associated γ -chains become phosphorylated by Src kinases like Lyn or Lck. This generates Src homology (SH)2-domain docking sites for Syk which in turn activates a number of other signal transduction molecules like phosphatidylinositol 3-kinase (PI3K) and SOS (son of sevenless). The generation of phosphatidylinositol 3-phosphate (PI_3) recruits Btk and phospholipase- $C\gamma$ (PLC γ) which lead to activation of downstream kinases and the release of calcium from internal storage sites like the endoplasmic reticulum (ER). DAG, diacylglycerol; ITAM, immunoreceptor tyrosine-based activation motifs

enables members of the Syk-kinase family to bind and to recruit and phosphorylate a number of downstream targets including the linker for activation of T cells (LAT); multi-molecular adaptor complexes consisting of Cbl, Slp-76, Grb-2, Shc, Sos, SHIP; and members of the Btk and Tec kinase family (Gulle et al. 1998; Launay et al. 1998; Park et al. 1999). Important downstream events are triggered by Syk-mediated activation of phosphatidylinositol 3-kinase (PI3K) and phospholipase-C γ (PLC γ). PI3K generated phosphatidylinositol polyphosphates, such as phosphatidylinositol 3-phosphate (PIP₃), allow pleckstrin homology (PH) domain containing proteins such as Btk and PLC γ to bind to the plasma membrane. Phospholipase C γ hydrolyzes membrane bound phosphatidylinositol phosphates into diacylglycerol (DAG) and inositol-triphosphate (IP₃) which induces a sustained calcium release and protein kinase C (PKC) activation (reviewed in Ravetch 2003). Moreover, the Ras–Raf–MAPK pathway becomes activated through Sos present in the multimolecular adaptor complex (Fig. 2).

Effector responses triggered by these activating signals most prominently include degranulation of mast cells and neutrophils, release of cytotoxic mediators and inflammatory cytokines, antibody dependent cellular cytotoxicity (ADCC), antigen presentation and phagocytosis. Moreover, activating signals delivered by Fc receptors can induce dendritic cell maturation which will be discussed in greater detail later in this chapter.

6.3.2 *Inhibitory Signaling Pathways*

For the generation of a balanced immune response activating signals need to be regulated to prevent uncontrolled inflammation (Ravetch 2003; Ravetch and Lanier 2000). A hallmark of Fc receptor biology is that activating receptors are usually coexpressed with their inhibitory counterpart, Fc γ RIIB. Therefore, Fc γ RIIB is ubiquitously expressed throughout the hematopoietic system. Exceptions to this rule are NK cells that solely express the activating Fc γ RIII and T cells that lack Fc receptor expression.

Fc γ RIIB is a single chain receptor that contains an ITIM in its cytosolic domain. This motif consists of the 13-amino acid sequence AENTITYSLLKHP which is necessary and sufficient for the inhibitory activity of Fc γ RIIB. Depending on the cell type alternatively spliced forms of Fc γ RIIB have been described that show a differential capacity to endocytose bound ICs (termed Fc γ RIIB-1 and Fc γ RIIB-2). All of these splice forms, however, contain the ITIM motif. On B cells Fc γ RIIB regulates activating signals transmitted by the B-cell receptor (BCR), whereas on mast cells, neutrophils or macrophages it balances activating signals triggered by Fc ϵ - or Fc γ -receptors.

Upon coaggregation with its activating counterpart, Lyn phosphorylates the ITIM-motif, which leads to the recruitment of SHIP (SH2-domain containing inositol 5'-phosphatase) (Fig. 3). SHIP activation leads to enhanced hydrolysis of phosphatidylinositol intermediates and thereby interferes with the membrane recruitment of Btk and PLC γ , resulting in inhibition of ITAM signaling mediated calcium release and downstream effector functions such as ADCC, cytokine secre-

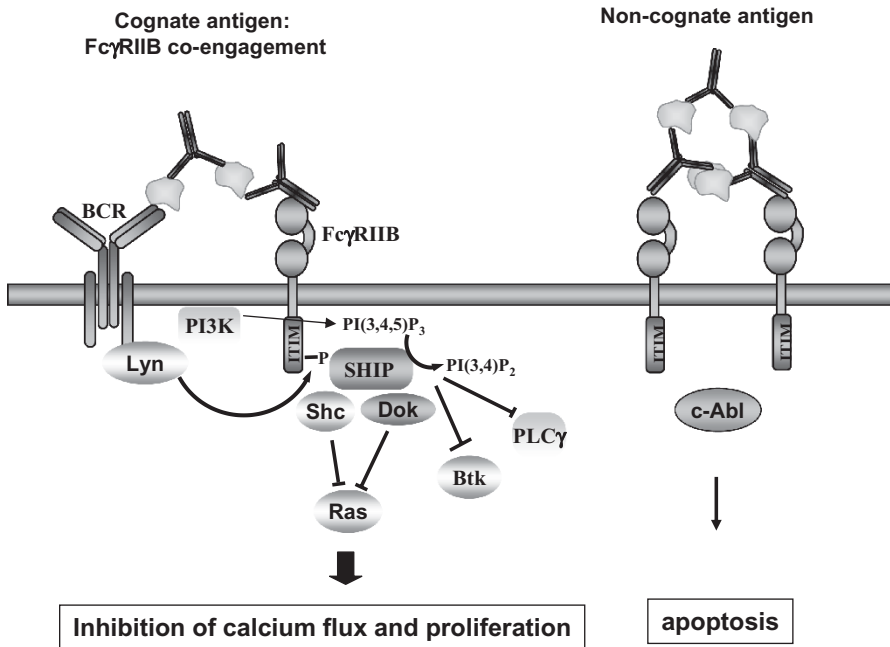


Fig. 3. Signaling pathways triggered by the inhibitory Fc receptor. Simultaneous crosslinking of activating receptors like the B-cell receptor (*BCR*) and the inhibitory Fcγ-receptor IIB (*FcγRIIB*) leads to phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (*ITIM*) in the cytoplasmic tail of *FcγRIIB* by *Lyn*. This results in the recruitment of SH2-domain containing inositol 5'-phosphatase (*SHIP*) and the hydrolysis of PIP_3 into PIP_2 , which ultimately inhibits recruitment of pleckstrin homology domain containing proteins like *Btk* and phospholipase- $C\gamma$ (*PLCγ*) (*left panel*). Isolated triggering of *FcγRIIB* leads to B-cell apoptosis via *ITIM* independent signaling pathways involving the *c-Abl* kinase family (*right panel*)

tion and release of inflammatory mediators. Moreover, tyrosine phosphorylated *SHIP* can bind to *Shc* and *Dok*, thereby inhibiting activation of the *Ras* pathway and ultimately cell proliferation. A third *ITIM* and *SHIP* independent signaling pathway has been described for crosslinking of *FcγRIIB* on B cells without concomitant activating signals by the *BCR*. This pathway leads to B-cell apoptosis via *Abl*-family kinase dependent pathways (Pearse et al. 1999; Tzeng et al. 2005). This situation may arise during the germinal center reaction when somatic hypermutation generates BCRs that lose specificity for their cognate antigen retained in the form of immune complexes on follicular dendritic cells. Thus, *FcγRIIB* has been suggested to be important for keeping tolerance.

6.4 Fc Receptor Biology In Vivo

After having described the basic signaling pathways and their interplay to achieve balanced immune responses, we will discuss other intrinsic and extrinsic factors that will determine whether more activating or inhibitory signals are triggered.

These factors include the actual affinity of antibody isotypes for the inhibitory and activating Fc receptors, the relative expression level of these receptors on individual immune effector cells and the local cytokine milieu that can change these relative expression levels. Furthermore, we will describe how this regulation impacts on the afferent and efferent phases of an immune response.

6.4.1 The Role of the Inhibitory Receptor in the Afferent and Efferent Immune Response

Fc γ RIIB belongs to the family of immune inhibitory receptors. Other prominent members of this family are PIR-B, KIRs, CTLA-4, PD-1, CD5, and CD22. These proteins carry ITIM motifs, are widely expressed on immune effector cells and are important regulators of their activating counterparts (Ravetch and Lanier 2000). The loss of these negative regulators leads to imbalanced immune responses resulting in autoimmunity and overt autoimmune disease (Bolland and Ravetch 2000; O'Keefe et al. 1999; Nishimura et al. 1999; Penninger et al. 1995; Takai et al. 1996; Tivol et al. 1995). Taking into account the cell types where Fc γ RIIB is expressed the loss or impairment of Fc γ RIIB mediated negative regulation would be expected to result in aberrant responses in the afferent as well as the efferent phases of an immune response. The generation of a mouse deficient in Fc γ RIIB over a decade ago has proven invaluable to study these predictions and the majority of the results were generated using this mouse model.

6.4.2 Fc γ RIIB and Dendritic Cells

Dendritic cells (DC) have long been recognized as central mediators that, depending on their activation state, determine whether an adaptive immune response or tolerance is induced (reviewed in Steinman et al. 2003). Several groups have shown that immune complexes are potent activators of DCs and are important for efficient cross-presentation of endocytosed antigen in the form of ICs on MHC class I molecules to CD8 $^{+}$ cytotoxic T cells (Dhodapkar et al. 2002; Groh et al. 2005; Rafiq et al. 2002; Regnault et al. 1999;). As low levels of ICs are constantly present in the serum, Fc γ RIIB is crucial to prevent spontaneous activation of DCs. In addition, expression of the inhibitory receptor on DCs present in epithelia has been implicated in establishing tolerance to air-borne and food allergens (Samson et al. 2005).

Although this negative regulation is essential to prevent expansion of autoreactive T cells during the steady state, it also limits immunotherapeutic approaches aimed at generating a strong response, e.g., during tumor therapy with monoclonal antibodies. Consistent with this, DCs derived from Fc γ RIIB deficient mice showed an enhanced potential to generate antigen-specific T-cell responses in vitro and in

vivo (Kalergis et al. 2002). These studies suggest that immunotherapeutic and vaccination approaches can be optimized by overcoming this negative regulatory effect of Fc γ RIIB on DCs. A recent study using human DCs and an Fc γ RIIB blocking antibody showed that blocking Fc γ RIIB was sufficient to induce DC maturation by immune complexes normally present in plasma. Besides upregulation of costimulatory molecules, these DCs were more potent in generating and activating tumor specific T cells (Boruchov et al. 2005; Dhodapkar et al. 2005). This supports the notion that blocking the inhibitory Fc receptor on DCs in vivo might indeed be a strategy for generating stronger and probably longer lasting immune responses.

Before using this strategy in patients it will be important to examine how systemic blocking of Fc γ RIIB-mediated negative signals impacts on the immune system in the steady state. As indicated before, immature DCs are continuously tolerizing self reactive T cells that escape negative selection in the thymus and Fc γ RIIB is crucial to control the expansion of autoreactive B cells. To test this hypothesis, the development of novel animal models with targeted deletion of Fc receptors in dendritic cells or other selective cell populations will be essential. Additionally, mice carrying the human Fc receptors instead of the mouse counterparts will become an important animal model for assessing the in vivo activity of blocking antibodies for human Fc receptors.

Whereas the essential role of dendritic cells in regulating T-cell responses is well accepted, it has only recently become clear that DCs are also important for the B-cell response (Bergtold et al. 2005; Kushnir et al 1998; Wykes et al. 1998). In contrast to macrophages, which rapidly degrade phagocytosed material, antigen taken up by DCs is degraded more slowly and therefore present in an intact form for prolonged times (Delamarre et al. 2005). It has been suggested that this allows transport of antigen from the periphery to lymphoid organs where it can be presented to B cells. DC-B-cell interactions have been observed in vivo and it has been suggested that this interaction is important for generation of an IgG response in vitro and in vivo (Wykes et al. 1998). The important role of Fc γ RIIB in this process is that ICs taken up via Fc γ RIIB are inefficiently degraded and recycled for cell surface presentation to B cells. In contrast uptake via Fc γ RIII results in a more rapid degradation of the antigen (Bergtold et al. 2005).

6.4.3 Loss of the Inhibitory Receptor on B Cells—Horror Autotoxicus

B-cell development proceeds through several stages and progression from one stage to another is tightly controlled by numerous checkpoints that ensure that B cells expressing a self-reactive receptor are eliminated (Goodnow et al. 2005; Grimaldi et al. 2005). As indicated before, Fc γ RIIB regulates activating signals triggered by the B-cell receptor, thus setting a threshold for B-cell activation. The strength of this signal will ultimately determine whether a B-cell proliferates, class switches and matures into an antibody secreting plasma cell. Loss of this negative regulator

was therefore predicted to result in uncontrolled B-cell activation. Especially the capacity of Fc γ RIIB to trigger B-cell apoptosis (Fig. 3) has been proposed to be an important mechanism to maintain self tolerance by deletion of low affinity or self reactive B cells. This was confirmed by the generation of Fc γ RIIB-deficient mice that spontaneously develop a lupus like disease characterized by the production of autoantibodies and premature death due to severe glomerulonephritis (Bolland and Ravetch 2000; Takai et al. 1996). This autoimmune phenotype is strain dependent—mice on the C57BL/6 but not the Balb/c background develop an autoimmune disease, suggesting that other epistatic modifiers are involved in disease susceptibility and severity (reviewed in Nguyen et al. 2002; Bolland and Ravetch 2002). Supporting this notion it was shown recently that Balb/c mice double deficient in programmed death 1 (PD-1) and Fc γ RIIB, but not the single knockout mice, developed severe autoimmune hydronephrosis (Okazaki et al. 2005). Moreover, Balb/c-Fcgr2b^{-/-} mice showed enhanced disease phenotypes in a model of pristane-induced lupus (Clynes et al. 2005).

Another line of evidence for the role of Fc γ RIIB as a gatekeeper of tolerance comes from autoimmune-prone mouse strains such as NZB, NOD, BXSB, and MRL that have been found to express reduced levels of Fc γ RIIB on activated and germinal-center B cells. It was suggested that this is due to a polymorphism in the Fc γ RIIB promoter (Jiang et al. 1999, 2000; Pritchard et al. 2000; Xiu et al. 2002). Similarly, a polymorphism in the human Fc γ RIIB promoter linked to lupus has been identified. This polymorphism leads to decreased transcription and surface expression of Fc γ RIIB on activated B cells of human lupus patients (Blank et al. 2005). Besides B cells, Fc γ RIIB is also expressed on other inflammatory immune effector cells such as neutrophils, monocytes and macrophages. As will be discussed below it is very likely, however, that the autoimmunity observed in C57BL/6- Fc γ RIIB-deficient animals is B-cell autonomous.

One important point in favor of this theory is that animals that only lack Fc γ RIIB expression on peripheral B cells develop autoimmunity. This was achieved by transferring Fc γ RIIB deficient bone marrow into irradiated B-cell deficient hosts (RAG or IgH knockouts). In these animals the monocytic compartment still expressed Fc γ RIIB while it was absent from peripheral B cells (Bolland and Ravetch 2000). Additionally, by doing the reverse experiment it was demonstrated that restoring Fc γ RIIB expression to wild-type levels in autoimmune prone mouse strains like NZM, BXSB and Fc γ RIIB knockout animals by retroviral transduction with Fc γ RIIB, tolerance could be restored (McGaha et al. 2005). Again, B cells represented the majority of cells that showed increased expression of Fc γ RIIB. A very important result of this study is that restoration of Fc γ RIIB expression on approximately 40% of peripheral B cells was sufficient to prevent the development of autoantibodies and autoimmune glomerulonephritis (McGaha et al. 2005). This highlights the threshold nature of autoimmunity and suggests that despite the complex nature of autoimmune diseases therapeutic effects are achievable by targeting specific cell populations. Besides polymorphisms that affect Fc γ RIIB promoter activity, there is evidence that mutations in the transmembrane region of the inhibitory receptor are linked to human lupus in certain racial groups (Chu et al.

2004; Kyogoku et al. 2004; Siriboonrit et al. 2003). Recent evidence suggests that this allelic variant loses its inhibitory capacity due to its inability to associate with lipid rafts (Floto et al. 2005; Kono et al. 2005).

The B-cell stage(s) at which Fc γ RIIB exerts its function as a gatekeeper of self tolerance has recently been defined. Autoreactive B cells can be generated at several stages during B-cell development (Grimaldi et al. 2005). There is accumulating evidence that Fc γ RIIB mediates its function during late stages of B-cell maturation thus representing a distal checkpoint. It has been suggested that B cells generating autoreactive or low-affinity BCRs during somatic hypermutation will lose BCR interactions with their cognate antigen retained in the form of immune complexes on follicular dendritic cells (FDC). This results in isolated triggering of Fc γ RIIB which induces apoptosis (Fig. 3) (Pearse et al. 1999; Ravetch and Bolland 2001). More recently it has been shown that in the absence of Fc γ RIIB IgG positive plasma cells secreting autoreactive antibody species can accumulate (Fukuyama et al. 2005). Fc γ RIIB deficiency did not impact on early events in the bone marrow like receptor editing nor did it prevent the development of IgM positive autoreactive B cells. After class switching to IgG, however, Fc γ RIIB was essential to prevent the expansion of autoreactive B cells and their maturation into plasma cells. Taking the considerably higher pathogenic potential of IgG compared to IgM antibody isotypes into account this relatively late stage of Fc γ RIIB mediated negative regulation might be sufficient to prevent the initiation of severe autoreactive processes.

These results would support a model in which several central and peripheral checkpoints prevent the emergence of autoreactive B cells and their maturation into plasma cells that could secrete pathogenic antibodies. Central checkpoints including receptor editing, deletion, and anergy of self-reactive BCR species ensure that the majority of B cells with an autoreactive BCR are deleted in the bone marrow (reviewed in Goodnow et al. 2005; Grimaldi et al. 2005; Meffre et al. 2000); this occurs independently of Fc γ RIIB. It is widely accepted, however, that this process is incomplete and self reactive cells can escape into the periphery, in a background dependent manner. Thus Balb/c are more efficient in editing than C57BL/6 mice, making the latter a more permissive strain for the development of autoimmunity. Consistent with this observation, Fc γ RIIB deficient mice on the Balb/c background did not develop spontaneous autoimmunity; in contrast, this deficiency of an inhibitory receptor on the C57BL/6 background resulted in the emergence of a highly penetrant, fatal lupus-like disease. Moreover, autoreactive B cells can be generated *de novo* in the periphery during the germinal center reaction (Ray et al. 1996; reviewed in Bona and Stevenson 2004). Therefore, additional checkpoints are of major importance to prevent the accumulation of autoreactive cells in the periphery. Furthermore, the expansion of class switched self reactive antibodies that can trigger a wide variety of inflammatory effector functions needs to be tightly regulated (Dijstelbloem et al. 2001; Ravetch and Bolland, 2001). Here Fc γ RIIB might serve as the final barrier to prevent these B cells with harmful BCR specificities from maturing into plasma cells that otherwise would induce tissue pathology by secretion of large amounts of self reactive antibodies.

6.4.4 The Role of Fc γ RIIB in the Efferent Response: Controlling Innate Immune Effector Cell Activation

Besides its autoregulatory role in the afferent response, Fc γ RIIB is an important modulator of inflammatory effector cells such as mast cells, neutrophils and macrophages during the efferent phase of an immune response (Dijstelbloem et al. 2001; Ravetch and Bolland 2001). On these cell types Fc γ RIIB is coexpressed with activating Fc receptors of varying affinities and isotype specificities and negatively regulates activating signals delivered by these receptors. Lack of Fc γ RIIB leads to elevated immune complex mediated inflammation and phagocytosis as demonstrated by an enhanced Arthus reaction, systemic anaphylaxis, anti-GBM glomerulonephritis, immunothrombocytopenia, hemolytic anemia, collagen-induced arthritis, and IgG-mediated clearance of pathogens and tumor cells (Ravetch 2003).

On allergic effector cells such as mast cells and basophils Fc γ RIIB regulates activating signals triggered by crosslinking Fc ϵ RI resulting in enhanced IgE-mediated anaphylaxis and heightened sensitivity to allergic rhinitis (Watanabe et al. 2004; reviewed in Kraft and Novak 2005). Moreover, Fc γ RIIB deficiency renders otherwise resistant mouse strains susceptible to development of certain forms of collagen induced arthritis (Takai 2002). In some of these models both increased autoantibody production due to Fc γ RIIB-deficiency on B cells and heightened effector cell responses are likely to contribute to the observed phenotype. As will be discussed below, the magnitude of Fc γ RIIB modulation is strictly isotype dependent and predictable based on the relative affinities of IgG subtypes for activating and inhibitory receptors.

6.4.5 The Activating Fc Receptors in the Efferent Response

Activating Fc receptors including Fc α R, Fc ϵ R, and the family of Fc γ -receptors (Fc γ RI, III, and IV) are expressed on a wide variety of immune effector cells including mast cells, basophils, monocytes, macrophages, neutrophils, and NK cells. The importance of these receptors for effector functions mediated by these cells has been demonstrated by genetic deletion of the common signaling γ -chain used by all of these receptors (Takai et al. 1994). In these animals, immune complex or allergen mediated effector functions, such as antibody dependent cellular cytotoxicity (ADCC), release of inflammatory mediators, cytokine release and phagocytosis of immune complexes are abrogated or heavily impaired. As many of these Fc receptors are coexpressed on the same cell subsequent deletion of the individual ligand binding α -chains was crucial to elucidate the role of the individual Fc receptors.

Expectedly, mice deficient in the high-affinity Fc ϵ RI, which is essential for cell surface binding of IgE to mast cells showed a dramatic reduction in IgE-mediated

anaphylaxis and allergic reactions. For the family of Fc γ Rs, however, the role of the individual receptors was less clear until recently. Here, deletion of the individual activating Fc γ -receptors I or III resulted in less pronounced phenotypes especially for effector responses involving the IgG2a and IgG2b antibody isotypes. This was surprising as *in vitro* studies had shown that the high-affinity Fc γ RI could bind to IgG2a and the low-affinity Fc γ RIII to IgG1, IgG2a, and IgG2b (reviewed in Hulett and Hogarth 1994; Ravetch and Kinet 1991). Although there is some evidence that Fc γ RI and III may participate in a limited fashion in IgG2a-mediated effector responses (Barnes et al. 2002; Ioan-Facsinay et al. 2002), the majority of studies concluded that IgG2a and IgG2b triggered effects occur independently of these two receptors, but in a γ -chain dependent manner (Fossati-Jimack et al. 2000; Hazenbos et al. 1996; Meyer et al. 1998; Nimmerjahn et al. 2005; Uchida et al. 2004). In contrast Fc γ RI, due to its high affinity for IgG2a (K_A : 10^8 – 10^9 M $^{-1}$), resulting in equal efficiency binding of monomeric IgG2a and immune complexes (ICs), might not be available for newly generated ICs (Fig. 4).

An alternative theory suggested that other effector mechanisms, such as activation of the complement cascade, might mediate the *in vivo* effects of these isotypes. Indeed, IgG2a and IgG2b can efficiently activate the complement cascade *in vitro* (Duncan and Winter 1988). However, several studies using mice deficient in a variety of complement proteins such as C3, C4 or CR2 failed to demonstrate a major involvement of the complement cascade (reviewed in Ravetch and Clynes 1998; Uchida et al. 2004). On the other hand, deletion of the γ -chain abrogated

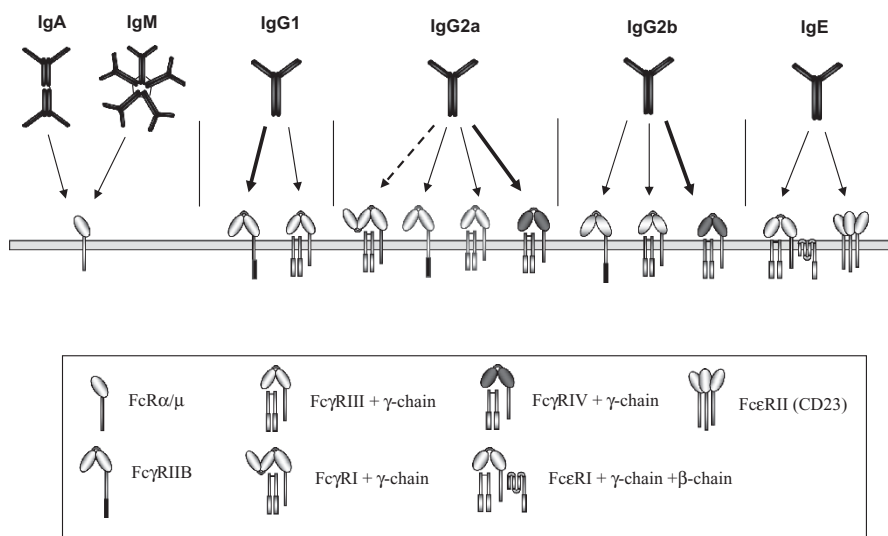


Fig. 4. Antibody isotype binding to Fc receptors. Shown is the interaction of different antibody isotypes (IgA, IgM, IgE, IgG1, IgG2a, and IgG2b) with their respective Fc receptors. Arrows indicate interactions and the *thickness of the arrows* indicates preferential interactions. A *broken arrow* indicates that the interaction is hypothetical (see text for details)

IgG2a and IgG2b effector functions strongly arguing for the existence of other γ -chain dependent Fc receptors.

In contrast to the unclear situation for IgG2a and IgG2b, IgG1-mediated effector functions were abrogated in the absence of the low-affinity Fc γ RIII. These results were confirmed in a variety of models like arthritis, glomerulonephritis, IgG-dependent anaphylaxis, IgG mediated hemolytic anemia and immunothrombocytopenia (ITP) (Bruhns et al. 2003; Fossati-Jimack et al. 2000; Fuji et al. 2003; Hazenbos et al. 1996; Ji et al. 2002; Meyer et al. 1998; Nimmerjahn et al. 2005). However, the most potent antibody isotypes for protection against bacterial or viral infections (Coutelier et al. 1987; Markine-Goranyoff and Coutelier 2002; Schlageter and Kozel 1990; Taborda et al. 2003), antibody-mediated cytotoxicity or antibody-based therapy (Fossati-Jimack et al. 2000; Kipps et al. 1985; Nimmerjahn et al. 2005 Uchida et al. 2004;) were of the IgG2a and IgG2b isotype. Therefore, a thorough understanding of how these isotypes exert their function is essential.

6.4.6 The Missing Piece in the IgG-Puzzle: Identification of Fc γ RIV

To identify new Fc receptor homologous or related proteins, several groups started genome database searches on the basis of conserved sequences among the classical Fc receptors. During this search one gene was identified that showed 63% overall amino-acid identity to human Fc γ RIIIA and an even greater identity in the antibody binding extracellular domain and was called Fc receptor like 3 (Fcrl3), CD16–2, or Fc γ RIV (Davis et al. 2002; Mechetina et al. 2002; Nimmerjahn et al. 2005). To be consistent with the current nomenclature system for mouse Fc γ -receptors and to avoid confusion with other Fcrl-proteins, we suggest calling this protein Fc γ RIV.

The Fc γ RIV gene is located on mouse chromosome 1, tightly linked to Fc γ RIIB and Fc γ RIII. A prediction of orthologue proteins in other species shows that there are related proteins in humans (Fc γ RIIIA), chimpanzees, macaques, rats, dogs, cats, pigs and cows (Fig. 1 and not shown) with the highest level of similarity to the rat (80%) and the human orthologue. As has been described for other γ -chain dependent Fc receptors, cross-linking of Fc γ RIV by immune complexes induces activating signaling pathways leading to sustained calcium flux (reviewed in Nimmerjahn et al. 2005; Ravetch and Bolland 2001).

Regarding cell type restriction, Fc γ RIV is highly expressed on neutrophils, monocytes, macrophages and dendritic cells and undetectable on mast cells, NK cells, T and B cells. Similar to other activating Fc receptors, inflammatory stimuli (lipopolysaccharide; LPS) and Th-1 cytokines (interferon gamma; IFN- γ) can upregulate Fc γ RIV; in contrast Th-2 cytokines interleukin (IL)-4, IL-10, or transforming growth factor beta (TGF- β) downregulate Fc γ RIV cell surface expression (Nimmerjahn et al. 2005). After induction of DC maturation Fc γ RIV together with other activating Fc receptors is downregulated. An important difference between

mouse Fc γ RIV and the human Fc γ RIIIA is that Fc γ RIV is not expressed on NK cells. Human neutrophils do not express Fc γ RIIIA but rather Fc γ RIIA as their dominant activating FcR.

In vitro analysis revealed that Fc γ RIV bound IgG2a and IgG2b with intermediate affinity (K_A : $2\text{--}3 \times 10^7 \text{ M}^{-1}$), but not IgG1 or IgG3 antibody isotypes. In contrast to the high-affinity Fc γ RI, this affinity was not sufficient to enable stable binding to monomeric IgG, leaving it accessible for immune complex binding. Remarkably its higher affinity for IgG2a and IgG2b compared to the inhibitory Fc γ RIIB predicted that it would be less sensitive to Fc γ RIIB-mediated negative regulation (Table 1). More importantly, even if coexpressed with Fc γ RIII, IgG2a and IgG2b ICs would preferentially engage Fc γ RIV due to its 20–40 times higher affinity and due to the strong Fc γ RIIB imposed negative regulation of Fc γ RIII. Consistent with this notion, blocking Fc γ RIV function in vivo greatly impairs the pathogenic effects of IgG2a and IgG2b antibodies in passive models of antibody mediated platelet depletion or tumor cell destruction (Hamaguchi et al. in press; Nimmerjahn and Ravetch 2005; Nimmerjahn et al. 2005) and in an active model of glomerulonephritis induced by administration of nephrotoxic serum (Kaneko et al. 2006). Neither deficiency in members of the complement cascade nor in Fc γ RI or III had a significant effect on IgG2a/2b mediated responses.

6.4.7 Isotype Specific Fc γ -Receptor Engagement and Differential Regulation by Fc γ RIIB

Taken together, these studies suggest that even if several activating Fc receptors with the same isotype specificity are present on the same cell only those Fc receptors will be engaged that show the optimal affinity for the respective isotype (Fig. 4). Therefore, IgG1 immune complexes will only trigger Fc γ RIII as it is the only activating Fc receptor that can bind IgG1 (Hazenbos et al. 1996; Meyer et al. 1998; Nimmerjahn et al. 2005; Takai 1994); IgG2a and IgG2b, despite their ability to bind Fc γ RI and Fc γ RIII, respectively, will mainly engage Fc γ RIV as Fc γ RI will be occupied by monomeric IgG2a. Interestingly, human Fc γ RIIIA also has a higher affinity for IgG compared to human Fc γ RIIA, indicating that the above rules might also apply for the human system. It should be noted, however, that allelic Fc receptor variants that show differential affinities for the specific antibody isotypes exist in humans, which creates a more complex scenario (Dijstelbloem et al. 2001).

If antibody activity in vivo is mostly dependent on the interaction with activating and inhibitory Fc receptors, antibody activity might be predictable if one knows the affinity differences (A/I-ratio) of antibody isotypes for the individual activating and the inhibitory FcR. This question was addressed recently and it was shown that individual antibody isotypes have significantly different A/I ratios (Table 2). IgG1 for example had an A/I ratio of 0.1, indicating a strong influence of the inhibitory receptor on antibody activity. In contrast, IgG2a and IgG2b had A/I ratios of 7 and 70 making them less sensitive to Fc γ RIIB-mediated negative regulation

Table 2. Activating Fc receptors are differentially regulated by Fc γ RIIB

	Ratio of activating to inhibitory receptor(A/I)	
	Fc γ RIII/Fc γ RIIB	Fc γ RIV/Fc γ RIIB
IgG1	0.1 ^a	n.b.
IgG2a	1.6	70
IgG2b	0.5	7
IgG3	n.b.	n.b.

^a A/I ratios were calculated based on the affinities of the indicated Fc receptors as described (Nimmerjahn et al. 2005). n.b. indicates that the isotype is not binding to the indicated Fc receptor

(Nimmerjahn et al. 2005). Testing these predictions in in vivo model systems of antibody-mediated platelet depletion or tumor cell destruction with antibody switch variants showed that IgG2a and IgG2b antibodies were the most efficient isotypes and that deletion of the inhibitory receptor impacted most strongly on IgG1 activity (Clynes et al. 2000; Nimmerjahn and Ravetch 2005). This hierarchy of antibody isotype activity has been observed in other model systems where isotype switch variants were used showing that IgG2a and IgG2b variants were more potent than IgG1 or IgG3 (Fossati-Jimack et al. 2000; Kipps et al. 1985).

6.4.8 The Effect of Cytokines on FcR Expression

Many studies have addressed the question how cytokines can impact on Fc receptor expression. It was shown that depending on cell type and Fc receptor these effects can be different. Frequently cytokines regulate expression of the associated signaling adaptors (β - and γ -chains), which leads to a concomitant change in α -chain expression, as shown for TGF- β , IL-4, and IL-10 for example (Gillespie et al. 2004; Tridandapani et al. 2003). Moreover, inflammatory cytokines/stimuli, such as tumor necrosis factor (TNF)- α or LPS, tend to upregulate activating receptors, such as Fc α -, Fc ϵ - and Fc γ -receptors, whereas TGF- β , IL-4, and IL-10 seem to have the opposite effect (Nimmerjahn et al. 2005; Otten and van Egmond 2004; Tridandapani et al. 2003). Importantly, these effects can be cell type specific. Interleukin-4 for example upregulates the inhibitory Fc γ RIIB on myeloid cells. On activated B cells, however, this cytokine downregulates receptor expression (Rudge et al. 2002).

Regarding the different IgG isotypes it is interesting to consider how cytokines will influence the basic A/I ratios discussed before. It seems likely that a change in basal expression levels of activating versus inhibitory receptors will impact differentially on the various IgG isotypes. Thus, IgG2a antibodies are relatively insensitive to these effects, while IgG1 is quite sensitive to modest changes in A/I ratios. For example, in active models of antibody mediated inflammation the steady state ratios will be changed in favor of the activating Fc receptors. Inflammatory mediators, such as IFN- γ and C5a, can upregulate activating Fc γ -receptors and at

the same time reduce Fc γ RIIB expression levels (Guyre et al. 1983; Shushakova et al. 2002). Under these circumstances autoreactive IgG1 antibodies are capable of triggering severe damage. In contrast Th-2 cytokines like IL-4, IL-10, or TGF- β upregulate the inhibitory Fc γ -receptor and decrease expression of the activating Fc γ -receptors (Nimmerjahn et al. 2005; Okayama et al. 2000; Pricop et al. 2001; Radeke et al. 2002; Tridandapani et al. 2003). Under these conditions isotypes that have a low or moderate A/I ratio (IgG1 and IgG2b) would be expected to lose more activity than those with a high ratio, such as IgG2a. High-dose intravenous gamma globulin (IVIG) provides a compelling example of the validity of this approach. The anti-inflammatory activity of this preparation has recently been shown to be linked to its ability to upregulate Fc γ RIIB expression on effector macrophages in models of ITP, rheumatoid arthritis (RA) and glomerulonephritis (Bruhns et al. 2003; Kaneko et al. 2006; Samuelsson et al. 2001). For IgG1, this modulation alone is sufficient to convert a pathogenic antibody to a nonpathogenic isotype, consistent with the low A/I ratio for this subclass in which modest changes in Fc γ RIIB expression will raise the threshold required for effective IgG1 crosslinking of Fc γ RIII. In contrast, in an active model of nephrotoxic nephritis, IgG2b antibodies were demonstrated to mediate the inflammatory response. IVIG protects in this disease model by upregulating Fc γ RIIB, as observed previously, and by downregulating Fc γ RIV, thus altering the A/I ratio to a level where inhibition dominates and inflammation is blocked. Fc γ RIIB modulation alone is insufficient to provide protection for this subclass, requiring the additional contribution of Fc γ RIV downregulation to lower the threshold to prevent IgG2b activation of Fc γ RIV (Kaneko et al. 2006).

Interestingly there is evidence that this paradigm holds true for human IgG antibodies as well. Human clinical trials with antibodies directed against tumor cell surface proteins, such as CD20, revealed that patients with the high-affinity Fc γ RIIIA allele resulting in a higher A/I ratio responded significantly better to antibody therapy with increased survival and time to relapse (Cartron et al. 2002; Weng and Levy 2003; Weng et al. 2004). The challenge for future antibody based immunotherapeutic approaches will be to select, or preferably design antibody isotypes that can trigger effector functions such as ADCC reactions even under unfavorable A/I ratios. A critical factor in such Fc engineering is knowledge of the FcR expression profile of the relevant effector cells mediating the biologically relevant response and the impact of both disease and therapy on the modulation of these receptors. Moreover, knowledge of the dominant antibody isotypes involved in an autoimmune disease might be predictive for the involvement of a specific activating Fc receptor, thereby allowing the identification of therapeutic targets. In line with this a recent study using a nephrotoxic nephritis model has shown that the predominant pathogenic antibody isotype under these experimental conditions was IgG2b (Kaneko et al. 2006). Despite the possibility that this subclass could bind Fc γ RIII and Fc γ RIV, only Fc γ RIV was found to be responsible for triggering the pathogenic effects; blocking of RIV abrogated disease. Similarly, since murine lupus models display a dominance of IgG2a and IgG2b subclasses, it is tempting to speculate that Fc γ RIV is the responsible activating Fc receptor in these disease models, as

well. The regulation of FcR expression by cytokines is coupled to the regulation of isotypes by these same cytokines. Thus, Th1 cytokines such as IFN- γ induce class switching to IgG2a whereas Th2-type cytokines (IL-4) induce class switching to IgG1; TGF- β in contrast will induce switching to IgG2b (Coffman et al. 1989; Finkelman et al. 1990). As these cytokines also influence Fc receptor expression the pathogenicity of an autoimmune response will be determined by both cytokine mediated regulation of class-switching and the changes of expression levels of the responsible activating versus inhibitory Fc receptors.

6.5 Summary

Every day the human immune system is confronted with billions of pathogenic and nonpathogenic microorganisms. The decision when to react and with what strength is crucial for survival of the host. Therefore, the immune system needs to distinguish self from nonself and especially from dangerous nonself. Research in the field of Fc receptor biology over the last decade has established the important role of these receptors for making such decisions. There are several levels of regulation that will determine whether Fc receptor triggering on immune cells will result in cell activation or inhibition. The most important is the relative affinity of individual antibody isotypes for activating and inhibitory receptors (their A/I ratio), the relative expression level of activating and inhibitory receptors and the cytokine environment which can influence this expression level. Factors that impair this threshold established by the delicate balance of activating and inhibitory signals often result in uncontrolled immune cell activation ultimately leading to destruction of self tissues and autoimmune disease. Thus, Fc receptors together with other proteins such as Toll-like receptors, NK cell receptors or costimulatory molecules provide the basis for distinguishing between self and nonself and for the generation of a well balanced immune response that destroys pathogens without concomitant damage to the host

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Self and Nonself Recognition by Coreceptors on B Lymphocytes: Regulation of B Lymphocytes by CD19, CD21, CD22, and CD72

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7.1 Introduction

B-cell antigen receptor (BCR) signaling plays an essential role in regulation of the development and function of B lymphocytes (see Chapter 4). These signals are modulated by coreceptors expressed on the surface of B cells, including CD19, CD21 (complement receptor 2, CR2), CD22, CD72 (Lyb-2), and low-affinity receptor for IgG (Fc γ RII) (see Chapter 6 on Fc receptors). When CD19/CD21 complex is colligated with BCR, this complex positively regulates BCR signaling through the recruitment of signaling molecules, such as a phosphatidylinositol 3-kinase (PI3K), to their phosphorylated tyrosines. On the other hand, CD22 and CD72 negatively regulate BCR signaling through the recruitment of the SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1) to their phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs). From the characterization of each of these coreceptors deficient mice, now it is clear that the regulations by these coreceptors physiologically play a significant role in the development, activation, proliferation and differentiation of B cells. Importantly, these coreceptors are able to detect and respond to extracellular environment and modulate BCR signaling by their ligand bindings. These functions contribute to the recognition of self- and nonself-antigens (Ags), in some cases, in cooperation with the innate immune system, such as the recognition of activated complement fragments by CD21 (see Chapter 1 on innate immunity). In this chapter, we focus on the physiological function of the CD19, CD21, CD22, and CD72 and their molecular mechanisms involving signal transduction by these coreceptors, which will help us to understand how B cells recognize self and nonself.

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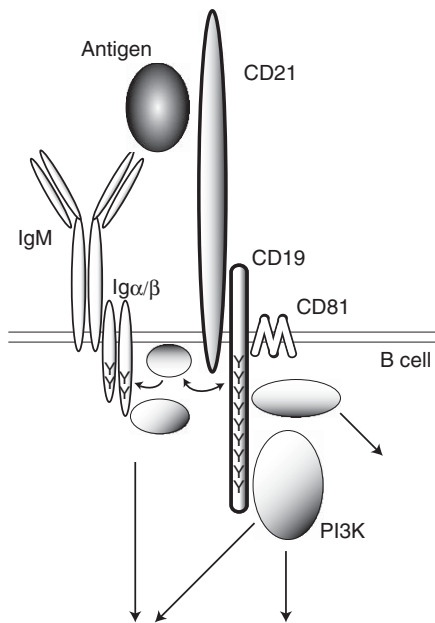
7.2 Regulation of B Cells by CD19/CD21 Complex

7.2.1 Structure and Expression of CD19 and CD21

CD19 is an approximately 95 kDa membrane protein and its extracellular region contains two or three immunoglobulin (Ig) like domains (Stamenkovic and Seed 1988; Tedder and Isaacs 1989) and its cytoplasmic region is extensively conserved among human, mouse and pig, including nine conserved tyrosine residues (Tedder and Isaacs 1989; Zhou et al. 1991), which is consistent with its critical role in CD19 function (as described in section 7.2.5). Expression of human CD19 is restricted to B lineage cells and follicular dendritic cells (FDCs) (Tedder et al. 1997) and its expression is regulated by B-cell-specific transcription factor BSAP (Kozmik et al. 1992). CD19 interacts with CD21 and other proteins (Fig. 1).

CD21, an approximately 150kDa protein, possesses 15 repeating structures termed short consensus repeat sequences (SCRs) in its extracellular domain and a 34-amino-acid short cytoplasmic domain. In mice both CD21 and CD35 (complement receptor 1, CR1) are encoded by Cr2 gene and mouse CD21 and CD35 are produced by alternative splicing. However, in human, distinct genes encode CD21 and CD35 (Kurtz et al. 1990; Molina et al. 1990). Murine CD35 shares 15 SCRs with CD21 and possess 6 additional SCRs. Expression of human CD21 is not restricted to B lineage cells. FDCs (Reynes et al. 1985), a subset of T lymphocytes (Fischer et al. 1991) and epithelial cells (Birkenbach et al. 1992), also express CD21. Thus, CD21 expression is different from CD19 and is regulated by promoter

Fig. 1. CD19/CD21 complex-mediated positive regulation of B-cell antigen receptor (BCR) signaling. Complement receptor CD21 interacts with activated C3 fragments that are bound to antigens. This coligation of BCR and CD19/CD21 complex by antigens induces activation of protein tyrosine kinases and also induces tyrosine phosphorylation of CD19. Tyrosine phosphorylation of CD19 induces recruitment of SH2-domain containing proteins, including phosphatidylinositol 3-kinase (PI3K), and activation of these proteins. The protein kinases and PI3K activated by CD19 positively regulates BCR signaling. PI3K also promotes cell survival. CD19/CD21 complex contains CD81, which is required for efficient cell surface expression of CD19



and intronic sequences of CD21 in mice and humans (Hu et al. 1997; Makar et al. 1998). Pro-, pre- and immature B cells express CD19 but not CD21 (Tedder et al. 1984). In the plasma, soluble form of CD21 exists (Lowe et al. 1989), which is produced not by alternative splicing (Illges et al. 1997) but by proteolysis of its extracellular portion from peripheral B cells (Masilamani et al. 2003). Besides the complex formation with CD19, as described in section 7.2.3, CD21 acts as a complement receptor by recognizing cleavage fragments of the third complement component (C3), that is iC3b, C3dg and C3d.

7.2.2 CD19/CD21/CD81 Complex

To date, several components of the CD19/CD21 complex are reported. Interactions of some of them depend on detergents used for solubilization of cells and may depend on the developmental stage of B cells. CD19 is a part of the complex including CD21, CD81 (the target of an anti-proliferative antibody, TAPA-1), and Leu-13. CD19 directly interacts with CD21 and CD81 through its extracellular domain (Bradbury et al. 1992; Matsumoto et al. 1991, 1993). Moreover in B cell lines, treatments of monoclonal antibodies (mAbs) against these molecules enhance the increase in intracellular Ca^{2+} concentration following suboptimal dose of IgM crosslinking (Bradbury et al. 1992). In this complex, CD21 recognizes cleaved complement fragments, CD81 regulates localization of them and CD19 mediates intracellular signaling.

CD81, a member of the tetraspan family, is a ubiquitously expressed 26 kDa cell surface protein with four transmembrane domains and a short intracellular tail lacking signaling motifs. CD81 is expressed at all stages of B-cell development (Levy et al. 1998). On B cells, CD81 interacts with CD19, CD21, Leu-13, MHC class II, tetraspan family proteins including CD37, CD53, R2/C33, CD82, and some of the integrin family proteins (Angelisova et al. 1994; Horvath et al. 1998; Takahashi et al. 1990). In CD81 deficient mice, the development of B cells is normal but the expression of CD19 is reduced (Maecker and Levy 1997; Miyazaki et al. 1997; Tsitsikov et al. 1997). The reduction of CD19 does not depend on transcriptional regulation of CD19 gene because mRNA levels of CD19 are not affected and the reduction of CD19 rather depends on trafficking to surface membrane or instability on surface membrane (Miyazaki et al. 1997; Shoham et al. 2003). Recently, it has been demonstrated that coligation of BCR and CD19/CD21/CD81 complex induces rapid CD81 palmitoylation and this results in stable partitioning of colligated complex into membrane lipid rafts (Cherukuri et al. 2004a,b). This function of CD81 can contribute to the CD19/CD21 complex-mediated enhancement of BCR signaling. CD81 also regulates cellular adhesion of B cells (Behr and Schriever 1995; Mittelbrunn et al. 2002).

The earliest B-cell precursors express CD19 but not CD21 and expression of CD19 is molar excess of that of CD21 at all developmental stages of B cells. CD19 interacts with equimolar CD21. This implicates that most of CD21 forms complex

with CD19, but not necessarily all CD19 form complex with CD21 (Fearon and Carroll 2000) and that CD19 can act independently of CD21 as described in 7.2.6 in this chapter.

7.2.3 Complement Receptor CD21

CD21 acts as a complement receptor and it recognizes cleaved C3 fragments iC3b, C3d, and C3dg. In human, CD21 also acts as the Epstein–Barr virus receptor (Fingeroth et al. 1984) and has been reported to interact with the low affinity IgE receptor, CD23 (FcεRII) (Aubry et al. 1992). Many works show the biological significance of the interaction between CD21 and C3 fragments. In addition to an important role in innate immune responses, C3 also plays an important role in T-dependent (TD) Ag responses (Bottger et al. 1986). Characterization of C3 deficient mice demonstrated that impairment of TD Ag responses was not at T-cell but at B-cell level, and due to impaired retention of Ags by FDCs (Fischer et al. 1996). Mice deficient in *Cr2* gene which encodes both CD21 and CD35 as alternatively spliced gene products also exhibit the reduced responses to TD Ags, which is similar to the phenotype of C3 or C4 deficient animals (Ahearn et al. 1996; Croix et al. 1996; Molina et al. 1996). Moreover, B cells from C3 deficient mice show reduced antibody (Ab) responses and FDCs from C3 deficient mice are unable to promote Ab responses of B cells. These results can be explained by the notion that the C3 fragments that bound to Ags on FDCs interact with CD21 on B cells (Qin et al. 1998). Expression of CD21/CD35 on FDCs also plays an important role in immune complexes (ICs) binding to FDCs, IgG production and normal TD responses (Fang et al. 1998). CD35 on marginal zone (MZ) B cells also contributes to an initial phase of TD response by promoting efficient transport of IgM-containing ICs from MZ B cells to FDCs because the initial IgM-ICs binding to MZ B cells is dependent on complement and its receptor, CD21/CD35 (Ferguson et al. 2004).

It is noteworthy that murine systemic lupus erythematosus (SLE) susceptibility locus contains mutated *Cr2* and this mutation located in the C3d binding domain causes the reduction of ligand-binding ability (Boackle et al. 2001). Moreover, CD21/CD35 deficiency concomitant with Fas (Apo-1/CD95) defective *lpr* mutation in C57BL/6 background is sufficient to increase the serum concentrations of IgG1, IgG2b, antinuclear and anti-double stranded DNA (dsDNA) Abs (Wu et al. 2002). Fas, a member of the death receptor family, plays a role in induction of apoptosis and its defect is associated with SLE susceptibility.

The generation of C3 fragments that interact with CD21 is as follows. By the activation of the classical complement pathway, alternative pathway, or mannose-binding lectin pathway, C3 is cleaved to C3a and C3b. C3b is then converted to iC3b and subsequently converted to C3dg. These cleavages are promoted by CD35 (Rickert 2005). Cleavage of C3dg generates C3d. Because of the high reactivity of C3b, it promptly binds various molecules and cells with covalent bonds.

Activation of C3b bound to host cells is regulated by many factors that exist on surface of host cells and protect host cells from complement systems. On human B cells, part of CD21 interacts with CD35 (Delibrias et al. 1992) but CD35 is not a component of complex including CD19/CD21 (Tuveson et al. 1991). In contrast, on mouse B cells, CD19 interacts with both CD21 and CD35. This is probably dependent on structure of mouse CD35 that contains six additional SCRs to the amino-terminal end of the mouse CD21. The conversion of C3b to iC3b or C3dg decreases the affinity for CD35 and increases the affinity for CD21 (Kalli et al. 1991; Pramoonjago et al. 1993). So it is possible that the interaction between CD35 and CD21 may play a role in facilitating the transfer of C3b bound Ags that interact with CD35 to CD21.

7.2.4 Coligation of CD19/CD21 Complex with BCR

As we saw in the previous section, CD21 has ability to interact with the cleaved complement fragments. Importantly, this function of CD21 enables the coligation of CD19/CD21/CD81 complexes with BCR when C3d bound Ags are recognized by BCR. In fact, it has been demonstrated that crosslinking of CD19/CD21 with BCR on B cells using bi-specific Abs, which is able to interact with both CD19 or CD21 and BCR, enhances the elevation of intracellular Ca^{2+} concentrations and proliferation, and decreased the threshold for BCR-mediated stimulation by 100 fold (Carter et al. 1991; Carter and Fearon 1992). Similarly, C3d bound Ags become significantly immunogenic and enhance B-cell responses to these Ags (Dempsey et al. 1996). The BCR stimulation with anti-Ig Abs alone also induces tyrosine phosphorylation of CD19 (Chalupny et al. 1993) and recruitment of CD19 to BCR (Pesando et al. 1989). CD19 interacts directly with BCR through juxtamembrane region of its cytoplasmic domain (Carter et al. 1997). However, the effect of the BCR stimulation on B-cell response in the absence of CD19/CD21 complex coligation is quite small, compared to that of the stimulation causing direct CD19/CD21 complex coligation with BCR (Fearon and Carroll 2000).

7.2.5 CD19-Mediated Signal Transduction

We have described CD19/CD21/CD81 complexes, where CD81 contributes controlling the localization of them, CD21 detects extracellular environment by recognizing cleaved complement fragments, and CD19 enables the coligation of CD19/CD21 complex with BCR. Now we focus on the role of CD19 in this complex especially in signal transduction. CD19 possesses relatively large intracellular region and this region contains nine conserved tyrosine residues, through which CD19 interacts with various proteins and regulates the intracellular signaling pathways.

B-cell receptor antigen stimulation and/or CD19 complex ligation induces phosphorylation of these tyrosines and leads recruitment of Src homology 2 (SH2) domain-containing proteins. Y330 (in the human CD19 sequence) of CD19 binds Grb2, which forms a complex with Sos and activates Ras (Brooks et al. 2000), Y391 binds Vav, which is Rho-family guanine nucleotide exchange factor (O'Rourke et al. 1998), Y391 and Y421 bind both Vav and phospholipase C-gamma 2 (PLC γ 2) (Brooks et al. 2000), and Y403 and Y443 bind Fyn and Lyn, which are members of the Src-family protein tyrosine kinases (Chalupny et al. 1995). Y482 and Y513 bind PI3K (Chalupny et al. 1995; Tuveson et al. 1993). Importantly, the analysis of CD19 deficient mice expressing CD19 mutant transgene demonstrated the essential role of Y482 and Y513 for normal B-cell differentiation and function. In mice that have mutated Y482 and Y513 CD19 (Y482F/Y513F), normal B-cell differentiation and function are blocked and this is similar to the phenotype of CD19 deficient mice. In contrast, in mice that have mutated Y330 and Y360 CD19 or mutated Y391 and Y421 CD19, the differentiation of B cells, Ab responses and germinal center reaction is relatively normal and is similar to the phenotype of CD19 hetero deficient mice (Wang et al. 2002).

Several studies have also demonstrated the importance of Y482 and Y513 and have revealed the mechanisms of how these tyrosines regulate intracellular signaling. First, Y482 and Y513 preferentially interact with PI3K (Chalupny et al. 1995; Tuveson et al. 1993). Moreover, when the BCR stimulation that is independent of CD19/CD21 coligation induces PI3K activation, CD19 plays a critical role in PI3K activation and subsequent Bruton's tyrosine kinase (Btk) activation and these events are dependent on Y482 and Y513 (Buhl et al. 1997; Buhl and Cambier 1999). Btk is a critical kinase in BCR signaling, as indicated by X-linked immunodeficiency phenotype of mice that express mutant forms of the Btk and X-linked agammaglobulinemia phenotypes of men who also express the mutated Btk. PI3K is also known as a critical activator of Akt/PKB that provides survival signal and inhibits apoptosis. Consistently, CD19 is required for the enhanced and prolonged activation of Akt following the BCR stimulation independent of CD19 coligation (Otero et al. 2001) and plays an important role in the BCR-mediated survival signaling (Barrington et al. 2005; Otero et al. 2003).

The results that PI3K, Vav1, and Vav2 play a critical role in the increase of intracellular Ca²⁺ concentration, and Vav1 and Vav2 play a critical role in the activation of PI3K following crosslinking of BCR and CD19, also support the significance of PI3K (Vigorito et al. 2004). Although strong BCR stimulation independent of CD19 coligation induces PI3K activation, coligation of BCR and CD19 induces the more efficient activation of PI3K. This study also indicates that the activation of PI3K following strong BCR stimulation independent of CD19 coligation does not require Vav (Vigorito et al. 2004).

Following BCR ligation, Lyn also phosphorylates Y513, subsequently Y482 of CD19, and then phosphorylated Y513 and Y482 provides a binding site for Lyn (Fujimoto et al. 2000). CD19 functions as a specialized adapter protein for the Src-family protein kinases and these kinases phosphorylates CD19 (Fujimoto et al. 1999, 2000; Hasegawa et al. 2001). However, there is room for the consideration

on the connection of Lyn and CD19. Whereas one report indicated that the activity of Lyn is diminished following BCR ligation in CD19 deficient mice (Fujimoto et al. 1999), another report indicated that the phosphorylation of CD19 after BCR ligation was not affected in Lyn deficient mice, and Lyn kinase activity is not affected in CD19 deficient B cells, either (Xu et al. 2002).

7.2.6 Regulation of B Cells by CD19 and CD21

As the result of the cooperation of CD19 and CD21, CD19/CD21 complex acts as a complement receptor and regulates intracellular signaling following coligation of this complex with BCR. This notion is supported by many studies although a part of the CD19 function is considered to be independent of CD21. For example, the characterizations of CD19 and CD21/CD35 deficient mice have revealed that CD19 and CD21/CD35 play a crucial role in TD Ag responses by lowering the threshold of BCR activation by several fold (Ahearn et al. 1996; Chen et al. 2000; Croix et al. 1996; Engel et al. 1995a,b; Molina et al. 1996; Rickert et al. 1995; Sato et al. 1995). In contrast to TD Ag responses, T-cell independent (TI) type II (TI-II) Ag responses are not significantly affected in CD19 deficient mice (Rickert et al. 1995; Sato et al. 1995). In CD21/CD35 deficient mice, however, TI responses to low dose Ags are significantly reduced including IgG3 Ab responses, which is important in response to TI-II Ags (Haas et al. 2002).

CD19 plays a critical role in the development of B-1 B cells. In CD19-deficient mice, B-1 cells are significantly reduced, early B-cell precursors are not significantly different and the number of peripheral B cells is reduced compared with those of wild-type mice (Engel et al. 1995a,b; Rickert et al. 1995). As for CD21, one line of CD21/CD35-deficient mice exhibit reduction of peritoneal B-1 cells (Ahearn et al. 1996) and demonstrated the importance of CD21/CD35 in maintenance of the B-1 cell repertoire to some, but not all, specificities (Reid et al. 2002), whereas another line of CD21/CD35 deficient mice exhibit normal number of peritoneal B-1 cells (Molina et al. 1996).

Proliferation of B cells in germinal centers (GCs) also requires CD19 and CD21. GCs play an important role in generation of memory B cells and Ab-forming cells (AFCs). The characterizations of CD21/CD35 deficient mice have demonstrated that even high-affinity antigen-activated B cells require CD21/CD35 for proliferation and survival in Germinal centers (GCs) (Fischer et al. 1998) and that CD21/CD35 is required for maintenance of serum Abs and long-lived AFCs (Chen et al. 2000) and the induction of Blimp-1, XBP-1, and Bcl-2 (Gatto et al. 2005). Blimp-1 and XBP-1 drive plasma cell differentiation, and Bcl-2 is important for inhibition of apoptosis. In addition, the analysis of CD19 deficient mice that express mutated Y482 and Y513 CD19 also indicates that normal function of CD19 is required for expansion and positive selection of GC B cells (Callea et al. 1997). Clonal selection of B cells in GCs requires a death receptor Fas that induces apoptosis (Takahashi et al. 2001), and deficiency of CD21/CD35 results in reduction of c-FLIP, which

inhibits death receptor-mediated apoptosis, and induction of Fas-mediated apoptosis (Barrington et al. 2005).

CD19 contributes to B-cell generation in the absence of CD21 at the early Ag-independent stages of B-cell development, which is consistent with its early expression in B-cell development. Bone marrow reconstitution experiments demonstrated competitive disadvantage of CD19 deficient early B cells in mixed bone marrow chimeras and in CD19 deficient mice: pre-B-cell proliferation is reduced after sublethal irradiation and pre-BCR signaling is impaired (Otero and Rickert 2003). The experiments using mAb to CD19 also indicate the involvement of CD19 to pre-BCR signaling (Krop et al. 1996).

7.3 Regulation of B Cells by CD22

7.3.1 Structure and Expression of CD22

CD22 is an approximately 140 kDa type I membrane protein and its expression is B-cell specific. CD22-deficient mice show the inhibitory function of CD22 in BCR signaling (O'Keefe et al. 1996; Nitschke et al. 1997; Otipoby et al. 1996; Sato et al. 1996). CD22 is a member of the Siglecs, sialic acid-binding Ig-like lectins. CD22 contains seven Ig-like domains in extracellular region and six tyrosines in cytoplasmic region (Wilson et al. 1991) and three of these tyrosines resides in ITIM sequence (Fig. 2). Although the form termed CD22 α containing five Ig-like domains

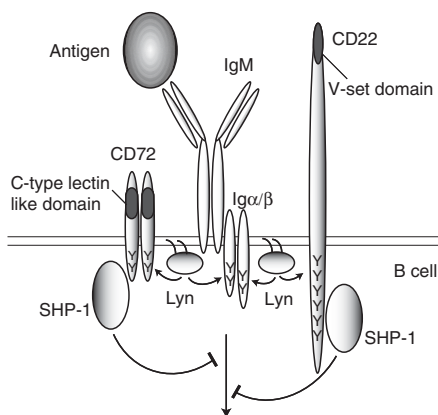


Fig. 2. B-cell antigen receptor (BCR) signaling inhibition by CD22 and CD72. Following antigen ligation, protein kinases, including Lyn, are activated. Lyn phosphorylates tyrosines in CD22 and CD72. This phosphorylation leads SH2-domain-containing protein tyrosine phosphatase-1 (*SHP-1*) recruitment to CD22 and CD72. *SHP-1* negatively regulates BCR signaling by dephosphorylating intracellular signaling molecules. CD22 can interact with sialic acid containing proteins including IgM by recognizing sialic acids. V-set domain in CD22, which is conserved in the sialic acid-binding Ig-like lectins (siglecs), plays an essential role in interaction with sialic acids. CD72 contains a C-type lectin like domain. However, its lectin activity is not yet clear

exists (Stamenkovic and Seed 1990), the form termed CD22 β containing seven Ig-like domains is dominant. Murine CD22 is expressed at high levels on mature recirculating B cells and in the peripheral B-cell subsets including follicular, marginal zone, B1, and class-switched B cells, at low levels on immature IgM^{hi} B cells, but is absent on pro-B cells, pre-B cells, newly emerging IgM⁺ B cells, and plasma cells (Erickson et al. 1996). Another work demonstrated the low level murine CD22 surface expression from Pre-B cells (Nitschke et al. 1997). The cell surface expression pattern of human CD22 is similar to that of mouse CD22, although human CD22 is detected in cytoplasm of pro-B and pre-B cells (Dorken et al. 1986).

7.3.2 Interaction of CD22 with Sialic Acid

It has been demonstrated that CD22 specifically interacts with Neu5Ac α 2–6Gal β 1–4GlcNAc or α 2,6-linked sialic acid (α 2–6 sialic acid) (Powell and Varki 1994). Sialic acids include *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), 3-deoxy-D-glycero-D-galacto-nonulo-pyranosonic acid (KDN), and their alkyl, acyl, and deoxy derivatives. Using CD22-Ig fusion protein (CD22RG) and truncating the side chain of sialic acids by mild periodate oxidation, CD22 was demonstrated to be a sialic acid binding-lectin (Sgroi et al. 1993). Then, it was demonstrated that oligosaccharides that are able to bind CD22 contain α 2–6 sialic acids (Powell et al. 1993). Furthermore, the oligosaccharide binding specificity of CD22 has been demonstrated by the study using CD22RG conjugated column and a number of naturally and enzymatically sialylated oligosaccharides and sialoglycoproteins. CD22 recognizes Neu5Ac α 2–6Gal β 1–4GlcNAc as a minimal structure. The recognition by CD22 is not affected by the presence or absence of α 2–3 sialic acids (Powell and Varki 1994), whereas 9-*O*-acetylation of sialic acids, a natural modification of sialic acid, was demonstrated to prevent CD22 recognition (Sjoberg et al. 1994). Moreover, the subsequent study using equilibrium dialysis and enzyme-linked immunosorbent assay (ELISA) demonstrated that dimeric CD22RG has two sialic acid binding sites and the apparent binding affinity for α 2–6 sialyl-lactose is low (32 μ M) (Powell et al. 1995). Another study using mAbs against CD22 demonstrated that a single region of CD22 mediates adhesion of B cells to T cells, monocytes and erythrocytes, and a treatment with neuraminidase, which catalyzes hydrolysis of *N*-acetylneuraminic acid residues from glycoproteins and oligosaccharides, inhibits these adhesions (Engel et al. 1993). Subsequently, the membrane-distal parts of CD22, the first and/or the second Ig-like domains of CD22, each corresponds to V-set domain and C2-set domain, respectively, were identified as ligand binding domains (Engel et al. 1995a,b; Law et al. 1995), and residues including arginine in V-set domain were demonstrated to be essential for CD22 lectin activity (van der Merwe et al. 1996). The V-set domain, including a key arginine residue, and the C2 set domain are conserved in the siglec family. In contrast to

human CD22, mouse CD22 strongly prefers a Neu5Gc form of the ligand. In human Neu5Gc is absent and only Neu5Ac forms of the ligands are expressed because of a mutation in CMP-sialic acid hydroxylase (Brinkman-Van der Linden et al. 2000).

7.3.3 Sialic Acid-Mediated Glycoprotein Interaction of CD22

Although CD22 specifically interacts with $\alpha 2-6$ sialic acids, it must be noted that this structure of oligosaccharides is common to many glycoprotein. So far, several proteins including CD45, soluble IgM, haptoglobin, Ly-6 have been reported as CD22 ligands (Hanasaki et al. 1995a,b; Pflugh et al. 2002; Stamenkovic et al. 1991). Importantly, CD22 interacts with surface IgM (Leprince et al. 1993; Peaker and Neuberger 1993; Zhang and Varki 2004). In addition, CD22 interacts with oligosaccharides of other CD22 or itself, which was suggested by the experiments that shared $\alpha 2,6$ -sialyltransferase-mediated sialylation of CD22RG abrogates CD22RG-ligand interaction (Braesch-Andersen and Stamenkovic 1994). The recent study also suggests that CD22 interacts with oligosaccharides of neighboring CD22 and forms homomultimeric complexes by photoaffinity crosslinking of oligosaccharide ligands to CD22 (Han et al. 2005). On the ligand binding preference of CD22, it is suggested that interaction between CD22 and CD45 is not dependent on higher affinity but on multiple ligand existence on CD45 because the binding affinity of CD22 to native CD45 and a synthetic $\alpha 2-6$ sialoglycoconjugate is similar (Bakker et al. 2002). On the other hand, it has been demonstrated that the interaction of CD22 with surface IgM and/or CD45 and the internalization of CD22 are independent of sialic acid (Zhang and Varki 2004). It has been suggested that *cis* ligands, ligands on the same cellular surface, regulate the ligand activity of CD22. The *cis* ligands on B cells can occupy the binding site of CD22 and this occupancy results in masking of CD22. Ligand interaction of CD22 is affected by induction of $\alpha 2-6$ sialic acid on B cells and target cells (Hanasaki et al. 1995a,b). *Cis* interaction restricts CD22 lectin activity in human resting B cells; however in vitro activation of these cells induces partial desialylation and unmasking of CD22 (Razi and Varki 1998, 1999). Other works have demonstrated that masked CD22 by *cis* ligands on resting B cells redistributes to the site of contact with other B or T cells and this redistribution is dependent on *trans* ligands and is independent of CD45 (Collins et al. 2004) although CD45 was reported as both a *cis* and *trans* ligand of CD22.

7.3.4 Ligand Binding of CD22 and Its Effects

CD22 interacts with sialic acid containing proteins and this function of CD22 plays an important role in the regulation of B cells. Although early works using anti-CD22 mAbs reported that simultaneous treatment with anti-CD22 mAb and anti-Ig

Ab or anti-CD22 mAb treatment prior to anti-Ig Ab stimulation enhances B-cell proliferation, the elevation in intracellular Ca^{2+} concentration and decreases the threshold for BCR stimulation (Doody et al. 1995; Pezzutto et al. 1987, 1988). Later studies demonstrated the significance of ligand binding activity of CD22 in negative regulation of BCR signaling by the experiments using synthetic sialic acids (Kelm et al. 2002) or cell lines expressing mutant CD22 that was not able to interact with sialic acids (Jin et al. 2002). On the other hand, in vivo study using CD22-deficient mice expressing mutant CD22 demonstrated that the ligand binding ability is not required for negative regulation of BCR signaling, although it should be considered that IgM and CD22 expression is decreased while MHC class II expression on B cells are increased due to ligand binding inability of mutant CD22 (Poe et al. 2004).

Importantly, B-cell activation is depressed when antigen-presenting cells express $\alpha 2-6$ sialic acid abundantly, and this depression is dependent on CD22. This may help the recognition of self and nonself because sialylation is a feature of higher eukaryotes (Lanoue et al. 2002).

7.3.5 CD22-Mediated Signal Transduction

CD22 contains three ITIMs and two immunoreceptor tyrosine-based activation motif (ITAM)-like regions. Through these regions CD22 has been demonstrated to interact with various proteins and regulate the intracellular signaling. Following BCR stimulation, CD22 cytoplasmic tyrosines are rapidly phosphorylated (Schulte et al. 1992) and recruit SHP-1 (Campbell and Klinman 1995; Doody et al. 1995; Lankester et al. 1995), which negatively regulates BCR signaling. In this signaling pathway, BCR-associated tyrosine kinase Lyn is required for the tyrosine phosphorylation and the inhibitory function of CD22 (Cornall et al. 1998; Smith et al. 1998). For the SHP-1 recruitment, carboxyl-terminal two tyrosines in ITIMs of CD22 are required (Otipoby et al. 2001). Furthermore, CD22 is reported to interact with plasma membrane calcium-ATPase (PMCA) and to augment calcium efflux after BCR crosslinking by potentiating PMCA. This interaction requires cytoplasmic tyrosine residues of CD22, and CD22-mediated calcium efflux requires SHP-1 (Chen et al. 2004). In addition to SHP-1, CD22 also interacts with Syk, $\text{PLC}\gamma$, PI3K, Grb2, Shc and SH2-domain-containing inositol 5-phosphatase-1 (SHIP) (Law et al. 1996; Poe et al. 2000; Wienands et al. 1995; Yohannan et al. 1999). Although the overall function of CD22 in BCR signaling is inhibitory as demonstrated by CD22-deficient mice, B cells in CD22-deficient mice also show a shorter life span, reduced proliferation, and enhanced apoptosis following BCR stimulation (Nitschke et al. 1997; O'Keefe et al. 1996; Otipoby et al. 1996; Sato et al. 1996). Thus, it is possible that the interactions of some of these molecules with CD22 may also function in positive regulation of BCR signaling and contribute to promoting cell survival and enhancing proliferation.

7.3.6 Regulation of B Cells by CD22

The characterization of CD22-deficient mice demonstrated that CD22 plays an important role in negative regulation of BCR signaling in mature B cells whereas the development of B cells in CD22-deficient mice is relatively normal. In CD22-deficient mice, the increase in intracellular Ca^{2+} concentration following BCR stimulation and tyrosine phosphorylation is enhanced (Nitschke et al. 1997; O'Keefe et al. 1996; Otipoby et al. 1996; Sato et al. 1996). On the other hand, CD22-deficient mice show reduced response to TI-II Ags and CD22-deficient B cells show the shorter life span, reduced proliferation and enhanced apoptosis following BCR stimulation. CD22-deficient mice also display decreased number of recirculating B cells (Nitschke et al. 1997; Otipoby et al. 1996) and reduction of MZ B cells (Samardzic et al. 2002; Sato et al. 1996). Since MZ B cells perform TI-II responses, it explains the reduced TI-II responses in CD22-deficient mice, and the maintenance of MZ B cells requires CD22 ligand-binding activity (Poe et al. 2004). Furthermore, hyper-responsiveness of B cells in CD22-deficient mice is correlated with the development of serum IgG against double-stranded DNA (O'Keefe et al. 1999). Importantly, negative regulation of BCR signaling by CD22 is dependent on the BCR isotype. Although CD22 negatively regulates signaling through IgM or IgD, CD22 is not phosphorylated on ligation of membrane bound IgG by Ags and does not inhibit signaling through IgG because of an IgG cytoplasmic tail. In contrast to IgM and IgD, which contain only three amino acids in their cytoplasmic domains, IgG contains 28 amino acid residues in its cytoplasmic tail. This BCR-isotype specific regulation is not the case of inhibitory coreceptor CD72. CD72 negatively regulates signaling through IgG as well as IgM and IgD (Wakabayashi et al. 2002). In fact, IgG-positive B cells show enhanced clonal expansion compared to IgM-positive B cells (Martin and Goodnow 2002). Thus, in addition to other factors, BCR isotype-specific regulation of CD22 may be involved in efficient switching from IgM to IgG production at the cellular level and the efficient response of IgG-positive memory B cells.

In mice, the deficiency of ST6Gal sialyltransferase, which appears to be solely responsible for producing the $\alpha 2-6$ sialic acids terminus on various *N*-glycans, causes a severe and widespread immunodeficiency, including reduced serum IgM levels and attenuated Ab production to TI and TD Ags, unlike CD22-deficient mice. These results indicate that ST6Gal sialyltransferase have additional function other than the CD22 ligand production and regulation of CD22 (Hennet et al. 1998).

7.4 Regulation of B Cells by CD72

7.4.1 Ligand Binding of CD72

Inhibitory coreceptor CD72 (Lyb-2) is an approximately 45 kDa type II membrane protein and is a member of C-type lectin superfamily (Fig. 2). CD72 contains

C-type lectin like domain in the extracellular region and an ITIM and an ITIM-like sequence in the cytoplasmic region. However, lectin activity of CD72 is not yet clear. CD72 forms a homodimer by disulfide linking. CD72 is expressed on all B cells but is downregulated on terminally differentiated plasma cells (Nakayama et al. 1989) and is also expressed on dendritic cells. Although CD72 was suggested to be a ligand of CD5 (Van de Velde et al. 1991), other works have demonstrated that CD5-Ig fusion protein containing an extracellular domain of CD5 does not bind CD72 (Biancone et al. 1996; Bikah et al. 1998). As a CD72 ligand, CD100/Sema4D, a member of the semaphorin family, was identified (Kumanogoh et al. 2000). In addition to nervous systems, CD100 is also abundantly expressed on T cells (Furuyama et al. 1996; Hall et al. 1996). CD100 can be released from the surface of T cells by proteolysis (Elhabazi et al. 2001). Interaction of CD72 with membrane anchored CD100 or soluble CD100 appears to inhibit negative signaling by CD72 because this interaction causes the reduction of both CD72 phosphorylation and recruitment of SHP-1 to CD72 (Kumanogoh et al. 2000; Shi et al. 2000). This resembles the treatment with anti-CD72 Ab, which promotes B-cell activation and survival (Nomura et al. 1996; Subbarao and Mosier 1984) and inhibits both CD72 phosphorylation and recruitment of SH2-domain-containing protein tyrosine phosphatase-1 (SHP-1) following BCR stimulation (Hokazono et al. 2003; Nitschke and Tsubata 2004)

7.4.2 Signal Transduction by CD72

Early works using anti-CD72 mAb have suggested the positive role of CD72 in B-cell activation. However, the cytoplasmic region of CD72 contains an ITIM and an ITIM-like sequence. In fact, BCR stimulation enhances both the tyrosine phosphorylation of CD72 and recruitment of tyrosine phosphatase SHP-1 to tyrosine phosphorylated ITIM in CD72 (Adachi et al. 1998; Wu et al. 1998). CD72 inhibits extracellular signal-related kinase (ERK) activation and the increase in intracellular Ca^{2+} concentration depending on its ITIM following BCR stimulation (Adachi et al. 2000) probably by dephosphorylating $\text{Ig}\alpha/\beta$ and its down stream signaling molecules Syk and SLP-65 (Adachi et al. 2001). In consistent with above results, B cells from CD72-deficient mice are hyper-reactive to various stimuli and show enhanced Ca^{2+} mobilization following BCR stimulation (Pan et al. 1999). CD72 also interacts with Grb2 through its ITIM-like sequence and this region does not interact with SHP-1, although the functional significance of Grb2 binding is not yet clear (Wu et al. 1998). In vitro, Tyrosine kinase Lyn phosphorylates CD72 (Adachi et al. 1998). The phenotype of CD72-deficient mice is relatively similar to that of CD22-deficient mice and Lyn deficient mice (Chan et al. 1998; Hibbs et al. 1995; Nishizumi et al. 1995). In case of CD22, CD22 can interact with IgM by recognizing sialic acids that exist on IgM. This interaction of CD22 with BCR seems to be important because CD22 is phosphorylated by BCR-associated kinase Lyn and this phosphorylation enables the recruitment of tyrosine phosphatase

SHP-1 which negatively regulates BCR signaling upon BCR stimulation. However, as for CD72, it is not yet known how CD72 associates with BCR although the signal transduction mechanism for inhibitory function of CD72 is similar to that of CD22.

7.4.3 Regulation of B Cells by CD72

The characterization of CD72-deficient mice demonstrated that the development of B cells in CD72-deficient mice is largely normal and CD72 negatively regulates BCR signaling (Pan et al. 1999). In the bone marrow of CD72-deficient mice, the number of mature recirculating IgM⁺IgD⁺ B cells is reduced, whereas the number of IgM⁺IgD⁻ pre-B cells increased. This may reflect the role of CD72 in the transition from pre-B to immature B cells and from immature to mature B cells. In the periphery of CD72-deficient mice, there is a decrease in mature and long-lived follicular B cells and an increase in B-1 cells (Pan et al. 1999; Parnes and Pan 2000).

7.5 Self and Nonself Recognition by Coreceptors on B cells

Coreceptors on the surface of B cells modulate BCR signaling positively or negatively, and control the outcome of an immune response by setting thresholds for B-cell activation. Importantly, this modulation by coreceptors is regulated by ligand bindings to coreceptors and ligands for coreceptors include *cis* ligands on B cells and *trans* ligands on targets. For example, CD19/CD21 complex interacts with both IgM and C3 fragments and positively regulates BCR signaling in the presence of C3 fragments conjugated Ags. This function facilitates B-cell activation to nonself-Ags. CD22 interacts with both IgM and other proteins by sialic acids recognition and negatively regulates BCR signaling. CD22 has been demonstrated to negatively regulate BCR signaling when Ag-presenting cells express sialic acids abundantly. This CD22 function may contribute inhibition of B-cell activation by self-Ags. CD72 also regulates BCR signaling negatively. CD72 interacts with CD100 and this interaction appears to prevent the negative function of CD72. These *cis*- and *trans*-ligand-mediated regulation of coreceptors have an advantage in selective regulation of immune response of B cells because of the function of recognizing extracellular environment, which enables the recognition of self- and nonself-Ag, in some cases, by cooperating with other mechanisms. However, details of the mechanisms of ligand bindings to coreceptors are not yet clear and many things on the mechanism of ligand bindings, especially on inhibitory coreceptors, remain to be elucidated.

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Co-Receptors in the Positive and Negative Regulation of T-Cell Immunity

Helga Schneider and Christopher E. Rudd

8.1 Introduction

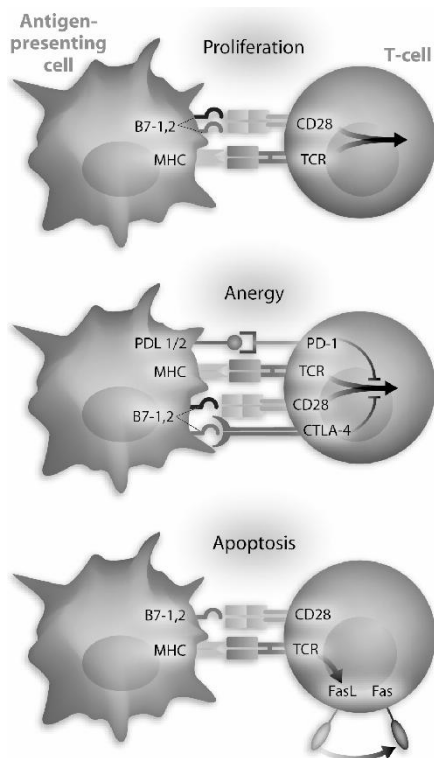
Engagement of the antigen-receptor complex (TcR ζ /CD3) on T cells is insufficient for the development of an optimal response against foreign antigen. Instead, T cells require at least two signals for optimal T-cell expansion (Bretscher 1999). Peptides presented to antigen-specific T cells in the context of major histocompatibility complex (MHC) molecules deliver signal 1, whereas a co-stimulatory signal through a distinct T-cell surface molecule triggers signal 2. Signaling through signal 1, in the absence of co-stimulation, leads to aborted activation and often a state of anergy or clonal unresponsiveness (Schwartz 1990). T-cell activation can occur in the absence of signal 2 under conditions where the TcR signal is very strong (i.e., high avidity peptide). With the advancement of the field, it is now apparent that there exists an array of different co-receptors on T cells (Fig. 1). These include CD28, inducible T-cell co-stimulator (ICOS) and cytotoxic T-cell antigen (CTLA-4), programmed death-1 (PD-1), B and T lymphocyte attenuator (BTLA) and T-cell immunoglobulin and mucin-domain-containing molecule- 3 (Tim-3). As will be outlined in this review, each of these receptors provides unique and overlapping signals that modulate different aspects of T-cell immunity.

CD28 is one of the best-characterized co-receptors on T cells (June et al. 1994). It comprises a single immunoglobulin domain, and is expressed on resting and activated T cells. During antigen-presentation, the TcR ζ /CD3 complexes bind to peptides as presented by MHC antigens. The CD4 and CD8 receptors also engage MHC antigens leading to the activation of the associated src kinase p56lck and the initiation of the tyrosine phosphorylation activation cascade (Rudd 1990). At the same time, CD28 binds to ligands B7-1 (CD80) and B7-2 (CD86) on antigen-

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Fig. 1. Co-receptors determine multiple aspects of T-cell function. *Upper panel*, co-ligation of CD28 with TcR/CD3 lowers the threshold of TcR signaling needed to induce T-cell activation. This leads to increased cytokine production and proliferation. *Middle panel*, ligation of the TcR in the absence of CD28 can lead to an induction of anergy or non-responsiveness. By contrast, expression of CTLA-4 is needed for the induction of anergy, while programmed death-1 (*PD-1*) ligation by programmed death-ligand 1/ligand 2 (*PD-L1/PD-L2*) can inhibit T-cell proliferation and reduces cytokine production. This can lead to peripheral tolerance. *Lower panel*, TcR stimulation without engagement of CD28 leads to apoptosis via the Fas/FasL pathway. (See Color Plates)



presenting cells (APCs). B7-2 is constitutively expressed at low levels and rapidly up-regulated, whereas B7-1 is inducibly expressed later than B7-2. The ligation of the TcR ζ /CD3 complex and CD28 provides the optimal signals for interleukin-2 (IL-2) production and proliferation (Fig. 1, upper panel). By contrast, engagement of TcR ζ /CD3 in the absence of CD28 can cause non-responsiveness or anergy (Fig. 1, middle panel). The related family members ICOS and CTLA-4 are expressed only on activated cells (Fig. 2). In this way, they are thought to play specialized roles in the function of activated and effector T cells. While CTLA-4 also binds to CD80 and CD86, ICOS lacks the so-called MYPPPY sequence needed for CD80/86 binding, and instead binds to ICOSL (B7h GL50, B7RP-1, LICOS) (Ling et al. 2000; Swallow et al. 1999; Wang et al. 2000; Yoshinaga et al. 2000).

While CD28 provides signals from the general activation of T cells, ICOS is preferentially expressed on T-helper 2 (Th2) cells and provides positive signals for various aspects of Th2 cell function (Coyle et al. 2000; Dong et al. 2001; McAdam et al. 2000, 2001; Tafuri et al. 2001; Tesciuba et al. 2001). Within the T-cell compartment exist T helper 1 (Th1) and Th2 subsets that express different cytokines and regulate distinct functions. Th1 cells produce interferon (IFN)- γ , interleukin (IL)-2 and tumor necrosis factor (TNF)- α , and are involved in cell-mediated immu-

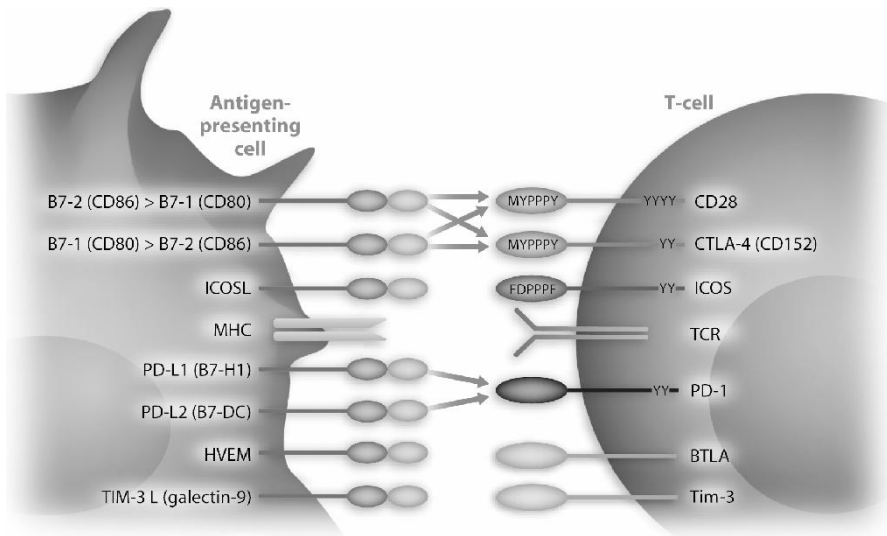


Fig. 2. Receptor–ligand pairs during the conjugate formation between T cells and antigen-presenting cells. T cells express an array of co-receptors that include CD28, inducible T-cell co-stimulator (*ICOS*), and cytotoxic T-cell antigen (*CTLA-4*) as well as co-receptors programmed death-1 (*PD-1*), B- and T-lymphocyte attenuator (*BTLA*), and T-cell immunoglobulin and mucin-domain-containing molecule-3 (*Tim-3*). CD28 and CTLA-4 bind to ligands B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells. Both possess MYPPPY sequence needed for B7-1/B7-2 binding. By contrast, ICOS has a FDPPPF motif and binds to ICOSL but not to B7-1 and B7-2. PD-1 is a receptor for both PD-L1 and PD-L2. The herpes virus entry mediator (*HVEM*), a member of the tumor-necrosis factor receptors (TNFR) superfamily, is the unique ligand for BTLA. Tim 3 binds galectin-9, a member of the galectin family that is expressed on lymphocytes and other cell types

nity and delayed-type hypersensitivity reactions. Autoimmune and chronic inflammatory diseases such as multiple sclerosis, type II diabetes and rheumatoid arthritis have been described as Th1-dominant diseases. By contrast, Th2 cells are characterized by the production of interleukins IL-4, IL-5, IL-10, and IL-13, and regulate humoral responses. Th2 responses dominate in defense against parasitic infections, such as helminth infections, affect the activation of mast cells and eosinophils, and are associated with atopy and allergy. ICOS expression and regulation of Th2 cells implicates this co-receptor in the regulation of these functions (Coyle et al. 2000).

On the other hand, positive co-signals are counterbalanced by signals that dampen the immune responses. These are provided by CTLA-4, PD-1 and the recently described Ig superfamily members BTLA and Tim-3 (Fig. 2). Surface CTLA-4 can only be detected in activated T cells, and CTLA-4-deficient mice show a profound post-thymic autoimmune phenotype with death by 3 weeks due to massive tissue infiltration and organ destruction (Tivol et al. 1995; Waterhouse et al. 1995). Recently, it has been suggested that CTLA-4 is constitutively expressed on CD4+CD25+ regulatory T cells (Tregs) where it plays a role in regulating their

function (Read et al. 2000; Takahashi et al. 2000). However, the relationship is not obligatory since CTLA-4-deficient mice possess Tregs with normal function (Takahashi et al. 2000).

PD-1 can be induced not only on T cells, but also on B cells and myeloid cells, suggesting that it has broader roles on immune regulation (Agata et al. 1996; Nishimura and Honjo 2001). The ligands PD-L1 and PDL-2 are inductively expressed on the lymphoid system and constitutively on parenchymal cells (Freeman et al. 2000; Latchman et al. 2001). The inhibitory co-signal may suppress inflammatory responses and autoimmunity in various organs on which PD-1 ligands are expressed (Greenwald et al. 2005). BTLA exerts inhibitory effects on B and T lymphocytes. BTLA-deficient mice show increased humoral responses to T-cell dependent antigens, and increased susceptibility to peptide antigen-induced experimental autoimmune encephalomyelitis (EAE). The herpesvirus entry mediator (HVEM), a member of the TNFR superfamily, is the unique ligand for BTLA (Sedy et al. 2005).

The Ig superfamily member Tim-3 has recently been described as a novel trans-membrane protein that is preferentially expressed on differentiated Th1 cells and plays an important role in suppressing Th1 effector activation (Meyers et al. 2005). Galectin-9, a member of the galectin family which is expressed on lymphocytes and other cell types, has been identified as a ligand for Tim-3 (Zhu et al. 2005). Blockade of the Tim-3 pathway by treatment with anti-Tim-3 antibody or Tim-3Ig fusion proteins led to increased Th1 cell proliferation and cytokine responses, in addition to loss of tolerance (Sabatos et al. 2003).

Overall, co-receptors not only provide critical positive second signals that promote and sustain T-cell responses, but they can also down-regulate T-cell responses. These negative signals function to limit and terminate T-cell responses, and they appear to be especially important for regulating T-cell tolerance and autoimmunity. Any subtle imbalance can be exaggerated and lead to an inappropriate immune response, which may cause serious autoimmune diseases. The balance of stimulatory and inhibitory signals is crucial to maximize protective immune responses while maintaining immunological tolerance and preventing autoimmunity.

8.2 Positive Co-stimulatory Molecules

8.2.1 CD28

8.2.1.1 Expression and Function

Human CD28 is expressed constitutively on most CD4 positive T cells and approximately 50% of CD8 positive T cells. Murine CD28 is expressed on all T cells, and CD28 is not down-regulated with age or chronic disease (Riley and June 2005). The importance of CD28 has been underscored by the phenotype of the CD28-

deficient mouse. T cells from these mice are impaired in their ability to proliferate in vitro in response to anti-TcR/CD3 stimulation, allo-stimulation and stimulation with specific antigen. In vivo, CD28-deficient mice exhibit impaired T helper B-cell response and fail to form germinal centers after immunization with protein antigens (Ferguson et al. 1996). These cells have a reduced capacity to produce IL-2 and to proliferate on subsequent stimulations. However, longer-term engagement of the TcR ζ /CD3 complex with repeated antigen stimulation (i.e., high virulence infection, repeated injection of peptide) can bypass the requirement for CD28 (Kündig et al. 1996).

CD28 can regulate an array of events including cytokine production, proliferation, anergy, apoptosis, glucose metabolism, and Th1 vs. Th2 cell differentiation (Acuto and Michel 2003; Rudd and Schneider 2003). Enhanced cytokine production occurs via the potentiation of TcR signaling, the enhancement of NFAT activity as well as the stabilization of messenger RNA (Acuto et al. 2003; Riley and June 2005). The ability to augment glucose metabolism is related to the up-regulation of Glut 4 receptors on the surface of T cells (Frauwirth et al. 2002). Some reports indicate that CD28 ligation might skew the differentiation of T cells into IL-4-producing Th2 cells, although the exact molecular mechanism by which this occurs is not known (Schulze-Koops et al. 1998).

8.2.1.2 Signaling Pathways

CD28, ICOS, and CTLA-4 possess small cytoplasmic domains of 41, 35, and 36 residues, respectively (Fig. 3). Common to each is the presence of a YxxM consensus motif: a YNM motif for CD28, a YMF motif for ICOS and a YVK motif for CTLA-4. Phosphorylation of the tyrosine creates conditions for the binding of one of two SH2 domains of the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) (August and Dupont 1994; Coyle et al. 2000; Pages et al. 1994; Prasad et al. 1994; Truitt et al. 1994; Schneider et al. 1995b). Specificity is determined by residues adjacent to the tyrosine, specifically a methionine (M) in the plus 3 position. CD28 also carries an asparagine (N) in the plus 2 position (i.e., pYxNM) that is not found in ICOS or CTLA-4. This is a signature residue for SH2 domain binding of the adaptor Grb-2 (Kim et al. 1998; Okkenhaug and Rottapel 1998; Schneider et al. 1995a). Grb-2 is an adaptor protein that is comprised of one SH2 domain flanked by two SH3 domains that bind to the exchange factor Son of Sevenless (SOS), an activator of the GTPase p21ras. CD28 and CTLA-4 also carry proline residues that are not found in the cytoplasmic domain of ICOS. Classic RKxxPxxP and PxxPxR motifs serve as ligands for SH3 domains (Pawson et al. 2001). CD28 has two non-canonical PxxP motifs. The PRRP motif at residues 196 to 199 may bind to the tyrosine kinase, interleukin-2 inducible T-cell kinase (ITK) (Marengere et al. 1997), while a second C-terminal PYAP motif (residues 208 to 211) binds to the SH3 domain of p56lck (Holdorf et al. 1999). The Grb-2 SH3 domain can bind constitutively at low levels to the same motif, whereas the phospho-YNM motif facilitates tandem SH2-SH3 domain binding (Kim et al. 1998;



Fig. 3. Structure of the cytoplasmic domains of human CD28, ICOS, and CTLA-4. The cytoplasmic domains of CD28, ICOS, and CTLA-4 have a common YxxM motif that binds to the SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase (PI3K). At the same time, each co-receptor has unique binding residues. CD28 has an asparagine that is needed for Grb-2 SH2 domain binding. Proline sites allow for the further binding of the SH3 domains of Grb-2 and the protein tyrosine kinase p56lck. CTLA-4 has a unique YVKM motif that binds in its non-phosphorylated form to the clathrin adapters AP-1 and AP-2. ICOS possesses a YxxM motif, but is characterized by the absence of additional proline residues

Okkenhaug and Rottapel 1998). The presence of a shared YxxM motif for PI3K binding amongst the CD28 family of co-receptors suggests they share an overlapping function, while differences in other residues point to different functions.

In this regard, PI3K binding to CD28 has been implicated in IL-2 production and rescuing from cell death or apoptosis. Mutation of the Y (i.e., loss of Grb-2 and PI3K binding) and M residues (i.e., loss of PI3K binding alone) interferes with the induction of IL-2 in T-cell hybridomas (Cai et al. 1995; Cefai et al. 1996; Kim et al. 1998; Pages et al. 1994). Further, in vivo reconstitution of Y-FMNM mutants in CD28^{-/-} T cells showed a partial reduction of IL-2 production and graft-versus-host responses (Burr et al. 2001; Harada et al. 2001). The key connection appears to involve PI3K and its activation of the PDK-1/PKB/GSK-3 pathway. Serine/threonine kinases PDK1 and PKB (AKT) carry a pleckstrin homology (PH) domain which binds to phosphatidylinositol-triphosphate (PtdInsP₃) at the inner phase of the plasma membrane leading to the activation of these kinases. PDK1 phosphorylates various proteins such as PKC and S6 kinase, whereas the targets of PKB include pro-apoptotic molecules, transcription factors and cell-cycle regulators (Fig. 4). Glycogen synthase kinase-3 (GSK-3), a kinase that is active in resting T cells is inactivated by phosphorylation on serine 9 and 21 by PKB. Inactivation of GSK-3 can lead to prolonged residency of NFAT in the nucleus leading to enhanced IL-2 transcription. However, this pathway has not been studied in detail in the context of CD28 and T cells.

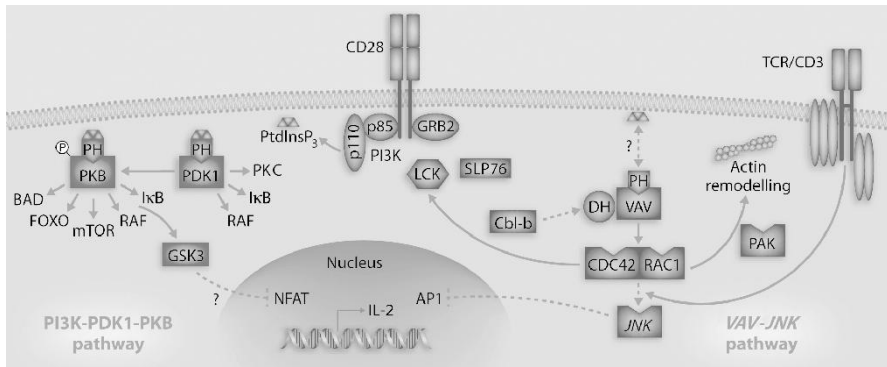


Fig. 4. CD28 pathways: PI3K–PDK1–PKB and CD28–VAV-1. Binding of PI3K to CD28 generates phosphatidylinositol phosphates that serve as binding sites for PH domains in an array of proteins. This localizes proteins such as inducible T-cell kinase (ITK) to the plasma membrane for interaction with other proteins. One key CD28 signaling pathway is mediated by binding to PI3K. In this scenario, PI3K activation is needed for PDK1 activation leading to the phosphorylation and activation of PKB (AKT). These kinases in turn can phosphorylate multiple downstream targets that are involved in protein translation and cellular metabolism. PKB can also inactivate GSK3 by phosphorylation that can prolong the nuclear residency of NFAT needed for IL-2 production. Another pathway involves the GTP–GDP exchange factor VAV-1 that connects CD28 with the activation of Rac and Cdc42 and the subsequent activation of MEK kinase 1 (MEKK1) and JNK. Activation of Rac, Cdc42, and PAK can also induce cytoskeleton remodeling

CD28 has long been known to rescue cells from TcR-driven antigen-induced cell death (AICD) (Burr et al. 2001; Kirchhoff et al. 2000; Okkenhaug et al. 2001). Apoptosis can be induced by two pathways, via mitochondria associated proteins (i.e., the Bcl family), and death receptors i.e., Fas (CD95)/Fas ligand (FasL (CD95L)) (Fig 1, lower panel). In the context of Fas/FasL, CD28 operates via at least three mechanisms: by decreasing FasL expression, increasing expression of FLICE-inhibitory protein c-FLIP short (Kirchhoff et al. 2000) and by interfering with the formation of the death-inducing signaling complex (DISC) (Jones et al. 2002). T cells expressing active PKB were found to have reduced caspase-8, BID and caspase 3 activation due to the impaired recruitment of caspase 3 to DISC (Jones et al. 2002).

In another pathway, CD28 also plays a role in up-regulating the pro-survival factors Bcl-2 and Bcl-XL. CD28-deficient mice reconstituted with a disrupted YNMN motif for PI3K binding were unable to up-regulate Bcl-XL (Okkenhaug et al. 2001). This is most probably due to the inability to recruit and activate PI3K and subsequently activate PKB. Transgenic expression of an activated form of PKB leads to constitutively elevated Bcl-XL expression (Jones et al. 2002). Lastly, the CD28-PI3K-AKT pathway plays an important role in the up-regulation of Glut4 receptor (increase in glucose transport) and glycolysis (Frauwirth et al. 2002).

8.2.1.3 CD28 and Disease States

CD28 modulation of T-cell function is reflected in its importance in various disease states. In EAE, a model for the human disease multiple sclerosis (MS), blockade of CD28/B7 interactions at the onset of the disease decreased the severity of EAE. Blockade of the CD28/B7 pathway during the acute or remission phases of EAE prevented further relapse (Miller et al. 1995; Perrin et al. 1999). By contrast, in the non-obese diabetic (NOD) mouse model (Lenschow et al. 1995), B7-1 and B7-2 seem to have different regulatory functions on the course of disease. Treatment of the NOD mouse with anti-B7-1 worsened the spontaneous diabetes, whereas anti-B7-2 had protective effects (Lenschow et al. 1995). Collagen-induced arthritis, experimental myasthenia gravis and autoimmune uveitis can be inhibited with anti-B7-2 (Salomon and Bluestone 2001).

8.2.2 Inducible T-Cell Costimulator (ICOS)

ICOS is expressed on CD4 and CD8 positive T cells following activation and remains present on effector and memory T cells (Coyle et al. 2000; Yoshinaga et al. 2000). This can be enhanced by CD28 co-ligation (Beier et al. 2000; Coyle et al. 2000; McAdam et al. 2000). Both Th1 and Th2 cells express ICOS during T-cell differentiation, though ICOS levels persists at higher levels on Th2 cells than on Th1 cells (Coyle et al. 2000; McAdam et al. 2000). ICOS is also expressed on activated NK cells, and it promotes NK cell function. Signaling via ICOS provides critical T helper function to B cells. Studies using pathway antagonists, transgenic mice, and knockout mice have revealed the important role of ICOS in B-cell differentiation, immunoglobulin class switching, germinal center formation, and memory B-cell development (Chapoval et al. 2001; Coyle et al. 2000; Hutloff et al. 1999).

Although ICOS and CD28 share an ability to induce IFN- γ , IL-4, and IL-10 production, they differ with respect to the regulation of IL-2 production. Little if any IL-2 is induced with ICOS (Arimura et al. 2002; Gonzalo et al. 2001a,b). In this context, ICOS possesses a YMFM motif that binds PI3K, but interestingly, lacks an intervening asparagine (N) that allows for Grb-2 binding (Coyle et al. 2000; Schneider et al. 1995a). The presence of this residue seems to be important since Jurkat T cells transfected with a mutant form of ICOS containing an asparagine in lieu of the phenylalanine was able to bind Grb-2 and concurrently activated the IL-2 promoter (Harada et al. 2003).

Overall, less is known about ICOS signaling than CD28 signaling. The regulation of Th2 cell function by ICOS has raised the question whether the co-receptor has a special connection with a unique Th2 signaling pathway, or whether it simply adds to the signals generated by CD28. In the latter scenario, the co-receptor would adjust the threshold of signaling by engaging ICOS ligand (B7h, GL50, B7RP-1, LICOS) expressed on APCs. This would produce D-3 lipids and as such should act

to recruit PH domains to the membrane (Coyle et al. 2000). On the other hand, it lacks additional proline residues that might provide additional signals. This suggests that ICOS may be more limited in its generation of signals.

8.2.2.1 ICOS and Disease States

Given the involvement of ICOS in B-cell differentiation, immunoglobulin class switching, germinal center formation, and memory B-cell development (Chapoval et al. 2001; Coyle et al. 2000; Hutloff et al. 1999), it is not surprising that it is associated with various disease states. Mice deficient in ICOS show a major reduction in IgE and Th2 cytokine production, as well as in the development of airway hyper-reactivity, which indicates a key role for ICOS in the development of allergic respiratory inflammatory responses (Gonzalo et al. 2001a,b; Umetsu et al. 2002). EAE is a Th1 disease mediated by myelin-specific CD4 positive T cells. ICOS blockade during induction of EAE exacerbated the disease and enhanced IFN- γ production (Rottman et al. 2001).

8.3 Negative Co-stimulatory Molecules

8.3.1 *CTLA-4 (CD152)*

8.3.1.1 Expression and Function

CTLA-4 is the most extensively characterized inhibitory receptor. Like CD28, it binds to B7-1 (CD80) and B7-2 (CD86) although with significantly higher affinity. Unlike CD28, CTLA-4 binds as a dimer creating a lattice as seen in its crystal structure (Ikemizu et al. 2000; Stamper et al. 2001). Resting T cells express little or no surface CTLA-4, but surface levels are increased upon activation, due to both redistribution of an intracellular pool and increased synthesis. CTLA-4 has potent negative effects on a number of parameters of T-cell function (Bluestone 1995; June et al. 1994). The strong inhibitory role for CTLA-4 is underscored by the phenotype of CTLA-4-deficient mice. They develop a massive lymphoproliferative disorder characterized by polyclonal T-cell proliferation and early lethality (Tivol et al. 1995; Waterhouse et al. 1995). In this way, the co-receptor is thought to modulate the threshold of signals needed for T-cell cytokine production and proliferation (Chambers et al. 1997; Thompson and Allison 1997). With this, CTLA-4 can inhibit T-cell activation by reducing IL-2 production and IL-2 receptor expression, and by arresting T cells at the G1 phase. In addition, CTLA-4 engagement of CD80/86 on dendritic cells can induce the release of indoleamine 2,3-dioxygenase (IDO) (Boasso et al. 2004; Fallarino et al. 2003), which has regulatory effects on T cells that result from tryptophan depletion. However, the degree to which this pathway plays a significant role in CTLA-4 function is uncertain since IDO^{-/-} mice

fail to develop auto immunify (Mellor et al. 2003). CD4+CD25+ regulatory T cells can modulate disease in the CTLA-4^{-/-} mouse (Eggena et al. 2004; Fehervari and Sakaguchi 2004; Lohr et al. 2003; Read et al. 2000).

8.3.1.2 Signaling Pathways

Reported mechanisms for CTLA-4 inhibition of T-cell responses include ectodomain competition for CD28 binding to CD80/86 (Masteller et al. 2000), disruption of CD28 localization at the immunological synapse (Pentcheva-Hoang et al. 2004), modulation of TcR signaling by phosphatases SH2-domain containing protein tyrosine phosphatase (SHP-2), and the serine-threonine phosphatase PP2A (Chuang et al. 1999, 2000; Cilio et al. 1998; Lee et al. 1998; Marengere et al. 1996) and interference with the expression or composition of lipid rafts on the surface of T cells (Chikuma et al. 2003; Darlington et al. 2002; Martin et al. 2001; Rudd et al. 2002). High avidity CTLA-4 binding to CD80/86 would almost certainly compete for CD28 binding in the limited interface between T-cell and APC. In support of this, expression of tailless CTLA-4 in CTLA-4^{-/-} mice can inhibit lymphocytic infiltration of organs (Masteller et al. 2000). However, the tailless mutant did not prevent lymphoproliferative disease indicating that intracellular signaling also plays a role in altering the threshold of activation. Further, CTLA-4 can inhibit T-cell responses in the absence of CD28 where competition for ligand did not occur (Fallarino et al. 1998; Lin et al. 1998). CTLA-4 function is not entirely cell autonomous as demonstrated in CTLA-4-deficient mice where reconstitution with a combination of deficient and normal T cells failed to develop a lymphoproliferative disease (Bachmann et al. 1999).

A clue to the importance of the cytoplasmic YVKM motif is evident from the swapping of the CD28 SDYMMN motif for the GVVVKM motif in CTLA-4, which can convert the co-receptor into a positive co-stimulatory receptor (Yin et al. 2003). Members of the Tec family or p59fyn can phosphorylate the motif (Miyatake et al. 1998; Schneider et al. 1998). CTLA-4 in turn has been reported to bind two phosphatases, SHP-2 and PP2A (Chuang et al. 2000; Lee et al. 1998; Marengere et al. 1996). SHP-2 binding depends on the YVKM motif (Lee et al. 1998; Marengere et al. 1996), although the interaction is indirect (Schneider and Rudd 2000). SHP-2 has generally been associated with positive signaling (Gadina et al. 1998; Hadari et al. 1998), and in certain systems, the mutation of the tyrosine in the YVKM motif has little effect on function (Baroja et al. 2000; Cinek et al. 2000; Nakaseko et al. 1999). Inhibition has also been observed without detectable SHP-2 binding. Conversely, CTLA-4 has been reported not to inhibit the function of naive CD8+ T cells, even with associated SHP-2 (Perez et al. 1997).

The PP2A binding site requires the YVKM motif (Chuang et al. 2000), and the membrane proximal lysine (K) residues (Baroja et al. 2000). PP2A has a well-established negative effect on differentiation and cell growth (Janssens and Goris 2001). PP2A also targets PKB (AKT), a key component in CD28 co-stimulation (Millward et al. 1999). This fits with the observed inhibition of CD28-PI3K/PDK1/

AKT up-regulation of glucose metabolism by CTLA-4 (Frauwirth et al. 2002). In one model, co-ligation of TcR/CTLA-4 leads to phosphorylation of PP2A that causes its dissociation from CTLA-4 and T-cell inactivation (Chuang et al. 2000; Teft et al. 2005). CD28 can also bind the phosphatase, but with different properties (Chuang et al. 2000). PP2A bound to CD28 in resting cells is enzymatically active, whereas dissociation of PP2A occurs when CD28 is tyrosine phosphorylated (Chuang et al. 2000; Teft et al. 2005). The phosphatase model involving SHP-2 or PP2A would be analogous to killer inhibitory receptors (KIRs) where the associated phosphatase dephosphorylates components needed for signaling. Supporting this, anti-CTLA-4 co-ligation can reduce linker of activation of T cells (LAT) phosphorylation (Lee et al. 1998), and various TcR signaling proteins are hyperphosphorylated in CTLA-4-deficient mice (Marengere et al. 1996; Lee et al. 1998).

An understanding of CTLA-4 signaling must also take into account its binding to PI3K (Schneider et al. 1995a,b). This implies that CTLA-4 may generate positive as well as negative signals. In agreement, CD4⁺ T cells deficient in CD80 and CD86 showed an increase in JNK activity (Schneider et al. 2002). At the same time, ERK activity was inhibited indicating that CTLA-4 can differentially regulate members of the MAPK family. The relationship of this finding to CTLA-4 function remains to be established. In this manner, the inhibition of ERK alone could account for negative signaling and CTLA-4 involvement in anergy (Perez et al. 1997). On the other hand, CD4⁺ T cells from unprimed mice include regulatory suppressor T cells (Tregs). Future studies will be needed to determine whether Tregs differ from other T-cell subsets in their response to CTLA-4. Regardless, the activation of JNK could not account for CTLA-4 induction of TGF- β production (Schneider et al. 2002). Further downstream, CTLA-4 has effects on the regulation of cell cycle, opposing the positive effects of CD28 and inhibiting transcription factors nuclear factor kappa-B (NF- κ B), NFAT, and AP-1 (Fraser et al. 1999; Olsson et al. 1999).

Major emphasis has recently been placed on the evaluation of dynamic changes in CTLA-4 expression, trafficking and degradation during T-cell activation. One of the unusual characteristics of CTLA-4 is the fact that it is primarily located in intracellular compartments (Alegre et al. 1996; Leung et al. 1995). Little of the co-receptor can be detected on the surface of cells, even during its optimal expression following T-cell activation. This tight regulation is presumably due to the need to tightly regulate the function of CTLA-4. Two pools of intracellular CTLA-4 have been described, one in the trans Golgi apparatus, and another in the endosomal and lysosomal compartments (Alegre et al. 1996; Iida et al. 2000; Leung et al. 1995; Linsley et al. 1996). TcR ligation and calcium ionophores can induce CTLA-4 release to the cell surface by an unknown mechanism (Linsley et al. 1996). Part of this regulation involves the YVKM motif since its disruption can increase the surface expression of CTLA-4 (Leung et al. 1995; Linsley et al. 1996). Recently, it was found that the adapter protein TRIM (T-cell receptor-interacting molecule) binds to CTLA-4 in the trans Golgi apparatus and promotes transport of CTLA-4 to the surface of T cells (Valk et al. 2006).

The internalization of CTLA-4 is mediated by a combination of tyrosine phosphorylation and the binding of the clathrin adapter complex AP-2 (Yi et al. 2004). Non-phosphorylated CTLA-4 can bind to AP-2 implicated in trafficking and endocytosis of CTLA-4, respectively (Bradshaw et al. 1997; Chuang et al. 1997; Schneider et al. 1999; Shiratori et al. 1997; Zhang and Allison 1997). Phosphorylation of CTLA-4 by the kinase p56lck, p59fyn, and Rlk inhibits these events (Bradshaw et al. 1997; Chuang et al. 1997; Miyatake et al. 1998; Schneider et al. 1998). Once dephosphorylated, the binding to AP-2 regulates its endocytosis (Shiratori et al. 1997; Zhang and Allison 1997). Similar processes occur to intracellular forms of CTLA-4 where AP-1 binding to CTLA-4 mediates its transport from the Golgi apparatus to the lysosomal compartment for degradation (Schneider et al. 1999).

Engagement of the TcR with antigen involves entry and aggregation of signaling proteins (i.e., LAT, PKC θ) into membrane compartments known as lipid rafts (Janes et al. 2000; Pearse and Robinson 1990; Samelson 2002; Xavier et al. 1998) and the formation of an immunological synapse (IS) between the T-cell and an APC. The IS is a characteristic structure in which an external leukocyte function-associated antigen-1 (LFA-1) ring (peripheral SMAC, pSMAC) surrounds central TcR clusters (central SMAC, cSMAC) (Dustin et al. 2001; Kupfer and Kupfer 2003). Viola and colleagues first demonstrated that CD28 coligation promotes the surface expression of lipid rafts (Viola et al. 1999), whereas Martin et al. showed that CTLA-4 can inhibit raft expression (Martin et al. 2001). Darlington et al. reported that CTLA-4 is recruited to lipid rafts during negative signaling and accumulates at the IS (Darlington et al. 2002). Further, recent work by Egen and Allison has suggested that the level of TcR signaling is directly related to the amount of CTLA-4 accumulated in the IS (Egen and Allison 2002). The level of CTLA-4 expression was increased in the IS under stronger T-cell activation and the majority of CTLA-4 in the cytoplasm was localized beneath the T-cell/APC contact site. Crystal structures of B7-ligated CTLA-4 suggest that it may form lattices within the IS (Schwartz et al. 2001; Stamper et al. 2001).

On another level, CTLA-4 can also influence the function of integrins such as LFA-1. Integrins play key roles in regulating T cell-APC conjugation and the migration of T cells (Hogg et al. 2002; Takagi and Springer 2002). A recent report by Schneider et al. have shown that CTLA-4 can generate inside-out signals that potently up-regulate LFA-1 clustering and adhesion on the surface of T cells (Schneider et al. 2005). The up-regulation of adhesion was observed under the same conditions that led to an inhibition of IL-2 production. The importance of the connection between CTLA-4 and LFA-1 adhesion was underscored by the fact that the loss of CTLA-4 expression in CTLA-4-deficient T cells resulted in an impairment of TcR/CD3 induced LFA-1 adhesion and clustering. This upregulation of adhesion was dependent on GTPase Rap-1 activation. Rap-1 is an allosteric regulatory element, switching between inactive GDP-bound and active GTP-bound conformations (Bos et al. 2003; Caron 2003). CTLA-4 activated Rap-1 at levels that were higher than those induced with anti-CD3 stimulation. The role for CTLA-4 in LFA-1 mediated adhesion provides an alternate route through which CTLA-4 may modulate T-cell immunity.

8.3.1.3 CTLA-4 and Disease States

Blockade of CTLA-4 has been employed in the therapeutic modulation of autoimmunity, transplantation and tumor immunotherapy (Hurwitz et al. 2002; Egen et al. 2002; Salomon and Bluestone 2001). CTLA-4 polymorphisms have been genetically linked to a number of human diseases (Kristiansen et al. 2000). Splice variants and mutations within the CTLA-4 gene have been identified as candidates for susceptibility to Grave's disease, autoimmune hypothyroidism and type 1 diabetes (Ueda et al. 2003). In the NOD mouse, disease susceptibility is associated with a reduced production of a splice form encoding a molecule lacking the ligand-binding domain (ligand independent CTLA-4; liCTLA-4) (Ueda et al. 2003). LiCTLA-4 is expressed in resting T cells and rapidly down-regulated during T-cell activation (Vijayakrishnan et al. 2004). Taken together, these findings suggest that genetic differences in CTLA-4 can contribute to the development of autoimmune diseases.

8.3.2 Programmed Death-1 (PD-1)

Another inhibitory member of the CD28 family is PD-1. As its name reflects, the PD-1 receptor was initially identified by subtractive hybridization studies using a T-cell hybridoma undergoing programmed cell death (Agata et al. 1996). Subsequent studies have questioned a direct role for PD-1 in cell death (Agata et al. 1996; Nishimura et al. 1996; Vibhakkar et al. 1997). PD-1 is a 55 kDa transmembrane protein that shares 24% amino acid homology in the extracellular domain to CTLA-4 (Ishida et al. 1992). It binds the distinct ligands PD-L1 and PD-L2 and is expressed on CD4 CD8 double negative cells and double negative $\gamma\delta$ thymocytes (Nishimura et al. 2000). It can also be induced on activated human CD4 and CD8 positive T cells, B cells and myeloid cells. This contrasts with the more restricted expression of CD28 and CTLA-4.

Engagement of PD-1 by PD-L1 or PD-L2 inhibits TcR-mediated proliferation and cytokine production by previously activated T cells. PD-L1 and PD-L2 strongly inhibit both T-cell proliferation and cytokine production at low antigen concentrations, even in the presence of strong B7-CD28 signals. By contrast, at high antigen concentrations, PD-L1 and PD-L2 do not inhibit T-cell proliferation but markedly reduce the production of multiple cytokines (Sharpe and Freeman 2002). Interestingly, there are also studies reporting that PD-L1 and PD-L2 can provide a co-stimulatory signal to suboptimally activated T cells (Tamura et al. 2001; Dong et al. 1999; Tseng et al. 2001).

8.3.2.1 Signaling Pathways

The cytoplasmic tail of PD-1 contains two tyrosines, one that constitutes an immunoreceptor tyrosine-based inhibition motif (ITIM) (Fig. 5). The ITIM sequence is

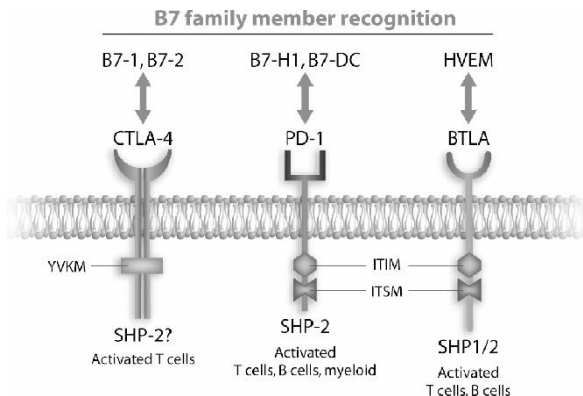


Fig. 5. Inhibitory co-receptors with binding sites for the phosphatases SHP-1 and SHP-2. Ligation-induced phosphorylation of immunoreceptor tyrosine-based inhibition motif (*ITIM*) and immunoreceptor tyrosine-based switch motif (*ITSM*) in the cytoplasmic tails of programmed death-1 (*PD-1*) and B and T lymphocyte attenuator (*BTLA*) results in the recruitment of phosphatases SHP-1 and SHP-2. Although cytotoxic T-cell antigen (*CTLA-4*) does not possess an *ITIM* or *ITSM* motif, it may indirectly bind to SHP-2 via its YVKM motif. Binding to these phosphatases leads to dephosphorylation of proximal signaling molecules

found in several classes of inhibitory receptors, including the killer inhibitory receptors found on NK cells and FcγRIIB on B cells, and functions by recruiting SH2-containing phosphatases (Vivier and Daeron 1997). The other tyrosine belongs to an immunoreceptor tyrosine-based switch motif (*ITSM*) (Shlapatska et al. 2001). Although both tyrosines are phosphorylated following PD-1 engagement, mutagenesis studies indicate that the tyrosine within the *ITSM* motif may be required for the inhibitory activity of PD-1, as opposed to the tyrosine in the *ITIM* that is more typically associated with inhibitory signaling (Okazaki et al. 2001). In T cells, phosphorylated *ITSM* recruits SHP-2 and SHP-1, whereas in B cells, only SHP-2 was recruited. In vitro studies have demonstrated that PD-L1 and PD-L2 can inhibit T-cell proliferation and cytokine production, others showed that the PD-1 ligands enhance T-cell activation. In addition to the possibility that there may be another ligand for PD-1, the *ITSM* motif might be relevant to the function of PD-1 ligands to deliver either positive or negative signals, dependent on the activation of the T-cell and therefore interaction with different phosphatases.

8.3.2.2 PD-1 and Disease States

The loss of negative regulation by PD-1 leads to the development of autoimmune diseases, and the genetic background of mice greatly affects the autoimmune phenotypes. PD-1-deficient mice of the C57BL/6 strain develop a late onset progressive arthritis and lupus-like glomerulonephritis with high levels of IgG3 deposition (Nishimura et al. 1999). By contrast, PD-1-deficient mice of the Balb/c strain

develop an early onset dilated cardiomyopathy with 50% mortality at 20 weeks of age. The affected hearts do not have IgG and complement deposition. Affected mice develop high titers of an autoantibody that recognizes a heart-specific 33 kDa protein on the surface of cardiomyocytes, but do not have other common autoantibodies (Nishimura et al. 2001).

Experiments in PD-1-deficient mice have also revealed a role for PD-1 in regulating CD8 positive T-cell responses. 2C-TCR transgenic T cells recognize cells bearing H-2L^d, an MHC class I molecule expressed in Balb/c and B10.D2 mice. 2C⁺ autoreactive T cells are negatively selected in the thymus and only a few 2C-TCR positive cells are found in the periphery, where they do not become activated or cause disease (Nishimura et al. 1999, 2000). In the absence of the PD-1 gene, however, these cells become activated and cause a lethal graft-versus-host disease. These results indicate that potentially autoreactive T cells in the periphery can become activated in the absence of PD-1 and implicate PD-1 in regulating peripheral T and/or B-cell tolerance (Sharpe and Freeman 2002).

8.3.3 T-Cell Immunoglobulin and Mucin-Domain-Containing Molecule-3 (Tim-3)

Tim-3 is another co-receptor with an inhibitory effect on immune responses, but via a fundamentally different mechanism. It is a type 1 membrane protein consisting of a single IgV-like domain and a highly glycosylated mucin-containing domain (Monney et al. 2002). Galectin-9, a member of the galectin family that is expressed on lymphocytes and other cell types, has been identified as a ligand for Tim-3. The first Tim family member to be identified was the human hepatitis A virus cell receptor, which exhibits 39% homology to Tim-3 and is now termed Tim-1 (Feigelsstock et al. 1998; Kaplan et al. 1996). Initial reports suggested that Tim-1 acts as a positive co-stimulatory molecule by stimulating cytokine production from Th2 cells without altering cytokine production from Th1 cells (Umetsu et al. 2005). The functional characterization of Tim-3 has identified its role in regulating the Th1 immune response. The Tim-3/galectin-9 pathway controls Th1 immunity by selective deletion of Tim-3 positive Th1 cells. Galectin-9 is highly expressed in the naïve immune system. This serves to inhibit the generation of Th1 responses, whereas loss of galectin-9 after activation allows the generation of Th1 cells. Although galectin-9 can induce both necrosis and apoptosis, it is believed that Tim-3/galectin-9 negatively regulate effector Th1 cells (Zhu et al. 2005).

8.3.3.1 Tim-3 and Disease States

In this context, the Tim-3/galectin pathway has also been shown to have a crucial role in the induction of peripheral tolerance. Tim-3 Ig treatment or the use of

Tim-3-deficient mice similarly prevented tolerance induction to MHC-mismatched allografts. Blockade of the pathway by treatment with anti-Tim-3 antibody or Tim-3Ig fusion proteins led to increased Th1 cell proliferation and cytokine responses, in addition to the loss of tolerance induction. Th1 cells are associated with the development of EAE, whereas the secretion of Th2 cytokines has been associated with regulation and disease recovery. The onset and progression of this autoimmune disease is dependent on a balance between the two responses. Tim-3-deficient animals also have increased severity in models of experimental autoimmune diabetes. Interestingly, Tim-3-deficient mice have increased lung inflammatory responses following allergen exposure (Coyle and Gutierrez-Ramos 2004).

8.3.4 *B and T Lymphocyte Attenuator (BTLA)*

BTLA is the most recently recognized member of the CD28 family. It is a type I transmembrane glycoprotein with an extracellular single IgV-like domain, a transmembrane and cytoplasmic domain. The latter contains three tyrosine residues which are part of tyrosine-based motifs (Grb-2 binding site, ITIM, ITSM) (Carreno and Collins 2003; Watanabe et al. 2003). BTLA interacts with the ligand HVEM, a member of the TNFR superfamily, and not with B7x as originally thought (Sedy et al. 2005; Watanabe et al. 2003). It is induced on T cells during activation, and remains expressed on Th1 but not Th2 cells. This suggests that BTLA may specifically down-regulate Th1-mediated inflammatory responses. In this way, the expression pattern contrasts with ICOS that remains elevated on Th2 cells, but is down-regulated on Th1 cells (Watanabe et al. 2003). BTLA also inhibits cytotoxic T lymphocyte (CTL) maturation and proliferation (Sica et al. 2003). T cells from BTLA-deficient mice have increased proliferation in response to antigen, and B cells from these mice have an increased response to anti-IgM. BTLA-deficient mice also show increased susceptibility to peptide antigen-induced EAE (Watanabe et al. 2003). BTLA-deficient mice have a more subtle phenotype than CTLA-4-deficient mice. One possibility for the lack of an obvious phenotype in BTLA-deficient mice compared to CTLA-4-deficient mice is that BTLA and PD-1 may have overlapping functions. Whether PD-1 and BTLA use similar or distinct mechanisms to block T-cell activation is less clear. Both PD-1 and BTLA have related and relatively long cytoplasmic tails that consist of an ITIM followed by a distal ITSM. BTLA also has a membrane-proximal Grb-2 binding site, but the functional importance of this motif is unknown since only mutation of the ITIM or ITSM blocked the association of SHP-1 and SHP-2 to the BTLA cytoplasmic tail (Gavrieli et al. 2003).

8.4 Conclusion

Signals provided by co-receptors act to fine-tune the response of T cells to antigen. The ability of co-receptors to modulate the level of intracellular signaling determines the degree to which the antigen-receptor complex can recognize self versus

nonself. These signals can either up-regulate or inhibit the immune response, depending on the activation status of the cell and its location in the peripheral immune system. The ligands for different co-receptors are expressed on cells in different regions of the peripheral compartment. Although many co-signaling events have been characterized, there remains much to be discovered regarding the entire range of their biological functions. A greater understanding will come as new techniques and approaches are applied to the field. New imaging technologies using new fluorescent labels and sensors and the use of more sophisticated computer software for image acquisition will further deepen our understanding of the assembly and expression of these co-receptors and their intracellular mediators. Such studies should lead to a better understanding of co-receptor functions and the development of new therapeutic targets for the treatment of infectious diseases, immunodeficiency disorders, autoimmunity and cancer.

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