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Volume 560

MECHANISMS OF LYMPHOCYTE ACTIVATION AND IMMUNE REGULATION X

Innate Immunity

Edited by
Sudhir Gupta,
William E. Paul,
and
Ralph Steinman

MECHANISMS OF LYMPHOCYTE ACTIVATION AND IMMUNE REGULATION X

Innate Immunity

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Innate Immunity

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PREFACE

Immunological science is an ever expanding enterprise in which new vistas continually open. Indeed, immunology represents one of the major scientific crossroads, linking human disease and fundamental biology. Yet even in a field as vigorous as this, the revolution touched off by the demonstration that the nature of innate immune responses determines the quality and the intensity of the adaptive response has been almost unprecedented.

Charles Janeway's prescient presentation at Cold Spring Harbor in 1989, in which he argued that there were receptors on critical cells in the immune system that were specific for certain invariant ligands expressed by microbial pathogens, planted a seed that has yielded a bumper crop. When he and Medzhitov showed that the Drosophila *toll* had a vertebrate homolog in the TLRs and that the TLRs were the (or one of the families of) *pattern recognition receptors* (PRRs) that he had postulated, there was no stopping the flood of highly innovative and important research that followed. Eleven different TLRs have been identified and the list of their ligands grows. Other molecules, some known for a long time, can also be validly considered PRRs.

The cellular and molecular details of how pathogens are detected by the immune system and how that detection is translated into the mounting of an immune response appropriate in quality to the lifestyle (disease style) of the pathogen are being rapidly unraveled. In many respects, this newly recognized "third law of immunology" — *the innate immune response directs the adaptive response* — has established itself fully beside the first law (*universality of immune recognition*) and the second law (*avoidance of self reactivity*).

The *Tenth International Conference on Lymphocyte Activation and Immune Regulation*, part of the biennial series held at Newport Beach California, dealt with this critical area. The presentations revolved about the topic of innate immunity, which was the title of the conference. Many of the leading figures in research in this subject were present. The presentations represented the cutting edge of contemporary research and inspired a spirited and highly useful discussion.

This volume, representing the papers that summarize the lectures, should provide the reader with a very good picture of this field in the midpoint of the first decade of the 21st century. The meeting and the book are organized along the main themes of the field, Toll Receptors and TLRs, NK cells, Dendritic Cells, and the Complement System.

Rather than reviewing the contents of the individual chapters, which the reader can do at his/her leisure, we emphasize the fact that this is a field in flux. Much has been

accomplished but the field has by no means become "mature." Areas of major controversy exist; progress is rapid; today's certainty may be the concept on tomorrow's dust heap. We can only say this to the reader: stay tuned, the best is yet to come.

The organizers are particularly grateful to all who played a role in making the meeting a success and the book a reality. We particularly thank Janet Nagurski for editorial assistance.

Sudhir Gupta
William E. Paul
Ralph Steinman

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1

TIR DOMAIN-CONTAINING ADAPTORS REGULATE TLR SIGNALING PATHWAYS

Masahiro Yamamoto[1] and Shizuo Akira[1,2]

1. INTRODUCTION

Against invading microorganisms, vertebrates including mammals develop innate immune systems, which are activated by microbial components possessing conserved structures called pathogen associated molecular patterns (PAMPs); including bacterial cell wall components, and viral genomic DNA and RNA. PAMPs are recognized by pattern recognition receptors mainly expressing on immune responsive cells. Toll-like receptors (TLRs)[1-3] are an example of pattern recognition receptors, and TLR family members are conserved among mammals. To date, 10 and 12 TLRs have been reported in human and mouse, respectively. Almost all TLRs have been shown to recognize PAMPs; TLR2 is the receptor for peptidoglycan and lipoprotein, including bacterial lipoprotein (BLP) and mycoplasmal lipoprotein (MALP-2)[2-5]. Especially, BLP and MALP-2 are reportedly recognized in the functional heterodimeric association of TLR2 with TLR1 and TLR6, respectively. TLR4 is involved in the recognition of a gram-negative cell wall component, lipopolysaccharide (LPS)[6,7]. TLR5 is a receptor for flagellin, a component of bacterial flagella[8]. TLR3 and TLR9 are receptors for double-stranded (ds) RNA and unmethylated CpG DNA, respectively[9,10]. Although the natural ligand for TLR7 is yet to be identified, the receptor has been shown to recognize imidazoquid or its derivative, R-848. Since they are utilized in the treatment of genital warts caused by human papilomavirus, the ligand for TLR7 seems to be a component of viruses[11]. Thus, accumulating evidence clearly demonstrates that TLRs serve as pattern recognition receptors to detect invading microbes (Figure 1).

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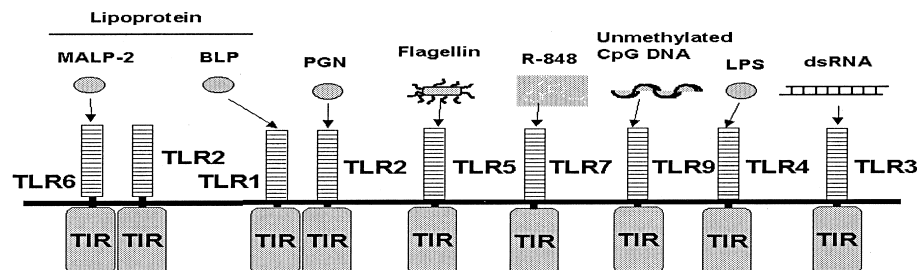

Figure 1. Summary of PAMPs that are recognized by TLRs

2. THE MYD88-DEPENDENT PATHWAYS AND THE MYD88-INDEPENDENT PATHWAYS

As a structural feature, all TLRs possess an extracellular domain rich in leucines, called the leucine-rich repeat (LRR), and an intracellular domain which shows substantial homology with the intracellular portion of IL-1 receptor (IL-1R) family members, the so called Toll-IL-1R (TIR) domain. LRR has been shown to be responsible for the interaction with PAMPs. Ligation of the extracellular domain of TLRs with microbial components activates intracellular signaling cascades, culminating in various immune responses such as proinflammatory cytokine production, B cell proliferation and dendritic cell (DC) maturation[1-3]. The signaling cascades originate from the TIR domain, which recruits an intracellular TIR domain-containing adaptor molecule, MyD88 (Myeloid Differentiation factor 88), which was originally identified as a myeloid differentiation primary response gene that is rapidly up-regulated upon IL-6-stimulated differentiation of M1 myeloleukemic cells into macrophages[12]. Subsequent *in vitro* studies showed overexpression of MyD88 leads to activation of NF-κB and MAPKs[13,14]. MyD88 possesses a TIR domain in the C-terminal portion and a Death Domain (DD) in the N-terminal portion, which interacts with DD-containing kinases such as IL-1R-associated kinase (IRAK) family members. Among them, IRAK-1 and IRAK-4 have been shown to participate in activation of IL-1R and TLR-mediated signaling pathways. Ligation of PAMPs with TLRs leads to phosphorylation of IRAK-1 and IRAK-4, and then activates NF-κB and MAPKs via TRAF6[15-17].

Evidence provided from studies using gene-targeted mice clearly demonstrates the significance of MyD88 in the activation of TLR signaling pathways. MyD88-deficient mice showed completely defective responses to IL-1 family members in terms of gene expression and activation of signaling molecules[18]. Moreover, responses to TLR1, TLR2, TLR5, TLR6, TLR7 and TLR9 ligands were also completely abolished in MyD88-deficient cells, indicating that MyD88 is an essential adaptor for those receptors[8,11,19-21].

However, MyD88-deficient mice retain certain types of responses, particularly observed in TLR3 and TLR4-mediated signaling pathways[9,22]. LPS and dsRNA still stimulated activation of NF-κB and MAPKs with delayed kinetics, even in the absence of MyD88[9,22]. In addition to those activations, IRF-3, a transcription factor crucial for the expression of IFN-β, was activated in response to LPS and poly(I:C) in MyD88-deficient mice[23,24]. Further analysis revealed that TLR3- and TLR4-mediated MyD88-independnent signaling pathways associate with IFN-β induction and subsequent expression of interferon-inducible genes such as IP-10, RANTES, and GARG-16[23,24]. Moreover, LPS and poly(I:C)-induced up-regulation of CD40,CD80, and CD86 in MyD88-deficient DCs was still observed, indicating that TLR signaling pathways were separated into two classes[25]. One is the MyD88-dependent pathway that leads to proinflammatory cytokine production, B cell activation; the other the MyD88-independent pathway observed in TLR3 and TLR4 signaling pathways, culminating in the expression of IFN-β and IFN-inducible genes. Several groups have attempted to elucidate the molecular mechanisms of the MyD88-independent pathway on the hypothesis that other TIR domain-containing adaptor molecules may regulate MyD88-independent pathways.

3. TIRAP: THE SECOND ADAPTOR MOLECULE

Two groups independently reported the identification of the second TIR domain-containing adaptor named TIRAP (for TIR domain-containing Adaptor Protein, also known as Mal)[26,27]. TIRAP possesses a TIR domain in the C-terminal portion. Ectopic expression of TIRAP strongly up-regulated the NF-κB-dependent promoter, and the dominant-negative form of TIRAP significantly inhibited TLR4-mediated, but not IL-1R- and TLR9-mediated, NF-κB activation. In addition, ectopic expression of TIRAP associated with the TIR domain of TLR4, but not TLR9. TIRAP inhibitory small peptide severely impaired LPS-induced DC maturation as well as proinflammatory cytokine production. Given that LPS-stimulated DC maturation is one of hallmarks of the MyD88-independent pathway, TIRAP may be involved in the TLR4-mediated MyD88-independent pathway.

However, analysis using TIRAP-deficient mice did not support the in vitro data; namely, the MyD88-independent signaling pathway in response to LPS was normal in TIRAP-deficient mice[28,29]. TIRAP-deficient mice showed normal induction of IFN-inducible genes in macrophages and similar levels of up-regulation of surface marker molecules in DCs in response to LPS. Furthermore, the MyD88-independent responses in MyD88/TIRAP-doubly deficient mice were also intact, indicating that TIRAP is dispensable for the MyD88-independent pathway[28]. However, LPS-stimulated proinflammatory cytokine production such as TNF-α, IL-6, and IL-12 p40 was not observed in TIRAP-deficient macrophages. In vivo responses to LPS in TIRAP-deficient mice were also severely impaired. TLR3, TLR5, TLR7, and TLR9-mediated proinflammatory cytokine production were normal; however, TLR2 (TLR1/TLR6)-mediated production was significantly perturbed in TIRAP-deficient mice. In terms of signal transduction, TLR2 and TLR4-mediated, but not TLR7-mediated, NF-κB and MAPKs activation were impaired in TIRAP-deficient cells. Especially, TLR4-mediated NF-κB and MAPKs were activated with delayed kinetics, suggesting that TIRAP is not involved in the MyD88-independent pathway. Instead, TIRAP is essential for the MyD88-dependent pathways

shared by TLR2 and TLR4. Taken together, these data indicate that molecules other than MyD88 and TIRAP are responsible for the MyD88-independent pathways.

4. TRIF: THE THIRD ADAPTOR MOLECULE

Further database searching and yeast two-hybrid analysis independently identified the third TIR domain-containing adaptor molecule, TRIF (for TIR domain-containing adaptor inducing IFN-β, also known as TICAM-1)[30,31]. Ectopic expression of TRIF significantly activated the NF-κB-dependent promoter, like MyD88 and TIRAP. Additionally, overexpression of TRIF strongly up-regulated the IFN-β promoter, unlike MyD88 and TIRAP. Biochemical analysis showed TRIF directly associated with the TIR domains of TLR3 and IRF-3, indicating that TRIF may be related to the TLR3-mediated MyD88-independent pathway, however, the involvement of TRIF in the TLR4-mediated MyD88-independent pathway remains to be determined.

TRIF-deficient mice and mice carrying a mutation in *Trif* gene (LPS2 mice) were generated and analyzed to examine the physiological function of the molecule [32,33]. TRIF-deficient mice showed defective responses to poly(I:C) including expression of IFN-β and IFN-inducible genes, and B cell activation. TLR3-mediated NF-κB and IRF-3 activation was also impaired in TRIF-deficient mice, indicating that TRIF is responsible for the TLR3-mediated MyD88-independent pathway *in vivo*. In the case of TLR4-mediated responses, LPS-induced expression of IFN-inducible genes was significantly reduced in TRIF-deficient cells; however, LPS-activated NF-κB and MAPKs were apparently intact. Even if TRIF-deficient cells may lack the MyD88-independent pathway, they retain MyD88-depednent NF-κB and MAPKs activation. This shows that the MyD88-depedent pathway may mask the loss of the MyD88-independent pathway. Indeed, LPS-induced NF-κB and MAPKs activation and the expression of IFN-inducible genes were completely abolished in MyD88/TRIF-doubly deficient mice, demonstrating that TRIF plays a central role in the TLR4-mediated MyD88-independent pathway as well as that of TLR3.

Further, TRIF-deficient mice showed defective proinflammatory cytokine production and B cell activation in response to the TLR4 ligand, but not to other TLR ligands. Although proinflammatory cytokine production has been considered to be MyD88-dependent, the impairment in TRIF-deficient mice suggested that, at least in the TLR4 signaling pathway, not only the MyD88-dependent pathway but also the MyD88-independent pathway may be required for proinflammatory cytokine production. *In vitro* studies using deletion mutants of TRIF indicated that the N-terminal and C-terminal portions of TRIF differentially take part in the activation of NF-κB. Furthermore, the N-terminal, but not the C-terminal, portion has been shown to potentiate the activation of the IFN-β promoter. These results suggest that the N-terminal portion of TRIF may be responsible for the MyD88-independent pathway. However, which portions of TRIF are required for proinflammatory cytokine production remains to be seen.

5. TRAM: THE FORTH ADAPTOR MOLECULE

To date, 2 more TIR domain-containing adaptor molecules have been registered in the database (Figure 2)[34]. One is TRAM (for TRIF-Related Adaptor Molecule, also

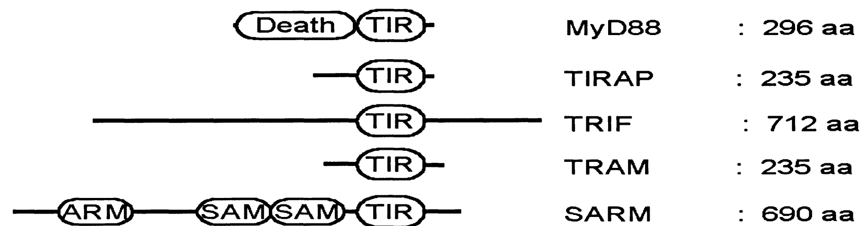

Figure 2. TIR domain-containing adaptor family consists of five members. To date, 5 molecules have been reported as TIR domain-containing adaptors. Death: the death domain. TIR: the Toll/IL-1 Receptor domain. SAM: the SAM domain. ARM: the armadillo repeat.

known as TICAM-2)[35-37]. TRAM is the fourth adaptor whose functions have been analyzed using gene-targeted mice and small inhibitory RNA (siRNA). TRAM possesses the TIR domain in the central portion and showed greater homology with TRIF than MyD88 and TIRAP. Ectopic expression of TRAM induced the activation of NF-κB and the IFN-β promoter, albeit at more reduced levels than MyD88 and TRIF, respectively.

In vivo analysis using TRAM-deficient mice showed that LPS-induced expression of IFN-β and IFN-inducible genes was not observed in TRAM-deficient macrophages[35]. However, poly(I:C)-induced expression of these molecules was intact. Moreover, LPS-mediated, but not other TLR ligand-mediated, production of proinflammatory cytokine, B cell activation and maturation were severely impaired in TRAM-deficient cells. In view of the activation of signaling molecules, TRAM-deficient cells showed defective levels of LPS-induced, but not poly(I:C)-induced, IRF-3 activation. This phenotype was reminiscent of that of TRIF-deficient mice in the TLR4 signaling pathway[32,33]. Moreover, LPS-mediated, but not poly(I:C)-mediated, activation of NF-κB and MAPKs was significantly abrogated, especially at later time points. Given that LPS-induced activation of the signaling cascade with delayed kinetics was the feature of the MyD88-independent pathway, the TLR4-mediated MyD88-independent pathway was affected by the TRAM deficiency. Compared with TRIF-deficient mice showing defective responses in TLR3- and TLR4-mediated MyD88-independent pathways, the TLR4-mediated MyD88-independent pathway was specifically blocked in TRAM-deficient mice. Further, biochemical analysis showed that TRAM associated with TRIF and TLR4, but not with TLR3 and other TLRs[36,37]. Together, these studies clearly demonstrated that TRAM is specifically involved in the MyD88-independent pathway of TLR4 signaling (Figure 3).

The other molecule registered in database is SARM[38], which was originally identified as containing an Armadillo motif and two SAM domains in the N-terminal portion; and

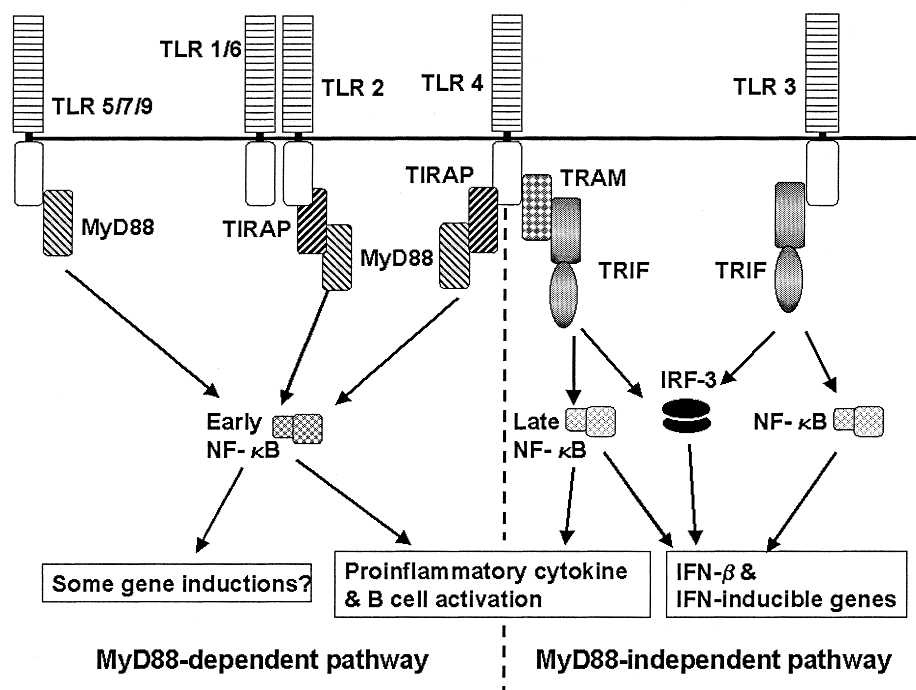

Figure 3. Participation of adaptors in TLR signaling pathways. MyD88 is essential for inflammatory cytokine production in response to all TLR ligands, except for the TLR3 ligand. The second adaptor, TIRAP/Mal, is required for TLR2/TLR1/TLR6- and TLR4-mediated MyD88-dependent pathways. The third adaptor, TRIF, is an essential signal transducer for both TLR3- and TLR4-mediated MyD88-independent pathways. The fourth adaptor, TRAM, is specifically involved in the TLR4-mediated MyD88-independent pathway. Other adaptor(s) may participate in the MyD88-dependent pathways via other TLRs such as TLR5, TLR7, and TLR9.

subsequently to possess the TIR domain in the C-terminal portion. The physiological functions of SARM in TLR signaling pathways or others remain unknown.

6. OTHER MOLECULES POSITIVELY REGULATING TLR SIGNALING

The analysis using TRIF-deficient mice showed TRIF is a key signal transducer in the MyD88-independent pathway leading to IRF-3[32,33]. Although IRF-3 was activated through the phosphorylation in the C-terminal portion, TRIF contains no kinase domain or kinase activity itself; indicating that there may exist a kinase(s) that bridges TRIF and IRF-3. Recent studies determined 2 candidates, IKKϵ and TBK1 (also known as IKK-*i* and NAK/T2K, respectively), out of a number of kinases[39,40]. Overexpression of IKKϵ or TBK1 strongly activated the IFN-β promoter, like TRIF and TRAM. Moreover, both IKKϵ and TBK1 phosphorylated IRF-3 *in vitro*. Analysis using siRNA targeted to IKKϵ

and TBK1 showed significant reduction in TRIF-mediated activation of the IFN-β promoter. Biochemically, both kinases associated with TRIF and IRF-3. Taken together, IKKϵ and TBK1 may act as IRF-3 kinases downstream of TLR3 and TLR4-mediated MyD88-independent pathways. Very recent studies using TBK1-deficient cells clearly demonstrated that TBK1 acts as an IRF-3 kinase downstream of TRIF[41].

Several molecules possibly relate to MyD88-dependent pathways downstream of MyD88. One is the Pellino family of proteins. In *Drosophila melanogaster*, Pellino has been initially identified as a molecule associating with Pelle, a Drosophila homologue of IRAK[42]. In mammals, the Pellino family has 3 members, Pellino1, 2, and 3. Pellino1 appears to activate NF-κB in the IL-1R signaling pathway through the integration of IL-1R-IRAK1-IRAK4-TRAF6 complex[43]. Pellino2 and Pellino3 promote activation of c-Jun and Elk-1 in the TLR-IL-1R signaling pathway[44-46]. However, the physiological roles of the Pellino family of proteins remain to be determined. Another is TRAF6. The significance of TRAF6 in the MyD88-dependent singling pathway has been well established. Indeed, TRAF6-deficient cells showed defective responses in IL-1 and LPS-induced cellular responses and activation of signaling molecules[47,48]. Surprisingly, TRAF6 has been shown to associate with TRIF and to be involved in the activation of NF-κB via the N-terminal portion of TRIF. Given that TRIF mediates the MyD88-independent late-phase NF-κB activation in the TLR4 signaling, TRAF6 may possibly participate not only in the MyD88-dependent pathway, but also in the MyD88-independent pathway via TRIF[49].

7. CONCLUDING REMARKS

MyD88 is a TIR domain-containing adaptor common to signaling pathways via the TLR family. However, MyD88-deficient mice show normal responses in TLR3-ligand stimulation and maintain certain types of cellular responses such as the production of IFN-β and IFN-inducible genes in TLR4-ligand stimulation; indicating that TLR3- and TLR4-mediated signaling may possess MyD88-independent pathways. The identification of other TIR domain-containing adaptors, TRIF, TIRAP and TRAM, revealed that TRIF is an essential signal transducer in TLR3- and TLR4-mediated MyD88-independent pathways. Moreover, TIRAP and TRAM provide specificity for the MyD88-dependent component of TLR2 and TLR4 signaling, and the MyD88-independent component of TLR4 signaling. Taken together, TIR domain-containing adaptors may account for specificity in the downstream signaling of individual TLRs.

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2

TOLL-LIKE RECEPTORS: LINKING INNATE AND ADAPTIVE IMMUNITY

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1. ABSTRACT

Work in recent years has shown an essential role for Toll-like receptors (TLRs) in the activation of innate and adaptive immunity in vertebrate animals. These germ-line encoded receptors, expressed on a diverse variety of cells and tissues, recognize conserved molecular products derived from various classes of pathogens, including Gram-positive and -negative bacteria, DNA and RNA viruses, fungi and protozoa. Ligand recognition induces a conserved host defense program, which includes production of inflammatory cytokines, upregulation of costimulatory molecules, and induction of antimicrobial defenses. Importantly, activation of dendritic cells by TLR ligands is necessary for their maturation and consequent ability to initiate adaptive immune responses. How responses are tailored by individual TLRs to contain specific classes of pathogens is not yet clear.

2. INTRODUCTION

In all animals, the innate immune system provides essential protection against invading pathogens. A key component of this system is a collection of germ-line encoded receptors called pathogen recognition receptors (PRRs), which recognize a highly conserved set of molecular structures specific to microbes (Pathogen associated molecular patterns, or PAMPs) [1]. In addition to this system, vertebrates have a second line of defense called the adaptive immune system, which employs a diverse set of somatically rearranged receptors (T- cell receptors [TCRs] and B-cell receptors [BCRs]) with the ability to recognize a large spectrum of antigens.

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The best understood and perhaps the most important subgroup of PRRs is the Toll-like receptor family. These receptors have the ability to recognize pathogens or pathogen derived products and initiate signaling events leading to activation of innate host defenses. Signaling by TLRs initiates acute inflammatory responses by induction of anti-microbial genes and inflammatory cytokines and chemokines [2,3]. In addition, TLRs have an important role in activation of adaptive immune responses [4,5]. Although T and B cells of the adaptive immune system express receptors of enormous diversity, activation of these cells depends on induction of co-stimulatory molecules and secretion of cytokines and chemokines by the cells of the innate immune system. Effective response to microbial infection requires several levels of interactions between innate and adaptive immune systems. A variety of cell surface receptors, secreted cytokines and chemokines participate in the induction of protective immunity. We will discuss here current paradigms of the importance of innate immune recognition by TLRs and the significance of that recognition for the outcome of adaptive immune responses. We will also discuss how inappropriate activation of TLRs under certain circumstances can lead to autoimmune diseases.

3. TLRs AND THEIR LIGANDS

The mammalian TLR family consists of 10 members with distinct ligand specificities and gene targets [2,3]. TLR4 recognizes lipopolysaccharide (LPS) [6,7] from gram-negative bacteria, TLR2 recognizes peptidoglycan from gram-positive bacteria [8], TLR3 recognizes double-stranded RNA from double stranded and negative strand viruses [9], TLR7 and 8 recognize RNA from single stranded viruses [10,11], and TLR9 recognizes unmethylated CpG DNA found abundantly in prokaryotic genomes and DNA viruses [12,13]. A comprehensive list of ligands and signaling events downstream of various TLRs is described elsewhere [3,14]. In addition, several reports have suggested that some TLRs can also recognize host-derived ligand. One example is the recognition of heat shock proteins by TLR2 and TLR4 [15-17]; however, it remains possible that the recombinant heat shock proteins used in these studies were contaminated with endotoxin (or other TLR ligands), consistent with more recent reports that more stringent purification of the hsps resulted in a loss of stimulatory activity [18-20]. Another example of recognition of a host ligand by a TLR (chromatin associated DNA by TLR9) is discussed in more detail below.

4. ADAPTIVE IMMUNE SYSTEM: MECHANISMS OF TOLERANCE AND IMPORTANCE OF INNATE IMMUNE RECOGNITION

Although most auto-reactive T and B lymphocytes undergo clonal deletion during their development in primary lymphoid organs (central tolerance), a few nevertheless escape into the periphery and must be held in check by mechanisms of peripheral tolerance [21].

Clonal deletion of auto-reactive B cells is achieved in the bone marrow. Immature B cells that interact with membrane bound self-antigens are promptly deleted, while those that interact with soluble self-antigens are subject to a much more prolonged process of anergy followed by apoptosis [22-25]. In the case of T cells, clonal deletion is the fate of thymocytes with high affinity to self-antigens expressed in the thymus [21]. To make this an effective process, it is important that genes that function only in peripheral tissues and organs are expressed in thymic epithelium and DCs resident in thymus. Recent findings identify a transcriptional regulator called autoimmune regulator (AIRE) that permits thymic expression of many genes that function or are otherwise expressed only in peripheral tissues and organs [26,27]. Mice and humans with defective AIRE have an increased number of self-reactive T cells, which underscores the important role of AIRE in central tolerance [26-28]. Because expression of co-stimulatory molecules on thymic APCs is essential for induction of negative selection, it will be interesting to address the role of TLRs in controlling co-stimulatory molecule upregulation in this context [29-32].

Tolerance to self-antigens in the peripheral tissues and secondary lymphoid organs is achieved by at least two distinct and equally important mechanisms. The first one is through controlled expression of co-stimulatory molecules on professional APCs. The second is by the suppressor activity of a specialized group of cells called suppressor or regulatory T cells (Tr cells). Priming of naive T cells can be induced only when TCR-MHC/peptide interaction is coupled with a second (co-stimulatory) signal provided by interaction between CD28 on T cells and CD80/CD86 molecules on APCs [33,34]. This second signal, a result of dendritic cell (DC) maturation [35], is regulated by Toll-like receptors, and in effect, flags the antigen as being pathogenic [36]. Another consequence of TLR-induced DC maturation is the elaboration of a variety of cytokines and chemokines [2,3]. These soluble signals play an important role in the outcome of adaptive immune responses. Some cytokines are particularly important for overcoming suppression mediated by Tr cells; others like IL-12 are responsible for directing the T cell responses towards a Th1 phenotype.

Recently, several studies have suggested that peripherally-resident DCs, in addition to TLR-induced migration to draining lymph nodes during infection, may also traffick to lymph nodes even in the absence of infection. This homeostatic migration enables DCs expressing self-peptides either to tolerize self-reactive T cells [37,38] or to promote these T cells to acquire properties of suppressor T cells [39,40], thereby promoting peripheral tolerance.

The second mechanism by which peripheral tolerance is maintained is through the activity of Tr cells. These cells can be divided into two major classes based on their function. The first class, which express CD4 and CD25, [41] reside in all the secondary lymphoid organs and develop in the thymus under control of a specialized transcription factor Foxp3 [42,43]. This class of suppressor cells may have evolved to prevent activation of auto-reactive T cells that escape thymic deletion; humans and mice with defective Foxp3 expression develop generalized, fatal autoimmune [42,44]. The second class is characterized by the secretion of either of two anti-inflammatory cytokines, IL-10 (Tr1) [45], or TGF-beta (Th3) [46]. These suppressor cells typically reside in mucosal tissues of the body such as the gut and lung, and may have evolved to prevent destruction of host tissues caused by chronic inflammatory processes.

5. ROLE OF TLRs IN PROTECTION FROM INFECTIONS

5.1. Induction of Innate Immune Responses

The primary function of pattern recognition receptors is to provide an immediate protection from invading pathogens [3,47]. This is achieved through activation of a plethora of defense mechanisms, including inflammatory cytokines, complement, phagocytosis, and killing via anti-microbial proteins and peptides [48]. In addition, many members of the TLR family are potent inducers of type I interferons and the consequent suite of interferon-inducible genes in response to viral DNA and RNA [9-11,13,49]. Below we will discuss data indicating an essential role for TLRs in control of adaptive immunity.

5.2. Induction of Adaptive Immune Responses: Role of Co-stimulation and Cytokines

As described earlier, TLR-induced DC maturation during infection is essential for naive T cell activation. This has been demonstrated in mice lacking MyD88, and adapter protein downstream of all TKRs. optimal activation of naive T cells requires signals through both TCR and CD28 molecules [33,34]. These requirements are met only by a fully mature DC exposed to TLR ligands either in the secondary lymphoid organ or the peripheral tissues. Engagement of TLRs on DCs by TLR ligands (presence of infection) leads to up-regulation of both MHC and co-stimulatory molecules [35]. Migration of this professional APC to the draining lymph node and subsequent interaction with naive T cells ensures that immune responses are mounted only to pathogen-derived antigens. As discussed earlier interaction of immature DC and naive T cells leads to tolerance or induction of suppressor T cells [50]. The scenario of exposure to pathogens is mimicked in most experimental conditions by use of TLR ligands as adjuvants. The hypothesis that induction of DC maturation by microbial stimuli is essential for naive T cell activation was experimentally confirmed using MyD-88 deficient mice. MyD88 is an adapter protein that functions downstream of all TLRs and deletion of this adapter abolish signal transduction downstream of most TLRs

As described earlier, TLR-induced DC maturation during infection is essential for naive T cell activation. This has been demonstrated in mice lacking MyD88, and adapter protein downstream of all TLRs. [3]. Unlike wild-type mice, MyD88-deficient mice failed to induce T cell activation and interferon-gamma production when immunized with antigens emulsified in complete Freund's adjuvant (CFA), which is essentially heat killed Mycobacterium and contains several TLR ligands [4]. Further analysis showed that mycobacterial extracts fail to induce maturation of DCs derived from MyD88-deficient mice [4], underscoring the importance of TLR-induced DC maturation for T cell priming.

In addition to controlling the co-stimulatory pathway, DCs seem to contribute to T cell activation by overcoming suppression mediated by Tr cells. This was shown in experiments using LPS-treated MyD88-deficient DCs, which can still undergo maturation, but cannot produce inflammatory cytokines [51]. Surprisingly, when these DCs were used as APCs in T cell priming assays, they failed to induce effective priming. Additional experiments revealed that the cytokine IL-6, produced by DCs upon TLR ligation, is essential to overcome the function of Tr suppressor T cells, by making responder T cells refractory to

suppression [5]. Importantly, TLR-induced cytokines, in the absence of co-stimulatory molecule induction, seem to be insufficient by themselves to induce T cell activation (unpublished observations, CP and RM), thereby ensuring that bystander T cells are not activated non-specifically by DCs during infection.

6. TLRs AND AUTOIMMUNITY

6.1. Control of B Cell Activation

In addition to their clonally-expressed B cell receptors, B lymphocytes express most known TLRs. TLR ligation on B cells induces polyclonal proliferation and expression of co-stimulatory molecules, and also promotes plasma cell differentiation, but the significance of these events (with regard to inducibility by TLR signaling) is not yet clear. However, that TLR activation on B cells can contribute to pathology in at least some contexts has been shown. In this study, linked recognition of ligands by BCR and TLR9 has been shown to initiate and exacerbate at least one autoimmune disease. While TLR9 evolved to recognize unmethylated CpG motifs found in bacterial genomes and DNA viruses, TLR9 can also be activated by self DNA under certain special circumstances. One such case occurs when apoptotic cells are inefficiently removed, leading to secondary necrosis and release of high quantities of chromatin-bound DNA fragments. B cells specific to these fragments efficient take them up via BCR-mediated endocytosis and deliver them to intracellular sites containing TLR9. Signaling through TLR9 in such a scenario can lead to activation of B cells and differentiation into plasma cells that secrete antibodies specific to chromatin or chromatin-associated antigens and lead to immune pathology. A mouse model has been described recently that demonstrates that uptake of such complexes by rheumatoid factor positive (RF+) B cells leads to highly efficient activation of such B cells [52]. This activation was shown to be dependent on TLR9-mediated recognition of DNA present in the mammalian chromatin. Dual signaling through BCR and TLR9 therefore led to enhanced production of RF antibodies and associated immune pathology. This example illustrates the potential danger inherent in expression of both BCRs and TLRs on B cells, and may explain why continuous signaling through the BCR, which may indicate engagement of self antigens, leads to inhibition of TLR9-triggered plasma cell differentiation [53].

6.2. Role of TLR-Induced Cytokines

As discussed earlier, cytokines secreted by DCs and macrophages in response to TLR ligands contribute significantly to induction of T cell responses. However, many of these cytokines, including IL-6, TNF, and interferons, can in some contexts contribute to autoimmunity [54]. IL-6 has been implicated in autoimmune diseases such as pristine induced lupus, collagen induced arthritis and experimental autoimmune encephalomyelitis (EAE) [55-58]. The fact that IL-6-deficient animals are resistant to several auto-immune diseases suggest that this may be, at least in part, due to the inability of its effector T cells to overcome suppression mediated by Tr cells. During chronic infections, persistence of pathogens and their products can lead to enhanced production of several pro-inflammatory

cytokines including IL-6, leading to a situation conducive to activation of self-reactive T cells. Indeed, several studies have shown a link between chronic infections and autoimmune diseases [59].

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3

ACTIVATION OF INSECT AND VERTEBRATE TOLL SIGNALING: FROM ENDOGENOUS CYTOKINE LIGAND TO DIRECT RECOGNITION OF PATHOGEN PATTERNS

Nicholas J. Gay, Alexander N. R. Weber and Monique Gangloff

1. INTRODUCTION

The Toll receptor families from insects and vertebrates have structural and evolutionary relationships and it was considered likely that they fulfilled similar functions in their respective organisms. Over the last two years, however, it has become clear that the way in which these receptors recognise pathogens in *Drosophila* and mammals is quite distinct. The completion of the genome sequences of *Drosophila*, human and mouse has revealed the presence of nine Toll receptors in the insect and probably ten or 11 in mammals[1,2]. As shown in Fig. 1, with the exception of dToll9, the *Drosophila* Tolls are more closely related to each other than they are to the human Toll-like receptors (hTlrs). All Tolls are type 1 transmembrane receptors: they have blocks of a widespread structural motif, the leucine rich repeat in their ectodomains[3], a single transmembrane spanning region and a cytoplasmic signalling module, the Toll/IL1R identity region (TIR). The leucine rich repeat is found in many intracellular and extracellular proteins and has structural features that have the potential to evolve a wide range of protein binding specificities.s

The first *Drosophila* Toll receptor was identified genetically in screens for developmental mutants and it is a critical component of the dorso-ventral patterning system in the pre-cellular embryo[4]. Activation of *Drosophila* Toll causes a ventral to dorsal gradient of the NF-κB related transcription factor dorsal in the nuclei of syncitial embryos. This gradient instructs the differentiation of the dorso-ventral axis in embryogenesis and regulates the expression of approximately 350 of the 13500 *Drosophila* genes[5]. These genes are activated by different threshold levels of dorsal and there may be up to seven distinct dorsal enhancers that respond to different concentrations of the transcription factor. In innate immune responses two other dorsal-related transcription factors, Dif and Relish, are involved in gene regulation and transcriptomic analysis has identified about 550 genes with significantly altered expression levels[6].

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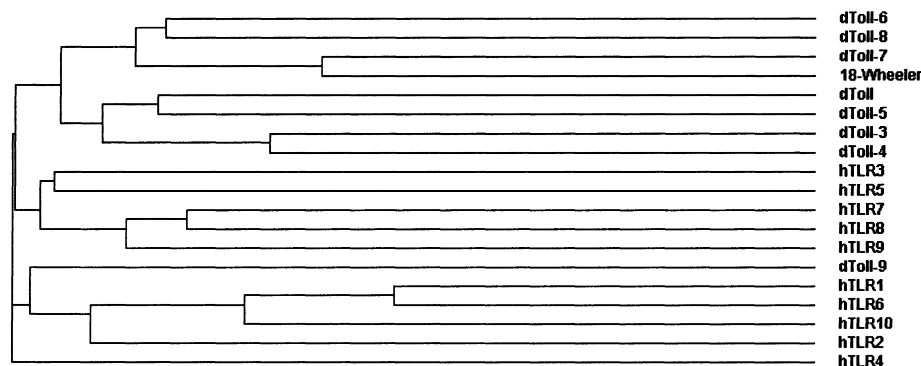

Figure 1. Phylogenetic relationships of ectodomains from *Drosophila* and human Toll receptors. Figure was generated by CLUSTALW[9].

In vertebrates, Tlrs do not function in development but do signal to NF-κB when exposed to pathogen associated molecular patterns[7]. The intracellular pathway leading to activation of NF-κB is significantly similar to that of the dorso-ventral patterning system and innate immune response of *Drosophila*. The adaptor MyD88 (dMyD88), the protein kinase IRAK4 (pelle) and IκB (cactus) are three examples of this conservation[8].

2. DROSOPHILA TOLL IS ACTIVATED BY A DIMERIC CYTOKINE LIGAND

Genetic studies implicated the product of the gene *Spätzle* as the activating ligand of the *Drosophila* Toll pathway in dorso-ventral and in innate immune responses to fungi and Gram-positive bacteria[10,11]. Spatial cues in the embryonic perivitelline membrane and pathogen patterns such as peptidoglycan from Gram-positive bacteria cause activation of a proteolytic cascade and the terminal member of this cascade cleaves an inactive pro-protein of Spätzle to produce an active C-terminal fragment, C-106. C-106 is a dimer and has a cystine knot fold similar to other signalling molecules such as nerve growth factor[12,13].

Recently, we were able to express and purify the pro-protein form of Spätzle and generate C-106, by partial proteolysis[14]. To address the binding characteristics of Spätzle and Toll, we used a derivative of the macrophage-like *Drosophila* S2 cell line carrying a reporter construct under the regulation of the *drosomycin* promoter. We first observed that addition of C-106 to these cells strongly activated the *drosomycin* promoter in a dose-dependent manner, with an EC_{50} of 0.5 nM, whereas the pro-protein was unable to establish signaling even at high concentrations (Figure 2a). Cell activation by C-106 was competed out by the addition to the assay of the ectodomains of either Toll full length or $Toll^{5B}$ (a truncated form[15]), which points to a direct interaction between C-106 and Toll. By contrast, the Spätzle pro-protein did not act as an antagonist of Toll signaling, suggesting that it does not bind to Toll (Figure 2b). Signaling by C-106 required Toll, as dsRNA knockdown of Toll suppressed cell activation by C-106 (Figure 2c). Furthermore,

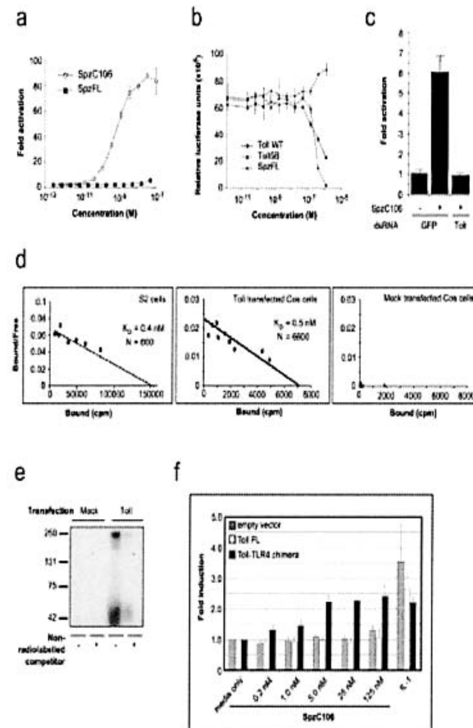

Figure 2. The processed form of Spätzle, but not the pro-protein, binds to and activates cells expressing the Toll receptor. (**a**) The cleaved form of Spätzle, but not the pro-protein, activates the *drosomycin* promoter in a dose-dependent manner in S2 cells. S2 cells expressing a *drosomycin-luciferase* reporter construct were cultured for 16 hours in tissue-culture medium supplemented with increasing concentrations of either the processed form (C-106) or the pro-protein form (FL) of Spätzle. (**b**) Recombinant soluble Toll ectodomains (wild-type (WT) or truncated (5B)), but not the Spätzle pro-protein, compete for *drosomycin* promoter activation. S2 cells expressing a *drosomycin-luciferase* reporter construct were cultured with 4 nM SpzC106 and increasing concentrations of Toll ectodomains or unprocessed Spätzle pro-protein. (**c**) Activation of the *drosomycin* promoter by SpzC106 in S2 cells is Toll dependent. S2 cells were co-transfected with a *drosomycin-luciferase* reporter construct and double-stranded RNA corresponding to GFP or Toll. 48 hours after transfection, the cells were stimulated with 1 nM SpzC106 (+) or left untreated (-) for 16 hours. (**d**) Scatchard analysis of ^{125}I-labelled SpzC106 binding to transfected Cos-7 cells and S2 cells. Binding experiments were performed by incubating Toll (middle) or mock (lower) transfected Cos-7 cells or S2 cells (upper panel) in the presence of increasing concentrations of radiolabelled SpzC106. (**e**) Cos-7 cells transfected with a Toll expression vector bind radiolabelled SpzC106. 3 x 10^6 cos-7 cells were transfected with an empty vector (mock) or a vector expressing Toll. 48 hours later, they were incubated with 5 nM radiolabelled SpzC106 alone (-) or in the presence of a 100-fold excess of cold competitor (+) for 2 hours followed by 45 minutes in the presence of 1mM EGS and BS3 cross-linking agents. (**f**) HEK293 cells were transfected with empty pCDNA3.1 vector or plasmids expressing full-length Toll or a Toll-TLR4 chimera (Toll extracellular domain and transmembrane region fused to the hTLR4 intracellular domain), respectively, as well as a luciferase reporter gene under the control of the NF-κB promoter (Promega). After transfection, cells were seeded and stimulated with media, Spätzle C106 dilutions (as indicated) or human IL-1 at 10 ng/ml 36 hours post-transfection.

inhibition of the other receptors of the Toll family in *Drosophila*, including Toll-5 and Toll-9, which have been shown to induce *drosomycin* in tissue culture, did not affect signaling by C-106. To measure the binding affinity of Spätzle to S2 cells we labeled C-

106 with ^{125}I. Scatchard analysis indicated that C-106 binds to S2 cells with a K_D of 0.4 nM and approximately 600 binding sites per cell (Figure 2d). These data show that signaling by C-106 involves binding to a specific membrane receptor. To confirm that this interaction is mediated by Toll, we examined binding of radiolabelled C-106 to Cos-7 cells transfected with a Toll expression vector. Cos-7 cells expressing Toll bound C-106 with an affinity similar to that of S2 cells, whereas no specific binding was detected on mock-transfected cells (Figure 2d). In addition, a large protein complex was detected after cross-linking iodinated C-106 to Cos-7 cells transfected with a Toll expression vector only (Figure 2e). To test whether binding of C-106 to Toll is sufficient to induce signaling, we constructed a mammalian expression vector expressing a chimeric receptor composed of the Toll ectodomain and transmembrane domain fused to the cytoplasmic domain of hTLR4 (see Methods). This plasmid was then tranfected into HEK293 cells and activation of NF-κB was assayed using a luciferase reporter gene. As shown in Figure 2f, C-106 induces activation of NF-κB in a dose dependent manner with maximum induction achieved at a C-106 concentration approximately ten times the K_d value determined for Spätzle binding to S2 cells. By contrast C-106 was unable to induce signaling in cells transfected with the full-length *Drosophila* Toll receptor or with empty vector. We therefore conclude that binding of C-106 to Toll is sufficient to directly establish signaling.

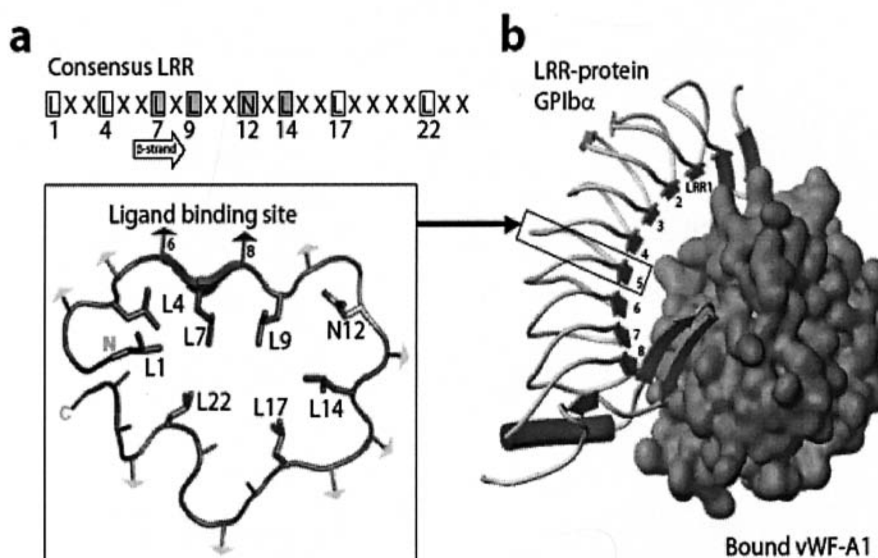

Figure 3. The leucine rich repeat (LRR) (a) the basic structural unit of the LRR. The conserved hydrophobic residues form the core and variable sidechains (mainly 6 and 8) make ligand specific interactions; (b) The LRRs assemble into an extended superhelix illustrated by the crystal structure of glycoprotein Ib α (GPIbα). The bound ligand, domain A1 of von Willebrand factor (vWF-A1) interacts with the concave β-sheet.

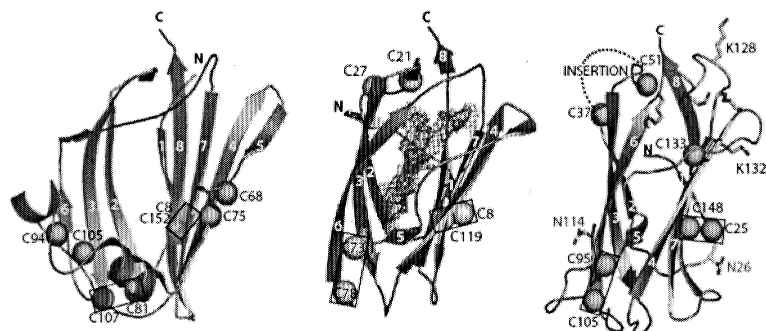

Figure 4. Structures of GM-2 activator protein, mite dust allergen and MD-2 (model) (from left to right). The positions of cysteine residues in the sequences and basic residues in the flexible loop at the entrance to the binding pocket are shown. Conserved cystine residues involved in disulphide bridges are boxed.

3. TOLL-LIKE RECEPTOR 4, MD-2 AND LPS SIGNALLING

The domain swap experiment described above and other experimental evidence[14,16] implies that receptor dimerization is required for signalling through the Tlr4 pathway. However it appears that although the two pathways use evolutionarily related signalling receptors, a different, more direct detection mechanism has evolved in mammals[17]. Consistent with this view, the human and mouse genome sequences do not appear to encode Spätzle related proteins whereas that of *Drosophila* contains at least five homologues.[18]

The ability of Toll ectodomains to evolve such diverse specificities is probably due to the structural properties of the leucine rich repeat[3]. Each unit is about 24 amino acids long and has a pattern of conserved hydrophobic residues, often leucines. The repeats have a short β-sheet, a turn and a region of variable secondary structure with the conserved residues forming a hydrophobic core (Figure 3a). Arrays of repeats form an extended, right-handed superhelix with a characteristic curvature (Figure 3b). The limited structural data available suggests that variable residues on the concave surface make ligand-specific interactions[19,20]. Taken together, these findings raise an important question: how does association of LPS with MD-2 induce a conformational rearrangement that results in dimerization or oligomerisation of the Tlr4 receptor and signal transduction?

4. MD-2 IS A MEMBER OF THE LIPID RECOGNITION FAMILY

A step towards answering this question has come from the discovery that MD-2 belongs to a small family of proteins, the MD-2 related lipid binding proteins (ML) all of which bind to lipids[21]. Members of this family are characterized by single domain architecture of about 150 residues preceeded by an amino-terminal signal peptide for secretion. Three-dimensional structures of four members of the ML family, Der f 2[22], Der p 2[23-25], Niemann-Pick disease type C2 protein (NPC2)[26] and ganglioside GM2 activator

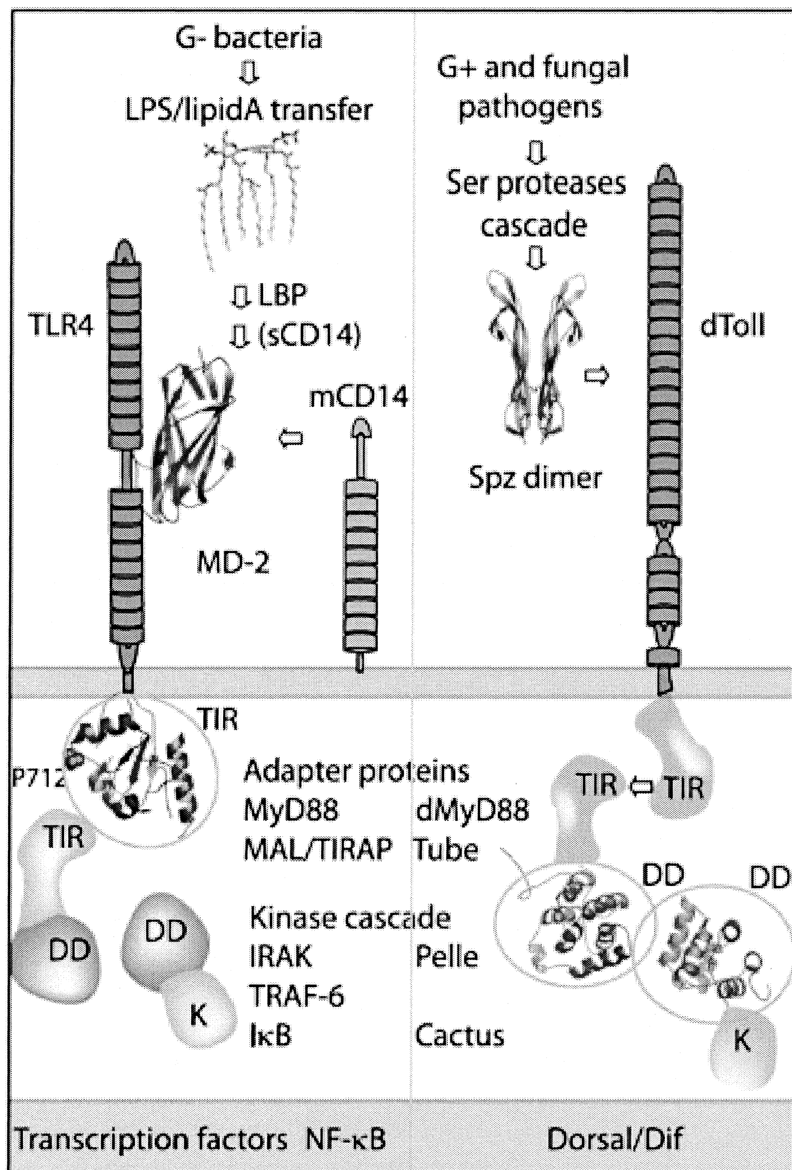

Figure 5. Comparison of receptor activation by *Drosophila* Toll and Tlr4.

precursor GM2-AP[27,28] (Figure 4b) have been solved and these structures allow us to build a homology model of MD-2 (Figure 4c). The ML family form a β-sandwich of at least 7 strands grouped in 2 β-sheets, displaying a greek key motif. A mechanism for ligand binding can be extrapolated from the information provided by the three-dimensional

structures of these proteins. Indeed a putative lipid-binding cavity is found in the crystal structure of the mite allergen protein Der p 2, the closest structural homolog of MD-2, according to the homology recognition software Fugue (Z-score of 5.7)[29]. The recent crystal structure of GM2-AP with bound lipids shows that the acyl chains are buried in the hydrophobic environment of the protein core, packed against the β-sheets, with a solvent-exposed polar head group[27]. Together these results suggest that one molecule of MD-2 can bind LPS by burying at least one of its diacyl moieties within its core and that this binding event is stabilised by ionic interactions between the conserved basic amino acids at the mouth of the cavity (Figure 4c) and the phosphate headgroups. The active lipid A component of LPS consists of three diacyl chains linked to a glucosamine phosphate headgroup, and the proposed mechanism of binding of LPS to MD-2 suggests two possible ways in which Tlr4 receptor dimerization might be achieved. Firstly, binding of LPS to the Tlr4/MD-2 heterodimer could induce a conformational change that allows a second heterodimer to be recruited. This process resembles that observed with the related cytokine receptor interleukin 1 receptor (IL-1R). Here binding of IL-1 and IL-1R to form a heterodimer enables the recruitment of an accessory transmembrane protein which by itself is unable to bind the cytokine[30]. Alternatively, different diacyl groups of lipid A might bind to separate molecules of MD-2 thereby bringing two Tlr4 receptor molecules into proximity in the membrane. A summary of Toll receptor activation by Spätzle on the one hand and LPS on the other is given in Figure 5.

5. DOES NF-κB REGULATE HIERARCHIES OF TARGET GENES?

Tlr4 is reported to support signalling by at least 15 distinct stimuli in addition to LPS, ranging from viral proteins to small molecules [31]. For example, a soluble secreted protein from the filarial nematode protein ES62 generates a low-level cytokine response and this response is absent in Tlr4 and MyD88 knockout mice (H. Goodridge, personal communication)[32]. This observation suggests that complexity in the innate immune response is generated by the induction of different threshold levels of NF-κB, in response to a large number of stimuli, acting not only through Tlr4 but other Tlrs and IL-1 family receptors as well. This is analogous to the situation described above in the *Drosophila* embryo during dorso-ventral pattern formation. Here a diffused gradient of the ligand Spätzle establishes a nuclear gradient of dorsal and different threshold levels of act on subsets of genes controlled by enhancers with variable affinities for the dorsal. The fate of cells located at different positions on the dorso-ventral axis is then determined by the combination of dorsal regulated genes that are expressed. Thus, it seems possible that there may be many distinct levels of NF-κB activation that depend on the initial stimulus detected by the Tlrs and that these initial events powerfully influence the subsequent development of the immune response.

6. CONCLUSIONS

In this article we have compared and contrasted the insect and vertebrate Toll receptor signalling pathways. While there are significant similarities in the intracellular arms of the pathways, the mechanism by which pathogen patterns are recognized appears to be radically different. The challenges for the future are to understand the activation

mechanisms at a structural level and to characterize the responses in cells of the immune system to different threshold levels of NF-κB.

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4

GENETIC ANALYSIS OF INNATE IMMUNITY: IDENTIFICATION AND FUNCTION OF THE TIR ADAPTER PROTEINS

Bruce Beutler, Kasper Hoebe, Philippe Georgel,
Koichi Tabeta, and Xin Du

1. SUMMARY

The innate immune system senses pathogens largely through signals initiated by a collection of phylogenetically related proteins known as "Toll-like receptors" (TLRs), of which ten representatives are encoded in the human genome. The sensing role of the TLRs first came to light when one member of this family, TLR4, was shown to serve the detection of endotoxin (lipopolysaccharide; LPS) in mice. This discovery was based upon positional cloning of a spontaneous mutation affecting a locus known as *Lps*. The recognition specificities of other TLRs have since been established by reverse genetic methods. The understanding of the biochemical circuitry that maintains the innate capacity for immune recognition and response has loomed as the next hurdle in the field. A total of five adapter proteins with cytoplasmic domain homology to the TLRs are known to exist in mammals. These proteins are not entirely promiscuous in their interaction with TLRs, but rather, show preferential association with individual family members, giving a particular character to the signals that distinct micro-organisms initiate. The adaptive immune response is dependent upon upregulation of costimulatory molecules (UCM) such as CD80 and CD86. Very recently, it has been shown that this upregulation is dependent upon an adapter encoded by a locus known as *Lps2*, known as Trif or Ticam-1, and upon type I interferon receptor signaling. LPS and dsRNA both signal via Trif, but dsRNA has an accessory pathway for UCM, that is independent of both Trif/Ticam-1 and the known dsRNA receptor, TLR3. Other key innate immunity genes have also been disclosed by germline mutagenesis, and are discussed in the present review.

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2. BACKGROUND

Although the receptors that are responsible for innate immune recognition were difficult to find, there is nothing fundamentally unusual about them. They detect neither "patterns," nor "danger." Rather, they detect molecules. The molecules that are detected have known structures, and represent components of microbes that cannot easily be eliminated by mutation. Direct interaction between the target molecules and the innate immune sensors occurs in order to elicit a signal.

The first inroad into the identification of innate immune receptors was made in 1965, with the fortuitous observation that mice of the C3H/HeJ strain were highly resistant to the lethal effect of LPS[1]. This observation was extended through the following decades, wherein it was realized that all cellular responses to LPS, including the adjuvant effect of LPS on adaptive immune responses[2-6], were impaired by a single mutation affecting a locus that was called *Lps*[7]. Further, the lethal effect of LPS was seen to depend upon mononuclear phagocytic cells of the host[8]. Moreover, a failure to sense LPS was associated with markedly enhanced susceptibility to infection by Gram-negative bacteria [9-12].

The *Lps* locus was resolved by positional cloning in 1998[13,14]. At that time, it became clear that all LPS sensing was dependent upon a single receptor protein known as TLR4. TLR4 was one of five paralogous proteins known to exist at that time, each with homology to the *Drosophila* Toll protein—a molecule known to fulfill a dual role in development and in immunity. Flies with mutations in Toll had been shown to be hypersusceptible to infection by fungal pathothens[15], and in the course of time, were also shown to be hypersusceptible to infection by Gram-positive bacteria[16]. The mammalian TLRs, first identified on the basis of homology searches as early as 1994, were initially thought to have developmental functions[17,18]. However, the identity of *Lps* and *Tlr4* proved that like Toll, at least one of the mammalian TLRs had a highly specific immune sensing function.

The discovery that *Lps* encoded TLR4 was a dramatic advance, for it suggested that each mammalian TLR might recognize a distinct microbial molecule, or at most, a small collection of such molecules. In the course of time, this hypothesis was proven correct. TLR2 (by itself or in conjunction with TLRs 1 or 6) serves as a sensor of bacterial lipopeptides[19], and glycans[20]; TLR3 recognizes double-stranded RNA[21]; TLR5 recognizes flagellin[22]; TLR9 recognizes unmethylated DNA[23]. The TLRs, collectively, respond to molecules produced by most microbes, and alert the host to the presence of infection. In some instances, specificity remains elusive. The natural ligand of TLR7 is still unknown, though this TLR senses small nucleotide-based drugs (imidazoquinolones)[24]. Human TLR10, which has no mouse counterpart but is closely related in structure to TLRs 1 and 6, also remains in search of a ligand. And three mouse TLRs (11, 12, and 13[25]) have no known ligands as yet.

3. THE BIOCHEMISTRY OF SIGNAL TRANSDUCTION: THE FUNCTIONS OF Myd88 AND MAL/Tirap

The mammalian TLRs comprise a major branch of a protein superfamily (Figure 1). Each TLR is a single-spanning type I transmembrane protein characterized by numerous leucine-rich repeat motifs in the ectodomain, and each is endowed with a single "Toll/IL-

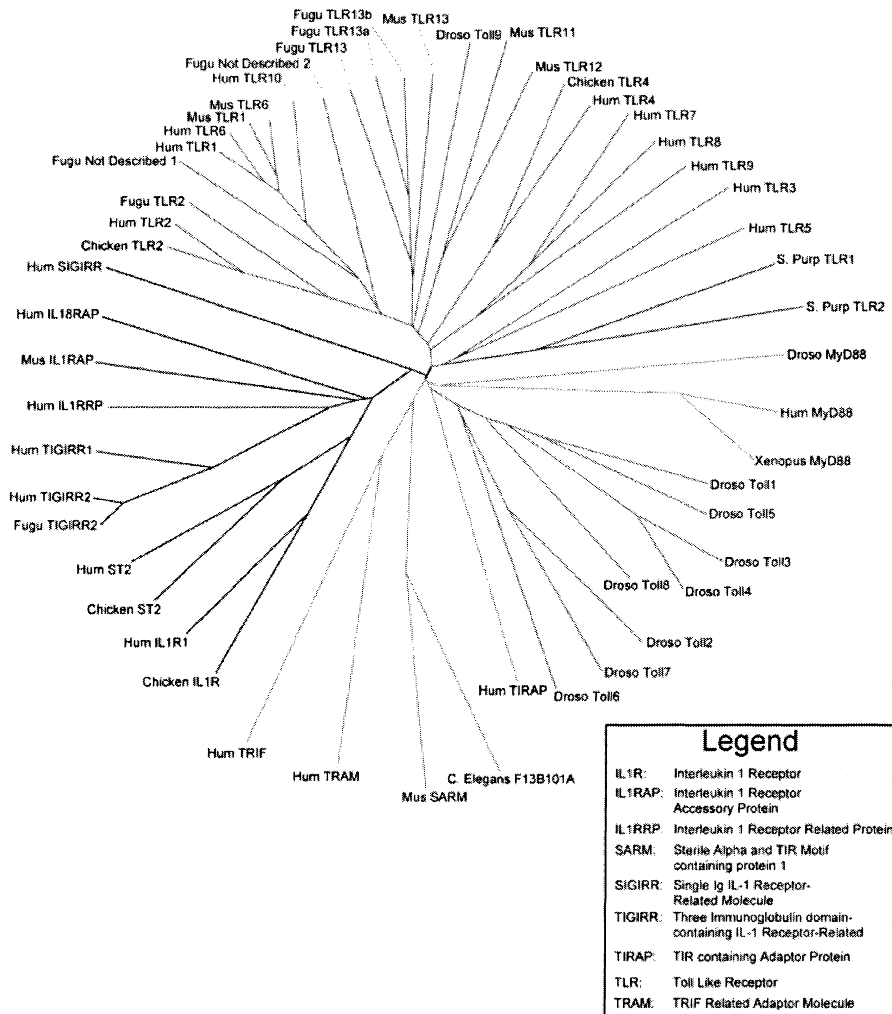


Figure 1. An unrooted tree of animal TIR domains shows the ancestry of the TLRs, IL-1R/IL-18R family members, and adapter proteins that serve them. Tree was generated using ClustalW. Where very sequences from close phylogenetic relatives were available (e.g., humans and mice), only a single sequence is represented in the tree in order to save space. Branch lengths are proportionate to times since divergence.

1R/Resistance" (TIR) motif that comprises the bulk of the cytoplasmic domain. The TIR motif is not represented only in TLRs, however. An ancient protein fold, the TIR motif is usually associated with an innate immune defensive function (even in plants, where it is an essential part of host resistance proteins). In mammals, TIR motifs are also present in proteins of the IL-1R/IL-18R family, which have immunoglobulin repeats in the ectodomain. Moreover, five cytoplasmic adapter proteins (MyD88, MAL, Trif, Tram, and Sarm) are known to have TIR motifs.

MyD88 was first identified as an intermediate in IL-1 receptor signaling. The IL-1 receptor had been identified as a homolog of Toll in 1990[26], and MyD88[27] as a related homolog in 1994[28]. It was logical to assume that heterotypic interaction between IL-1R and MyD88 might be required for effective signaling. The case was proved in with the observation that mice with targeted deletions of the MyD88 gene could not sense IL-1; neither could they sense IL-18[29]. The role of MyD88 in LPS signaling was established subsequently[30]. At present, it appears that all TLRs except TLR3 depend upon MyD88 for signaling, at least in part.

MyD88 displays an N-terminally placed death domain that serves to recruit the interleukin receptor associated kinase (IRAK)-4, a serine kinase, which in turn phosporylates IRAK-1 and IRAK-2. IRAK-1 and IRAK-2 may serve a scaffold function, and help to recruit TRAF-6, another scaffold protein that activates numerous downstream kinases, including members of the MAP kinase superfamily and components of the signalosome complex (IKKα, β, and γ) that is responsible for phosphorylation of IκB, and consequent activation of NF-κB.

In MyD88-deficient mice, it was noted that LPS signal transduction is only partially impaired[30]. There is tardive phosphorylation of MAP kinases, and there is tardive activation of NF-κB. Moreover, some events occur without any impediment at all: for example, the phosphorylation of IRF-3, a transcription factor required for interferon-β gene expression.

The fact that residual LPS signaling activity was observed in MyD88-deficient mice prompted speculation that TLR4 must engage more than one adapter protein in order to signal. Moreover, the fact that TLR3 did not require MyD88 suggested that still other adapters might exist. A second adapter (MAL, the "MyD88 Adapter Like" protein, also known as Tirap), was identified by blast searches of EST and genomic databases[31,32]. It was proposed that MAL/Tirap was responsible for MyD88-independent signaling[33]. However, this assertion proved to be incorrect, based as it was upon transfection rather than germline mutations. In 2002, the phenotype of the MAL/Tirap knockout was shown to be identical the that of the MyD88 knockout, where TLRs 2 and 4 were concerned[34]. For TLR2, all signaling potential was lost; for TLR4, only moderate impairment of signaling was observed.

4. FORWARD GENETIC ANALYSIS IDENTIFIES A THIRD ADAPTER REQUIRED FOR TLR3 AND TLR4 SIGNALING: THE *Lps2* LOCUS AND Trif/Ticam-1

The success of phenotype-driven gene discovery in the identification of the mammalian TLRs as primary sensors of infection led to the use of a germline mutagen, N-ethyl-N-nitrosourea (ENU) as a tool for the production of still other innate immunodeficiency phenotypes. Hoebe and colleagues produced mice with a defect in TLR3 and TLR4 signaling caused by a single point mutation[35], mapped to mouse chromosome 17. On 1567 meioses, this mutation (in the *Lps2* locus) was confined to a 216 kb interval and positionally cloned[36]. It was found to reside in the distal coding region of a third adapter protein, recently identified by homology searching and by use of the two-hybrid system, and respectively, called Trif[37], or Ticam-1[33].

While the Trif/Ticam-1 protein was believed to be capable of inducing interferon-β gene expression through interaction with IRF-3, there was disagreement as to which

TLRs it served. As has commonly been the case in the TLR field, the primacy of germline mutations over *in vitro* methods was demonstrated, as it was revealed that TLRs 3 and 4 (rather than most TLRs or TLR3 alone) depended upon Trif/Ticam-1 for effective signal transduction. Hoebe and colleagues showed that the *Lps2* mutation was required for effective antiviral responses, and for much of LPS toxicity *in vivo* (19129}. They also demonstrated that the protein was an integral component of the TLR3/4 → IRF-3 → interferon-β signaling axis[36]. Trif/Ticam-1 was also shown to mediate all of MyD88-independent signaling in that mice with mutations in both the Trif/Ticam-1 gene and the MyD88 gene showed no residual LPS responses at all[36].

The phenotype of homozygosity for the codominant *Lps2* allele was subsequently shown to be similar or identical to that of homozygosity for a Trif/Ticam-1 knockout allele [38]. However, an additional observation was made using *Lps2* homozygous mice that pointed to the existence of still another adapter, required for LPS signaling.

5. Trif/Ticam-1 INDEPENDENT CELLS: THE ROLE OF Tram

FACS analysis of TNF production in cells from wild type mice showed that virtually all peritoneal macrophages respond to LPS by producing TNF protein. On the other hand, no cells from MyD88-deficient mice produce TNF protein in any great quantity. Remarkably, cells from *Lps2* mutant homozygotes are of two types: some produce TNF in response to LPS and others do not[36]. The cells that show residual responsiveness are termed "Trif-independent." Trif independence cannot be attributable to MyD88 signaling, since MyD88 is represented in all cells, and if it were responsible for the "rescue" that is observed, rescue should be uniform (and not bimodal). Hence, the existence of an "adapter X" was posited, and further, it was suggested that adapter X was most likely identical to Tram, the TIR adapter protein that is the closest phylogenetic relative of Trif[36]. Evidence from the knockout of Tram suggests that this hypothesis is correct[39]. Tram functions as a component of the MyD88-dependent pathway downstream from TLR4, and in certain cells, can partly replace the function of Trif. However it does not transduce signals from TLR3.

6. Sarm

The fifth known TIR adapter protein is also endowed with Sterile Alpha Motif (SAM) domains, and at present, its function is entirely unknown. This is the most distant of the TIR adapters, displaced from the other members of the family by a great evolutionary distance. The Sarm TIR motif is most similar to a TIR motif observed in *C. elegans*.

7. WHICH TIR ADAPTERS SERVE THE ADAPTIVE IMMUNE RESPONSE?

The adjuvant effect of microbes has been known for more than eighty years, since Lewis and Loomis demonstrated the phenomenon of "allergic irritability"[40]. Later, it was found that heat-killed microbes were endowed with adjuvant activity: a fact that suggested that individual microbial molecules must function as adjuvants. By 1955, LPS was shown to be an adjuvant for adaptive immune responses[41], and as already noted, the

Lps locus was shown in 1975 to be required for this biological endpoint of LPS action[3]. Hence adjuvanticity depends upon TLR4[14], and a biochemical pathway for this effect would seem definable.

Adjuvanticity is dependent in large part upon the upregulation of costimulatory proteins (e.g., CD80, CD86, and CD40) that engage receptors on T cells and coordinate the mitogenic response to a specific antigen. LPS upregulates these costimulatory proteins on antigen presenting cells, and does so by engagement of TLR4. However, the initial suggestion that TLR-induced NF-κB activation was responsible for upregulation[42] proved to be incorrect. In MyD88-deficient cells, upregulation proceeds unimpeded[43,44]. On the contrary, in Trif-mutant cells, despite persistent activation of NF-κB, upregulation by of costimulatory proteins by LPS is abolished[45].

Recently, Hoebe et al. have demonstrated that LPS-induced upregulation of costimulatory proteins proceeds directly through the TLR4 → Trif → TBK1 → IRF-3 → IFNb axis, and depends upon activation of the type I interferon receptor[45]. On the other hand, upregulation of costimulatory proteins may be achieved through two alternative pathways when the inducer is dsRNA. One pathway is dependent upon TLR3, Trif and its downstream signaling partners. The other pathway is obscure, but is TLR3- and Trif-independent (Figure 2). A quantitative trait locus on chromosome 7 (designated *dsRNA1*) defines the alternative pathway, and is presently being mapped.

Jiang and colleagues[46] have shown that TLR3 signaling (now known to occur via Trif) entails activation of Traf-6, which in turn leads to IκB degradation, bypassing the MyD88-dependent activation pathway. However, MyD88-dependent signaling is not required for the upregulation of costimulatory proteins.

The second MyD88-independent adapter, Tram, appears to cooperate with Trif in all of Trif's LPS responses, including the upregulation of costimulatory proteins. But plays no part in the upregulation of costimulatory proteins initiated at the level of TLR3[39]. A plausible model of TIR adapter function would hold that all of the adapters function as homodimers or heterodimers, much as all of the TIR-domain receptors are believed to function (Figure 3a and 3b).

8. ADDITIONAL MUTATIONS AND WHAT THEY IMPLY

Recently, the ENU-induced phenotype *CpG1* was positionally cloned in our laboratory, and proved to be caused by a point mutation in the TLR9 ectodomain[25]. The molecular domain affected by the mutation is shared with TLRs 7 and 8, but not other TLRs. This mutation was identified in two phenotypic screens. First, macrophages from *CpG1* homozygotes do not respond to unmethylated DNA oligonucleotides bearing CpG motifs. Second, mice homozygous for the *CpG1* mutation are strikingly susceptible to infection by mouse cytomegalovirus (MCMV). So, too, are mice lacking MyD88. As mentioned already, $Trif^{Lps2}$ homozygotes are susceptible to MCMV. To an almost identical degree, so are mice lacking TLR3. In all four kinds of homozygous mutant, both type I interferon production and NK cell activation are defective *in vivo*. This suggests that the NK cell depends upon type I interferon stimulation as well as activation via the m157 → Ly49H pathway[47,48]. Both the TLR9 → MyD88 axis and the TLR3 → Trif axis are required for antiviral defense: the former more than the latter[25]. Moreover, it appears that defense against both viral and bacterial pathogens depends upon TLR sensing, which occurs via shared adapter proteins.

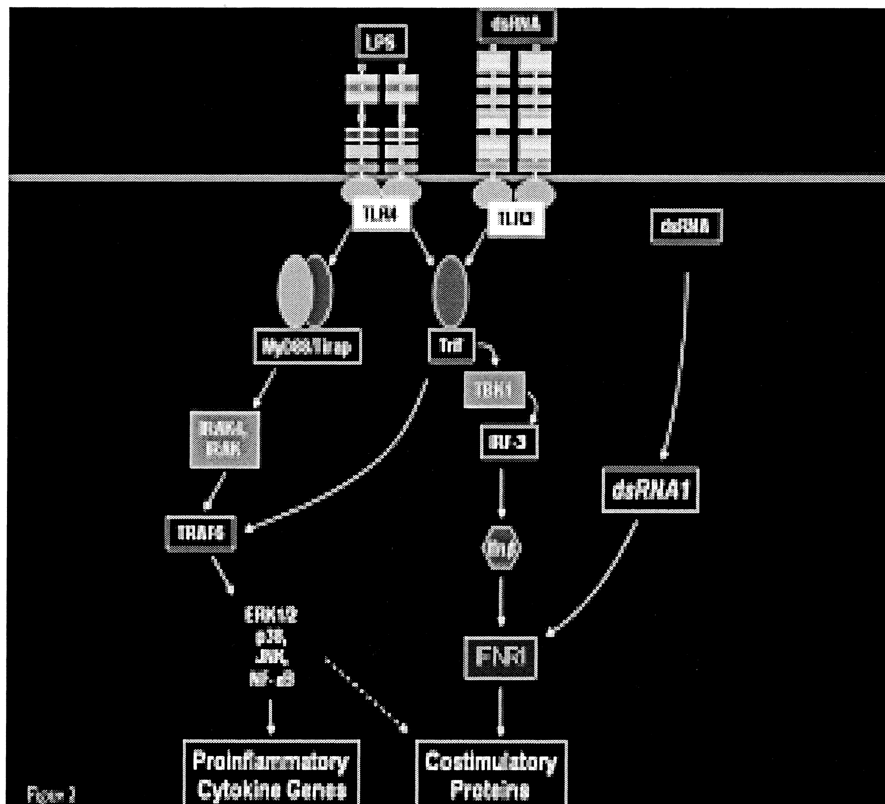
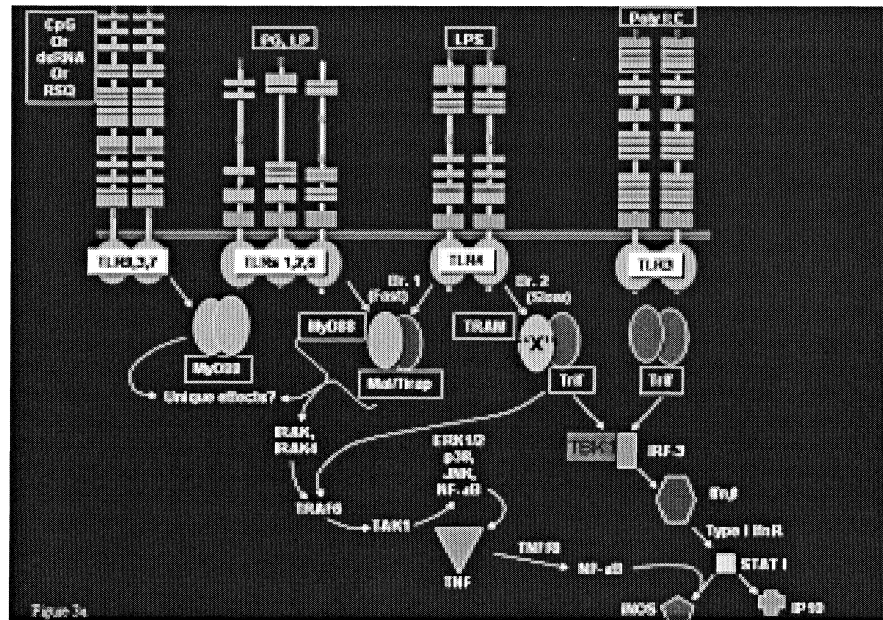


Figure 2. Two pathways for detection of dsRNA. The TLR3 6 Trif pathway offers one mode of detection. However, even in mice that lack TLR3 or Trif, dsRNA sensing and transduction of signals that lead to upregulation of costimulatory molecules can still occur. The alternative sensing pathway is dependent upon a locus called dsRNA1, which has been mapped to mouse chromosome 7.

PanR1, a dominant phenotype in which TNF production in response to all microbial inducers is markedly diminished, has been traced to a mutation affecting a surface residue of the TNF trimer (Hoebe, et al., unpublished data).

Oblivious, a phenotype in which mice show marked susceptibility to *Staphylococcal* infections and fail to adequately sense microbial diacylglycerides including MALP-2 and lipoteichoic acid, has also been positionally cloned. The gene in question seemingly encodes a coreceptor for microbial sensing that permits activation of TLR2/6 heteromers (Hoebe, et al., unpublished data). *3D*, a phenotype in which there is defective sensing of dsRNA, resiquimod, and CpG oligonucleotides, has been tightly mapped but not yet identified (Tabeta, et al., unpublished data). Other immune defects (*Achtung* and *Achtung2*, respectively), have been ascribed to *Edar* and *Edaradd* loci. The *Ugly* mutation has been tracked to a gene encoding an enzyme previously known for its role in lipid metabolism, but now recognized to be required for immunity to Gram-positive skin infections (Georgel, et al., unpublished data). Still another mutation (sootie) appears to be a novel allele of the *Lyst* gene, defective in Chediak-Higashi syndrome.



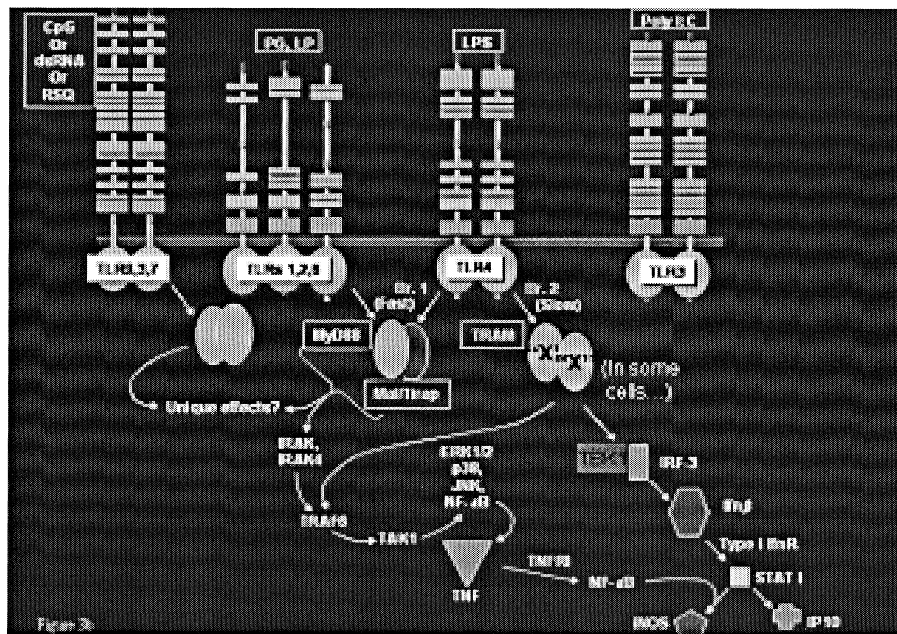


Figure 3. **A**. Key signaling pathways used by the human TLRs. TIR adapters are shown as ovals. A total of four TIR adapters serve the LPS receptor, TLR4. Homodimers of Trif alone serve TLR3. Heterodimers of MyD88 and MAL/Tirap serve TLRs 1, 2, and 6. And MyD88 alone serves TLRs 7, 8, and 9. **B**. The basis of Trif-independent LPS signaling to elicit TNF synthesis. Adapter X (Tram) homodimers are able to substitute for Trif-Tram heteromers in some (but not all) macrophages.

9. CONCLUSIONS: EVALUATION OF THE FORWARD GENETIC APPROACH

Forward genetic analysis may at times break open a field, revealing pathways that were once unknown, or disclosing novel functions of known proteins in a dramatic fashion. This was the case when the function of TLR4, and its transducer Trif, were discovered. In both cases, understanding based on point mutations anticipated understanding based on reverse genetic analysis (i.e., gene knockout). On the other hand, a focused approach, in which every paralog in a family of proteins is deliberately deleted, may yield a comprehensive picture of function that would take many years to develop by random mutagenesis.

A total of 33 ENU-induced immunodeficiency mutations have been identified by screening, and are in various stages of mapping or cloning. On the basis of present experience, it is believed that about half of them will prove to be functionally "new;" that is, they will disclose the function of proteins, rather than representing novel ENU induced alleles of known immune competence genes. In this sense, ENU mutagenesis may be viewed as a cutting-edge method, and one that is very much complementary to gene targeting.

10. ACKNOWLEDGEMENTS

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5

NF-κB, AN EVOLUTIONARILY CONSERVED MEDIATOR OF IMMUNE AND INFLAMMATORY RESPONSES

Changchun Xiao and Sankar Ghosh

NF-κB is a family of structurally related and evolutionarily conserved transcription factors[1]. There are five NF-κB proteins in mammals: RelA/p65, RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100); and three in flies: Dorsal, Dif, and Relish. All NFκB proteins contain a N-terminal 300 amino acid rel homology domain, which is responsible for DNA binding, dimerization, and interaction with the inhibitors of NF-κB, the IκB family proteins. RelA, RelB, c-Rel, Dorsal, and Dif have a transcription activation domain at their C-termini, where p100, p105, and Relish contain ankyrin repeats, signature structures of IκB proteins. NF-κB proteins form hetero- or homodimers and are retained in the cytoplasm by IκBs. There are five IκB proteins in mammals: IκBα, IκBβ, IκBγ, IκBε, and Bcl-3; and one IκB protein in fly: Cactus. IκBα and IκBβ share a tripartite organization: an N-terminal domain that is phosphorylated in response to signals, a central ankyrin repeat domain, and a C-terminal PEST domain that is involved in the basal turnover of the protein. All other IκB proteins have central ankyrin repeat domain, but differ from IκBα and IκBβ at their N- and C-

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terminal domains. IκB proteins form complexes with NF-κB dimers, with ankyrin repeats in direct contact with rel homology domains. This interaction is essential to keep NF-κB dimers in the cytoplasm, thus physically sequestrating them from their transcriptional targets[1,2].

NF-κB proteins are ubiquitously expressed in adult tissues and coordinate the body's response to infection, stress, and injury. The key feature of the NF-κB transcription system is its inducibility. Many inducers lead to its activation and each of them signals to the cells that damage or infection has occurred. Some inducers are actually products of the body's response to damage or infection, such as inflammatory cytokines TNFα and IL-1[1].

A major group of NF-κB inducers are microbial components, the so-called pathogen associated molecular patterns (PAMPs). They are recognized by germline-coded receptors, the pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are a very important group of PRRs[3]. The Toll pathway was originally identified in Drosophila through genetic screens for mutants with embryo patterning deficiency. A key component of the pathway is the Toll receptor, whose engagement leads to the activation of transcription factors of the NF-κB family. Subsequent studies showed that the Toll pathway was also essential for host defense in the adult fly. At least eight Toll-related genes have been reported in Drosophila and most of them are implicated in host defense[4]. The homologous family of Toll-like receptors (TLRs) in mammals contains at least ten members[5], though evidence suggests more are encoded in the mouse and human genomes. Genetic evidence has demonstrated that TLRs recognize conserved pathogen-associated molecular patterns, e.g. lipopolysaccharide, double strand RNA, and CpG DNA, thus playing essential roles in innate immunity. The basic signal transduction pathway induced by the Toll receptors is homologous in Drosphila and mammals. Upon activation, TLRs recruit an adapter protein called MyD88, which subsequently recruits a serine-threonine kinase IRAK. IRAK binds to TRAF6, an adaptor protein of the tumor necrosis factor receptor associated factor (TRAF) family. The assembly of this receptor complex activates IRAK, which undergoes auto-phosphorylation. Phosphorylated IRAK, together with TRAF6, detaches from the receptor complex and transduces the signal downstream, ultimately leading to activation of the IκB kinase (IKK) complex. The IKK complex phosphorylates IκB, causing its ubiquitination and degradation. This process frees NF-κB and allows it to translocate into the nucleus, where it helps coordinate immune responses[3].

Two pathways have been proposed to bridge the signal from TRAF6 to the IKK complex. One pathway is through TAK1 and its associated adaptor proteins TAB1 and TAB2, while the other one goes through Ecsit and MEKK1 or other MAP3K kinases[6-8]. Recent gene targeting results show that TAB2 is not required for NF-κB activation in response to signaling through the TLRs[9]. TAB1 knockout leads to embryonic lethality between embryonic day (E) 15.5-18.5, and mutant embryos exhibit extensive edema and hemorrhage, probably due to deficiency in activating the kinase activity of TAK1 and thus defective TGFβ signaling[10]. However, activation of NF-κB by TLRs in the absence of TAB1 has not been analyzed yet. We have generated TAK1 mutant mice by inserting promoter less βgeo, a chimera of β-galactosidase and the neomycin-resistant gene, into the first intron of the *Tak1* gene. Homozygous mutants are embryonic lethal around E9.5, exhibiting an open neural tube and significantly reduced embryo size (Xiao C.,

Steckel M., and Ghosh S., unpublished result). This early lethality has prevented us as yet from establishing *Tak1-/-* cells to analyze the TLR-induced NFκB activation.

Ecsit is a TRAF6-interacting protein that was discovered in a yeast two-hybrid screen using TRAF6 as bait [6]. The interaction between TRAF6 and Ecsit is conserved in Drosophila. Ecsit also interacts with MEKK1, a MAP3K kinase that can phosphorylate and activate the IKK complex. Expression of a dominant negative mutant of Ecsit specifically blocks signaling from TLRs, but not from the TNF receptor. Therefore Ecsit may transduce the signal from Toll receptors by bridging TRAF6 to the IKK complex [6]. To determine whether the TAK1/TAB1/TAB2 proteins can substitute for Ecsit in Toll signaling, and to further elucidate the physiological function of Ecsit, we deleted the *Ecsit* gene in embryonic stem cells and generated null mutant mice. *Ecsit-/-* mice died around E7.5 and analysis of the mutant embryos revealed a striking similarity to the phenotype of mice lacking *Bmpr1a* [11]. Further characterization showed that Ecsit is an obligatory intermediate in Bmp signaling that functions as a cofactor for Smad1/Smad4-dependent activation of specific Bmp target genes[12],[19]. In addition, ablation of Ecsit using shRNA results in the block of NF-κB activation by LPS, but not TNFα, demonstrating the specific involvement of Ecsit in Toll receptor signaling[19]. Therefore these studies show that Ecsit is an essential component in both Bmp and Toll signaling pathways and is required for early embryogenesis (Figure 1).

Cross talk between Toll pathway and TGFβ/Bmp pathways has been well documented in prior studies. At the syncytial stage of Drosophila embryo development, the expression of *decapentaplegic* (*Dpp*), a close homolog of mammalian *Bmp4* and a morphogen that specifies the dorsal fate of the Drosophila embryo, is under tight regulation by the Toll pathway. Dorsal, activated by Toll, binds directly to multiple sites in the *Dpp* gene and represses its expression in the ventral domain of the embryo [13]. The interaction between the Toll and Dpp pathways establishes the dorsoventral axis of Drosophila embryo [14].

There is also considerable evidence that the Toll and TGFβ pathways antagonize each other in the mammalian immune response. While Toll-like-receptors (TLRs), which recognize signature structures of various pathogens, induce inflammation and subsequently activate the adaptive immune responses, TGFβ generally plays an anti-inflammatory and immunosuppressive role [3,15]. Many important target genes of pro-inflammatory stimuli contain binding sites for both NF-κB and Smads in their promoters, which can be activated or repressed by the Toll pathway and the TGFβ pathway, respectively [16] [17]. An additional means through which antagonism between these pathways is mediated is through inhibitory Smads. Lipopolysaccharide (LPS) up-regulates Smad7 expression through TLR4-NF-κB pathway. Smad7 then suppresses TGFβ by its direct interaction with TGFβ type I receptor and blocking TGFβ–induced Smad phosphorylation [18].

Our current study adds a potential new mechanism for cross-regulating the TLR and Bmp pathways. By functioning as an essential component in both pathways, Ecsit might help determine which pathway functions at any one time. Our earlier studies had indicated that Ecsit is potentially modified during Toll signaling [6]. It is possible that modified Ecsit might not function in the Bmp pathway and would therefore inhibit Bmp/TGFβ signaling. Alternatively, Bmp/TGFβ signaling might suppress TRL signaling by sequestering Ecsit in nuclear Smad complexes. Determining the exact mechanism by which Ecsit mediates cross-talk between these pathways will require detailed biochemical

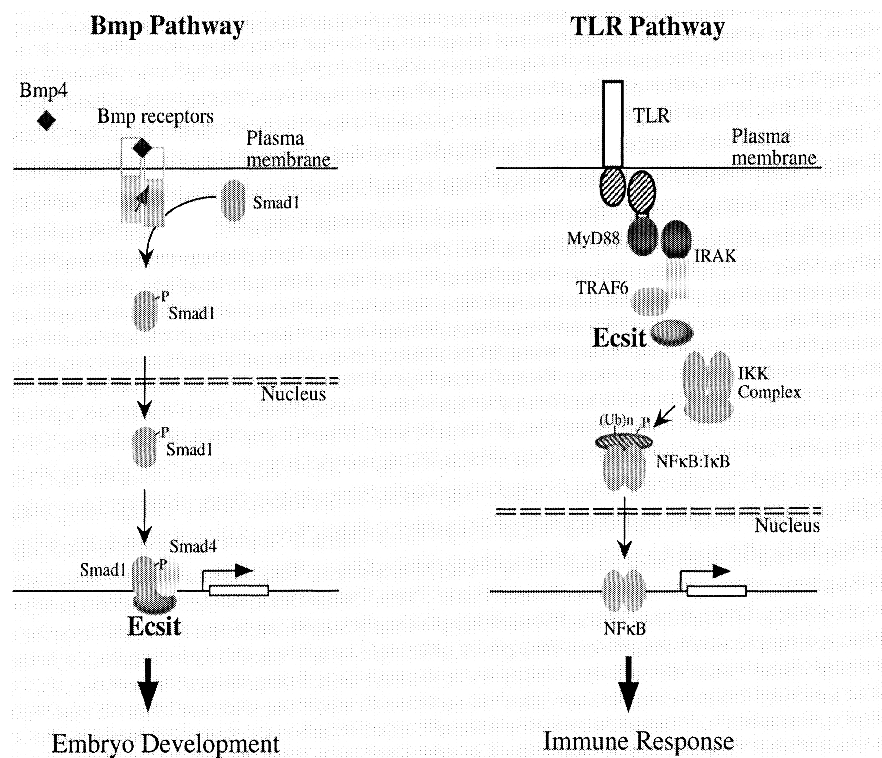

Figure 1. Proposed function of Ecsit in Bmp and TLR signaling pathways. Ecsit is localized to both cytoplasm and nucleus of unstimulated cells. In the nucleus, Ecsit and Smad4 bind to the promoters of Bmp target genes, but are not able to drive transcription. Upon Bmp stimulation, Smad1 is phosphorylated, translocates to the nucleus, and forms a complex with Smad4 and Ecsit on the target gene promoters, which is then able to drive transcription. Upon TLR activation, cytoplasmic Ecsit interacts with TRAF6 and transduces signals to the IKK complex, probably through activation of MAP3K kinases. The IKK complex then phosphorylates IκB, leading to its ubiquitination and degradation. Subsequently, NFκB is freed and translocated to the nucleus, where it regulates target gene expression. Red arrow indicates phosphorylation of the type I receptor by the type II receptor. P, phosphate group; (Ub)n, polyubiquitin chain.

analysis of Ecsit function in response to signaling through both pathways, and these studies will be significantly facilitated by the future availability of conditional knock-outs of *Ecsit*.

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6

INFLUENCE OF KIR DIVERSITY ON HUMAN IMMUNITY

Peter Parham

1. INTRODUCTION

Killer cell immunoglobulin-like receptors (KIR) are expressed on natural killer (NK) cells and on subpopulations of T cells, mostly CD8 cells, that have memory phenotype. KIR thus have the potential to contribute to both the innate immune response, through the action of NK cells, and the adaptive immune response, through the action of memory T cells. KIR were first defined functionally in the context of alloreactive human NK cells that showed specificity for polymorphic HLA class I determinants. Identified in this manner were inhibitory KIR with specificity for HLA-A, B and C determinants. Cloning of cDNA for these KIR led to the identification of additional KIR, some of which are activating receptors with HLA class I specificity and others — including both inhibitory and activating KIR — for which ligands have yet be defined (reviewed in [1]).

2. DIVERSITY OF KIR EXPRESSION WITHIN THE INDIVIDUAL

The human genes encoding KIR comprise a compact family which are part of the leukocyte receptor complex (LRC) on human chromosome 19 (reviewed in [2]). A consequence of the program of human NK-cell development is that individual NK cells express different numbers of KIR genes and in different combination [3]. Such patterns of expression appear stable over time and determined by the methylation status of the genes [4,5]. This differential expression of KIR genes creates heterogeneity of receptor expression within a person's NK-cell population: a repertoire that has the potential to impart clonal specificity to the NK-cell response to pathogens.

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3. DIVERSITY OF KIR GENOTYPE WITHIN THE HUMAN POPULATION

Human KIR haplotypes differ in gene content [6]. The genes that define the center of the locus (KIR2DL4) and the two ends (KIR3DL3 and KIR3DL2) are almost always present but that is not so for the other KIR genes. The number of KIR haplotypes defined by differences in gene content is approaching one hundred (reviewed in [7]. These haplotypes are further diversified by the allelic polymorphism that is a feature of most of the KIR genes [8]. The combined effect of diversity due to gene content and allelic polymorphism ensures that unrelated individuals rarely (<1%) have identical genotype. KIR genotype diversity thus individualizes NK-cell repertoires. The KIR haplotypes form two groups: the group A haplotypes are shorter having relatively few genes encoding activating receptors (0 or 1), whereas the group B haplotypes are longer because of the presence of more genes encoding activating receptors. Consequently, within the population there is considerable variation in the extent to which activating KIR are present, less so for the inhibitory KIR.

4. POPULATION DIFFERENCES IN KIR DIVERSITY

Populations differ in the relative frequency of group A and B KIR haplotypes: Caucasoid populations have even frequencies, while group A haplotypes predominate in the Japanese and group B haplotypes in Aboriginal Australians. In general there appears to be considerable population specificity in KIR genotype with relatively few KIR genotypes being common to populations and a majority being population-specific. Thus the KIR locus has undergone considerable evolution during the history of the human species (reviewed in [7]).

5. SPECIES-SPECIFICITY OF KIR

The picture of human KIR as a rapidly evolving system of immunoreceptors is fully endorsed by the analyses of KIR in other primate species. Comparison of human KIR with their counterparts in chimpanzee, bonobo, gorilla, orangutan and rhesus macaque shows that every species has a distinct set of KIR genes, with only a minority of them being common to any other species (see [9] and references therein). A major distinction can be made between the hominoids (humans and apes) and the Old World monkeys, represented by the rhesus macaque. Only KIR2DL4, the gene present in the middle of the human KIR locus, is shared by these species; the other rhesus macaque KIR representing a distinct lineage from those found in the hominoids. Given the extent of KIR diversification over relatively short periods of evolutionary time it now comes as little surprise that cattle and mouse KIR genes represent totally different lineages, which in the case of the mouse are known to be present on the X chromosome, not in the LRC [10]. Neither do they account for the dominant alloreactivities of mouse NK cells, a role that is played by the diverse and rapidly evolving lectin-like receptors encoded by the Ly49 genes of the natural killer complex (NKC) (reviewed in [11]).

6. KIR GENES EVOLVE RAPIDLY THROUGH RECOMBINATION

New KIR genes and haplotypes appear to be the work of asymmetric recombination, a mechanism that is likely facilitated by the high sequence similarity between KIR genes and the short intergenic regions that separate them. The only unique intergenic region is that separating the KIR2DL4 gene from the KIR3DP1 pseudogene, and which is of ~14kb. This regions appears a favored site for homologous recombination; it divides the locus roughly into two halves, within each of which there is high linkage disequilibrium (reviewed in [7]).

7. DIFFERENCES IN KIR REPERTOIRES OF EXPRESSION ARE DETERMINED PRINCIPALLY BY THE KIR GENES BUT WITH SOME INFLUENCE OF HLA GENES

When two siblings are genotyped for KIR and HLA they form four groups: KIR and HLA matched; KIR and HLA mismatched; KIR matched, HLA mismatched; and KIR mismatched, HLA matched. The patterns of KIR expression by the NK cells of siblings matched for KIR and HLA are very similar, whereas they range widely for siblings who are KIR and HLA mismatched. Most of the difference is due to the KIR genes, as evidenced by the similarity between KIR matched, HLA mismatched siblings, but there is a lesser effect due to HLA [12]. The latter contribution correlates with the observation that NK cell populations develop to be tolerant of all autologous, but not all allogeneic, HLA class I allotypes [3].

8. A MAJORITY OF ALLOGENEIC BONE MARROW TRANSPLANTS INVOLVE KIR MISMATCH

In matching donors and recipients for clinical allogeneic bone-marrow transplantation the 'gold standard' is for the donor to be a healthy, HLA-identical sibling (reviewed in [13]). Because the HLA and KIR loci are situated on different chromosomes, only one quarter of such gold-standard donors will also be matched for KIR. (For unrelated HLA-matched donors those that are KIR-matched number less than 1%.) When the bone marrow donor is HLA-identical and KIR mismatched the recipient reconstitutes an NK-cell repertoire like that of the donor and different from that of the patient prior to transplantation [14]. The kinetics of the reconstitution differ: some patients are fully reconstituted within one year while others take much longer. The results point to KIR mismatch being beneficial for both quicker recovery of KIR expression and good clinical outcome.

9. CONCLUSION

The extent of human KIR diversity and its rapid evolution point to these genes being subject to natural selection by pathogens. One likely contributor is balancing selection, which provides responsiveness to pathogens through activating receptors, while maintaining tolerance to self through inhibitory receptors. Consistent with this model are the studies correlating activating KIR both with better response to infection [15] and with

susceptibility to autoimmunity [16,17]. Another likely contributor is directional selection, which causes the system to continuously change. This can be conceived in terms of successive pathogen-specific selections. Variant activating receptors could be selected for their capacity to direct pathogen-specific NK-cell responses; variant inhibitory receptors could be selected for their capacity to engage variant HLA class I molecules that have themselves been selected for their capacity to direct pathogen-specific T-cell responses. Anothre and quite distinct biological function of NK cells also has a strong potential to select for improvements in NK-cell function. This is the function that NK cells in the decidua serve in implantation at the beginning of pregnancy (reviewed in [18]).

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7

NKG2D IN INNATE AND ADAPTIVE IMMUNITY

Lewis L. Lanier

1. INTRODUCTION

The term "NK receptors" has been applied to a growing number of cell surface receptors that were initially identified by their expression on NK cells. However, it is becoming increasingly obvious that few or none of the known "NK receptors" are completely restricted in expression to NK cells. For example, many of the inhibitory mouse Ly49 receptors were in fact originally cloned from T cell lines [1,2], the NKR-P1 [3,4] and CD94/NKG2A receptors are found on subsets of both human and mouse T cells [5-7], and KIR have been identified on human T cells [8,9]. Typically, these "NK receptors" are present on effector or memory T cells, most frequently on γδ-TcR+ T cells or CD8+ T cells, and are rarely observed on naive resting T cells. Thus, the expression of "NK receptors" on T cells implies a role in adaptive, as well as innate, immunity.

CD94, *NKR-P1* (CD161), *NKG2A* (CD159a), *NKG2C*, *NKG2D* and *NKG2E* are localized within the cluster of genes in the "NK complex" on human chromosome 12p13 and the syntenic region of mouse chromosome 6. The CD94, NKR-P1 and NKG2D receptors are expressed as disulfide-bonded homodimers, whereas CD94 may also form disulfide-bonded heterodimers with NKG2A or NKG2C (as yet whether human NKG2E pairs with CD94 has not been established conclusively). CD94/NKG2A and CD94/NKG2C (associated with DAP12) are inhibitory and activating receptors, respectively, recognizing HLA-E in humans and $Qa1^b$ in mice. In mice, different genes within the NKR-P1 family encode either inhibitory or activating receptors (reviewed in [10]). The function of the receptor encoded by the single human NKR-P1A (*KLRB1*) gene is not established in that there are neither known signaling motifs in the cytoplasmic region of this protein nor any associated adapter proteins identified [4]. Ligands have been identified for two of the mouse NKR-P1 receptors [11], but not for human NKR-P1A. While the physiological role of NKR-P1 has not been determined, the

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CD94/NKG2A and CD94/NKG2C receptors have been implicated in both innate and adaptive immunity against pathogens.

NKG2D differs fundamentally from the other "NK receptors" in that it is encoded by a single, essentially non-polymorphic gene that provides for immune activation, with no evidence for a related receptor with inhibitory function. Signaling occurs by the association of NKG2D with the DAP10 transmembrane adapter protein, which contains a YINM motif able to activate the PI3-kinase pathway [12,13]. In mice, a splice variant of NKG2D, designated NKG2D-S [14], can also associate with the DAP12 ITAM-bearing adapter protein, although this does not occur in humans. NKG2D is expressed constitutively on essentially all human and mouse NK cells, all human CD8+ T cells and most mouse and human γδ-TcR+ T cells [15,16]. It is not present on resting mouse CD8+ T cells or macrophages, but can be induced by activation [16].

NKG2D binds with high (nM) affinity to a family of MHC class I-like proteins. In humans, NKG2D was first shown to bind the MICA and MICB glycoproteins [15], encoded by genes within the human MHC and linked to HLA-B [17]. In addition, human NKG2D also recognizes another family of MHC class I-related molecules, which includes ULBP-1, -2, -3 and -4 [18,19] (also known as RAET1) (reviewed in [20]). While *MIC* genes do not exist in mice, mouse orthologs of the *RAET1* genes include mouse *RAE-1α,β,γ,δ,ε* and the related *H60* and *MULT-1* genes, all of which are linked on mouse chromosome 10 in a region syntenic to human chromosome 6q24.3 (location of the *RAET1* genes) (reviewed in [21]). All of the NKG2D ligand proteins share in common a general MHC class I-like structure with an α1 and α2 domain involved in NKG2D receptor binding, but unlike conventional MHC class I all lack the ability to present peptides or bind β2-microglobulin (reviewed in [22]). In general, the NKG2D ligand proteins are absent or expressed at only low levels on normal, healthy cells, but are induced or elevated on transformed or pathogen-infected cells (reviewed in [21]).

2. NKG2D IN INNATE IMMUNITY

NKG2D is constitutively expressed on human and mouse NK cells and has been implicated in NK cell-mediated responses against tumors and viral infection. In particular, it has been shown that ectopic expression of NKG2D ligands (including H60, RAE-1β, RAE-1δ, or RAE-1ε) can result in the rejection of the MHC class I-bearing lymphoma RMA [23,24]. This is noteworthy because the expression of H-2 on RMA tumor cells previously has been shown to prevent NK cell responses, as revealed by the ability of NK cells to reject the H-2-deficient variant of RMA, called RMA-S, but not the parental RMA cells [25]. Thus, in this situation signaling through NKG2D provides sufficiently strong activation to functionally override the inhibitory MHC receptor operational in the responding NK cells. Rejection of the RAE-1-bearing RMA tumors requires perforin, but not interferon-γ production by the NK cells [26]. Ectopic expression of NKG2D ligands in the B16 melanoma and EL4 thymoma has also been shown to induce NK cell-mediated protection against these transplanted tumors [24]. RAE-1 and H60 transcripts have been detected in the skin of mice exposed to chemical carcinogens and it has been suggested that this might play a role in the ability of γδ-TcR+ T cells to provide immune surveillance against nascent tumors [27]. Human tumors expressing NKG2D ligands are also susceptible to NK cell-mediated cytotoxicity *in vitro* [15,18]. The relative contribution of NKG2D to the NK cell response varies between

different tumors, likely due to the presence or absence of ligands for other activating NK cell receptors that are expressed on the individual tumor [28].

NKG2D has also been implicated in innate immunity against viruses, in particular cytomegalovirus (CMV). CMV induces transcription of genes encoding NKG2D ligands in cells infected with the virus [29]. Furthermore, treatment of mice infected with CMV with a neutralizing anti-NKG2D mAb increased viral titers early during infection, indicating a role for NKG2D in NK cell-mediated anti-viral responses [29]. Interestingly, the gp40 viral glycoprotein encoded by the *m152* gene in mouse CMV prevents cell surface expression of certain NKG2D ligands in the infected cells [29,30], due to the ability of gp40 to retain RAE-1 proteins in the cytoplasm [29]. Similarly, in human CMV the UL16 glycoprotein causes intracellular degradation of certain NKG2D ligands (MICB, ULBP1 and ULBP2). This partially protects CMV-infected cells from attack by human NK cells [31-34]. Recent studies have also suggested that expression of MICA and MICB on dendritic cells is impaired in patients with chronic hepatitis C infection, suggesting a potential role for NKG2D in this disease [35].

A link between NKG2D and bacterial infection is provided by the observation that interactions between AfaE on *Escherichia coli* and CD55 on human endothelial cells induces the expression of MICA, triggering the production of INF-g by NK cells [36]. Similarly, infection of dendritic cells and epithelial cells by *Mycobacteria tuberculosis* induces MICA, resulting in the activation of gd-TcR+ T cells [37]. A mechanism to account for bacterial or viral induction of NKG2D ligands is provided by our recent studies showing that signaling through Toll-like receptors (TLR) can induce transcription of the *RAE-1* family of genes in mouse macrophages, resulting in the activation and modulation of NKG2D on NK cells *in vivo* and *in vitro* [38]. In mice, macrophages, which constitutively express DAP10 and DAP12, have been shown to transcribe NKG2D after activation [14,39]. Since activated macrophages also express NKG2D ligands, it is possible that this provides an autocrine stimulation pathway for myeloid cells and contributes to their function in innate defense.

3. NKG2D IN ADAPTIVE IMMUNITY

Expression of NKG2D on CD8+ T cells implies a role in adaptive immunity. While expressed constitutively on all human CD8+ T cells [15], it only appears after TcR-dependent stimulation on mouse CD8+ T cells [16]. In contrast to the role of NKG2D in NK cells or activated macrophages, in T cells engagement of NKG2D alone is usually insufficient to trigger cytolytic function or cytokine production. Rather in T cells NKG2D may predominantly serve as a "costimulatory" receptor, functioning in conjunction with the TcR, although this may depend upon the activation state of the T cells. Groh and colleagues have reported that NKG2D functions to augment the response of CMV-specific human CTL, particularly when amounts of viral peptide antigen are limiting [40]. Exposure of human CD8+ T cells to IL-15 upregulates expression of NKG2D and co-crosslinking with anti-CD3 + anti-NKG2D mAb augments proliferation and cytokine production, even in CD8+ T cells lacking CD28 [41]. Similar results have been observed with mouse CD8+ T cells, although the costimulatory activity of NKG2D appears rather modest [16] compared with costimulation mediated by CD28. Based on these findings, it has been proposed that NKG2D may provide an alternative to CD28 for the costimulation of CD8+ T cells, particularly in human CD8+ T cells that lack CD28 and in situations where antigen-presenting cells or target cells bear NKG2D ligands.

A role for NKG2D costimulation in tumor immunity mediated by mouse CD8+ T cells is suggested by the finding that mice which rejected RAE-1-transfected RMA cells generated tumor-specific CTL [24]; however, this was not observed in other studies using RAE-1-transfected RMA tumors [23]. In human cancer patients expressing tumors bearing MICA, the levels of NKG2D on the CD8+ T cells in peripheral blood and within the tumor are lower than in healthy subjects and their NKG2D-dependent functions are impaired [42]. Interestingly, these tumors secreted MICA, which could be found in the sera of these patients and the soluble MICA was able to down-modulate NKG2D receptors on the lymphocytes [42]. NKG2D is also down-regulated by TGF-β [43], which is frequently over produced by certain tumors. These findings suggest mechanisms by which tumors, like viruses, may evade NKG2D-dependent immune surveillance.

While NKG2D may provide a beneficial role in anti-viral and anti-tumor immunity, this pathway may contribute to autoimmunity if NKG2D ligands are expressed by cells in the absence of infection or transformation. Although usually absent from CD4+ T cells, recent studies have documented NKG2D on CD4+ T cells in the synovial fluid of patients with rheumatoid arthritis [44]. IL-15 and TNFα, cytokines induced by inflammation, apparently were responsible for induction of NKG2D on the CD4+ T cells [44]. Recently, our lab has observed that *RAE-1* genes are inappropriately over-expressed in the pancreas of NOD mice, a strain of mouse that spontaneously develops autoimmune type I diabetes. Furthermore, blockade of NKG2D has a therapeutic benefit and prevents diabetes in these animals (Ogasawara and Lanier, unpublished). Therefore, there is emerging evidence that NKG2D may participate in both immunity and autoimmunity.

4. ACKNOWLEDGMENTS

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8

SPECIFIC AND NON-SPECIFIC NATURAL KILLER CELL RESPONSES TO VIRAL INFECTION

Wayne M. Yokoyama

1. INTRODUCTION

Initially identified by their ability to kill tumor cells without prior sensitization of the host, natural killer (NK) cells are now known to provide a crucial initial defense against pathological organisms. In particular, they play a critical role during the early phases of infection (days 0 to 5) while specific immunity develops (reviewed in [1]). Recent advances, however, indicate that NK cells specifically recognize virus-infected cells in a manner akin to their recognition of tumor cells, and also respond non-specifically to viral infections.

2. NK CELL RECOGNITION OF TUMOR TARGETS

Targets expressing major histocompatibility complex (MHC) class I molecules are generally more resistant to NK cell mediated killing than targets lacking MHC class I, an observation that led to the "missing-self" hypothesis [2]. This phenomenon is now explained by NK cell inhibitory receptors, specific for MHC class I, that belong to two structural categories, killer immunoglobulin-like receptors (KIRs), and lectin-like receptors such as CD94/NKG2 heterodimers, and Ly49. The inhibitory receptors recognize major histocompatibility complex (MHC) class I molecules on the target and contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon ligand binding the ITIMs are tyrosine phosphorylated leading to recruitment and activation of phosphatases that affect signaling through activation receptors. Less is known about NK cell activation receptors and their ligands [3-5] but many are highly related to the inhibitory receptors. However, the putative activation receptors lack ITIMs and instead contain charged residues in their transmembrane domains allowing interactions with signaling molecules (FcϵRγ, CD3ζ, DAP12) containing cytoplasmic immunoreceptor

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tyrosine-based activation motifs (ITAMs). As yet, many inhibitory and activation receptors have been tentatively identified by sequence similarity and motif analysis, though most are orphan receptors because their ligands and physiologic functions are unknown. Nevertheless, NK cell-mediated killing of tumor targets is regulated by the interplay of specific inhibitory and activation receptors and their ligands.

3. NK CELL RECOGNITION OF VIRUS-INFECTED CELLS

That NK cells must use similar recognition systems to detect virus infected cells is demonstrated by the complex viral mechanisms that appear to specifically thwart NK cell activation receptors; these studies also highlight the importance of NK cells in anti-viral defense [6,7]. Several viruses have evolved multiple mechanisms to evade MHC class I-restricted cytotoxic T lymphocytes (CTLs) by specifically downregulating MHC class I expression [7]. However, according to the missing-self hypothesis, this strategy should render infected cells more sensitive to NK cell lysis. To counter this, viruses have evolved mechanisms that appear to selectively inhibit NK cells. For example, human cytomegalovirus (CMV) contains an open reading frame (ORF)(UL40) that upregulates HLA-E surface expression, a ligand for the CD94/NKG2A, a lectin-like inhibitory receptor [8,9]. Furthermore, human CMV encodes UL18, an MHC class I mimic that is not affected by HCMV mechanisms that downregulate MHC class I molecules [10], and binds LIR-1 (ILT-2), an Ig-like inhibitory receptor [11]. Binding of either the lectin-like or Ig-like receptors results in inhibition of NK cell cytotoxic activities. Thus, these viral strategies appear to block the action of NK cell activation receptors that may be specifically involved in NK cell activity against viruses.

Recent advances support the concept that NK cell activation receptors are involved in anti-viral defense. In the mouse, the NK cell activation receptor, Ly49H, is the first NK cell activation receptor to be required for resistance to a specific viral infection *in vivo* [12-14]. This is bolstered by genetic and immunological data. The selective deletion of *Ly49h* resulted in susceptibility to murine CMV (MCMV) [12,14], and accounted for the *Cmv1* genetic locus within the NK gene complex that encodes lectin-like NK cell receptors [15,16]. Gene transfer of bacterial artificial chromosomes containing Ly49h rendered resistance to otherwise susceptible mice [17]. In genetically resistant mice, such as C57BL/6, the administration of monoclonal antibodies specific for Ly49H caused susceptibility to MCMV [12,13]. Signaling through Ly49H is mediated through a physically associated ITAM-containing molecule, DAP12 (also known as KARAP) [18,19]. The activation receptor role of Ly49H in MCMV resistance was confirmed with mice expressing Ly49H with mutant DAP12 molecules containing a nonfunctional ITAM [20]. Thus, the NK cell activation receptor Ly49H is responsible for genetic resistance to MCMV infections.

The ligand for Ly49H during MCMV infection was identified to be m157 by two independent groups [21,22]. Using reporter cells expressing transfected Ly49H, both groups determined that Ly49H specifically recognized m157, a molecule encoded in the MCMV genome, and that appears to have an MHC class I-like fold. A soluble m157 protein also bound Ly49H transfectants, indicating direct binding [21]. In 129 mice, m157 appears also to be recognized by an inhibitory receptor, Ly49I [21]. Furthermore, m157 belongs to the m145 family of related molecules, one of which (m152), has immune evasion properties because it prevents host cell MHC class I expression, suggesting that m157 may possess still other potential immune evasion functions.

The identification of the Ly49H and its ligand, m157, facilitated studies of specific NK cell responses to MCMV infection. Transfection of m157 into targets resulted in Ly49H-dependent killing [21,22]. Furthermore, intracellular staining for IFNγ and lymphotactin/ATAC revealed that Ly49H+ NK cells can be selectively activated early when co-incubated *in vitro* with the m157 transfectants or with MCMV-infected macrophages [22]. In addition, triggering of Ly49H+ NK cells by m157 transfectants led to coordinate expression of other chemokines, including MIP-1α, MIP-1β, and to a lesser extent, RANTES by individual activated NK cells, suggesting that NK cells direct early host inflammatory responses [23]. Thus, the NK cell activation receptor, Ly49H, directly recognizes an MCMV encoded MHC class I-like molecule.

4. SPECIFIC AND NON-SPECIFIC NK CELL RESPONSES *IN VIVO*

Interestingly, other pro-inflammatory cytokines, such as IL-2, IL-12, IL-15, and IL-18, also triggered the coordinate expression of the NK cell produced factors *in vitro* [23]. However, when NK cells were stimulated with cytokines, chemokine and cytokine production was not restricted to the Ly49H+ NK cell subset, indicating relatively "non-specific" (with respect to Ly49H) production. These latter findings likely reflect the non-specific stimulation of NK cells found during MCMV infection *in vivo* where IFNγ production also was not confined to the Ly49H+ NK cell subset [24]. In addition, FACS analysis of *in vivo* BrdU (bromo-deoxyuridine) incorporation [24] demonstrated that MCMV infection stimulated early (day 1 to 2 pos-infection), non-specific NK cell proliferation [25,26]. Thus, NK cells can be stimulated specifically (through Ly49H) and non-specifically (by cytokines).

These studies also demonstrated two phases of *in vivo* NK cell proliferation during MCMV infection [24]. Whereas initial NK cell proliferation was nonselective with respect to Ly49H and resembled the cytokine-driven "bystander proliferation" observed in T-cells in response to viral infections or stimulation with type I interferons [27], there followed a period of preferential proliferation of Ly49H+ NK cells peaking at days 4 to 6 of MCMV infection [24]. This phase of specific proliferation was blocked when anti-Ly49H antibody was administered suggesting that Ly49H recognition of MCMV-infected cells stimulates selective proliferation of Ly49H+ NK cells. Furthermore, the specific proliferation of Ly49H+ NK cells was virus-specific whereas the early non-specific phase was not virus-specific. Thus, there are two phases of NK cell responses to viral infection, early non-specific followed by a specific phase, and the initial specific NK cell responses may be masked by generic cytokine responses.

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9

ANTIGEN PROCESSING AND PRESENTATION BY DENDRITIC CELLS: CELL BIOLOGICAL MECHANISMS

Ira Mellman

1. INTRODUCTION

Dendritic cells (Dcs) are now widely understood to be perhaps the most efficient and critical of all antigen presenting cells. They are primarily responsible for acting as sentinels that detect and internalize foreign antigen in peripheral tissues and then conveying the antigen to lymphoid organs for presentation to T cells. Although B cells also exhibit an exquisite capacity for antigen presentation, they efficiently present only the single antigen recognized by the B cell receptor. DCs, on the other hand, can present a seemingly limitless array of complex protein, carbohydrate, and lipid antigens, and do so even if provided with only minute quantities. Moreover, DCs have a marked capacity to stimulate even immunologically naive T cells, and as such are increasingly thought to play a unique role in the initiation of all antigen-specific immune responses [1].

Interestingly, the role of DCs in initiating immunity appears to be balanced by their ability to maintain peripheral tolerance to self antigens [2]. Being that they are endowed with such a marked capacity for T cell stimulation, this is probably a good thing as DCs can, indeed, present self antigens quite effectively. In fact, DCs help control the quality of the immune response. In addition to being able to drive T cells toward tolerance or immunity, they can also determine whether immunostimulatory T cells differentiate into the various known subsets (eg Th1, Th2, or regulatory T cells) at the time of initial stimulation.

How DCs accomplish this wide array of activities is obviously a critical problem for immunologists. However, it is also emerging as a problem of equivalent interest to cell biologists since in the end, each of the specializations exhibited by these cells must reflect an underlying cell biological specialization. That has indeed proved to be the case,

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with the past several years having witnessed great progress in understanding the cell biological basis for DC function. To a large extent, this progress has reflected a fundamental and fascinating feature of DC biology, namely that nearly all of the cell's most important activities are controlled by a process of terminal differentiation called "maturation" [3]. Much in the same way that conditional mutants of yeast have aided the study of basic cell biological mechanisms, the ability to control the function and organization of DCs by controlling maturation has enabled us to identify and dissect a number important insights into how the DC accomplishes its various activities. Several new or unanticipated principles of cell biology have been uncovered by studying activities which, in other cells, would not have been expected to occur. Most pleasingly, uncovering this information has also allowed us to place fundamental cell biology in single cells in the context of a larger biological problem, namely the immune response. One can argue that this "bottom-up" approach, in which the classical strategies of reductionist cell biology are used to collect information that systematically builds a new understanding of the immune system, is "systems biology" in its purest or at least most immediately useful form.

2. FEATURES OF DC MATURATION

DC maturation must be viewed simultaneously in two ways: changes in phenotype and changes in function. Functionally, the transition from immature to mature DCs denotes a number of fundamental alterations. Immature DCs, generally the form found in peripheral tissues, are actively endocytic and thus well suited for antigen accumulation. They are less well suited, however, to antigen processing and presentation. That is the major functional attribute of mature DCs, often the DC type found in lymphoid organs. Mature DCs cannot internalize macromolecules or particles efficiently but do express high levels of MHC molecules, peptide-MHC, and costimulatory molecules. They are also highly motile and can track down and grab T cells in the hope of finding ones they can stimulate. This functional mature state, however, is far more complex than this simple description would suggest. Since DCs control the quality of the immune response, inducing naive T cells to Th1, Th2, T_{reg} etc. phenotypes, there must be important differences in the features among different "mature" DCs. Even more striking is the fact that DCs also appear to induce or maintain peripheral T cell tolerance, meaning that yet another functionally distinct phenotype is likely to exist.

The key is probably held by the factors that induce maturation. A wide array of such maturational stimuli are now known, perhaps the most popular of which are the ligands for Toll-like receptors (TLRs). However, these represent only a small fraction of the total number of possible inducers, with various inflammatory and non-inflammatory stimuli also clearly being potent maturation signals. Little is know yet regarding how these different stimuli transduce functionally different states of maturity is not known and represents one of the major challenges facing DC biology.

To a first approximation, however, the phenotypic or organizational features of immature vs. mature DCs are the same, regardless of functional attribute. Since cell biologists always attempt to take complex problems and seek simple solutions, it is this aspect of the problem to which our laboratory has devoted most of its effort. Hopefully, our attempts will not emerge, to paraphrase H.L. Mencken, as an attempt to provide simple solutions to complex problems, almost all of them wrong. Our interim studies, indeed, do appear to have generated some interesting information.

3. PHENOTYPIC MATURATION OF DCs

Immature DCs, whether differentiated in culture or taken from peripheral tissues, have a distinctive organization. The express high levels of MHC class II molecules, but they are largely concentrated intracellularly in lysosomal compartments reflecting the targeting of newly synthesized molecules to lysosomes rather than the plasma membrane [4]. Internalized antigen is also delivered to these compartments, yet it is used at best inefficiently for the formation of peptide-MHC class II complexes [5,6]. Upon receiving a maturation signal, endocytosis in these cells is rapidly down regulated (in <30 min) [7] and newly synthesized MHC class II is now transported from the Golgi complex to the cell surface [4,9]. Most importantly, even previously internalized antigen can now be mobilized for the formation of peptide-MHC class II complexes [5,6,9]. With time (~15 hr), the cells now translocate their intracellular MHC class II to the surface, which also extends numerous processes creating the canonical mature DC phenotype [4]. Transport from lysosomes to the plasma membrane has been imaged by live cell microscopy (using GFP-tagged MHC class II molecules) and involves the activation of a novel "retrograde" pathway from late endocytic structures to the surface. This involves the formation of tubular carriers (0.5-3 μm in length) that emanate from lysosomes and ultimately fuse with the plasma membrane, as detected by total internal reflectance microscopy [10].

In the end, the surface of the mature DC is exceedingly rich in MHC class II molecules and important co-stimulatory molecules (upregulated largely at the transcriptional level) that are needed for efficient T cell priming. The changes together comprise one of the most dramatic and beautiful re-organizations in all of cell biology.

4. MECHANISMS OF DC MATURATION

In seeking to understand these alterations, we have concentrated on changes that occur within endocytic compartments. While many details remain uncertain, a coherent picture is beginning to emerge.

To summarize our current concepts briefly, it would appear that maturation is linked to an overall activation of endosomal-lysosomal proteolytic capacity. We first found that lysosomes in immature DCs were rich in the anti-protease peptide cystatin C. A potent inhibitor of the cysteine protease cathepsin S (cat S), we obtained evidence that cystatin C attenuated the rate and efficiency of invariant chain processing, leading to the lysosomal targeting of $\alpha\beta$ dimers, much in the same way that pharmacologic anti-proteases such as leupeptin have been known to do for years if added to various MHC class II-positive cells [8]. Upon maturation, intracellular levels of cystatin C decrease, cat S is activating, invariant chain is more efficiently processed, and $\alpha\beta$ dimers are freed at the level of endosomes for delivery to the cell surface as opposed to transport to lysosomes (via the lysosomal targeting signal found in the invariant chain cytoplasmic tail).

More impressively, however, is the overall regulation of proteolytic activity. Antigen internalized by immature DCs is poorly degraded unless the cells receive a maturation stimulus [9]. The explanation for this phenomenon, however, does not lie with the obvious. Immature and mature DCs possess the same quantities of lysosomal enzymes and, moreover, these hydrolases are equivalently localized to MHC class II/antigen-containing lysosomal elements in both cases. *In vitro*, lysosomal extracts of immature and mature DCs degrade protein substrates with comparable abilities. What has changed? The

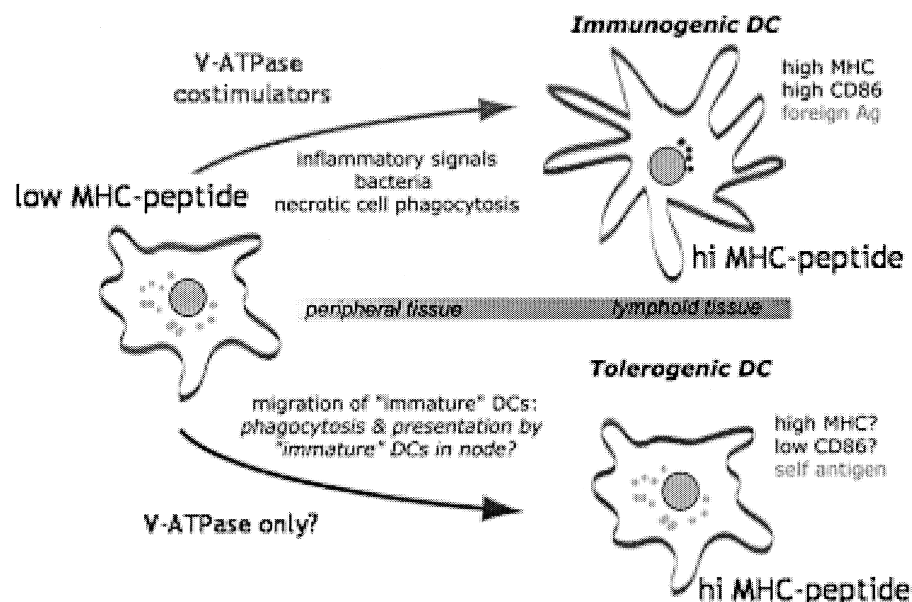

Figure 1. Immature DCs as vehicles for self antigen: induction of tolerance.

answer came from assays of lysosomal pH. Normally, lysosomes achieve an internal pH of ~4.5, conditions which are optimal for the activity of most lysosomal hydrolases. Although this pH value is found in mature DCs, immature DCs have an internal pH of about 1 unit higher: an alteration found (based on *in vitro* assays) to greatly slow the degradation of authentic protein substrates [9].

We have also explored how lysosomal pH is regulated in maturing DCs. Like other cells, lysosomes in DCs acidify due to the activity of an ATP-driven protein pump, the "vacuolar ATPase" (V-ATPase). An F1-F0-like enzyme, the V-ATPase consists of two subcomplexes: V1, which is a multiprotein complex consisting of soluble cytosolic proteins including the ATPase's catalytic subunit; and V0, a multiprotein complex consisting of integral membrane proteins, including the proton pore or proteolipid. In immature DCs, the V1 sector is largely cytosolic; in mature DCs, it is almost entirely membrane-bound, indicative of assembled and active V-ATPase. Consequently, it would appear then that DC maturation regulates lysosomal acidification by controlling the post-translational assembly of the V-ATPase. This, in turn, would be expected to regulate proteolytic activity, antigen processing, and thus peptide loading onto MHC class II molecules. Since our results also indicate that DCs are likely to have levels of lysosomal enzymes that are limiting in concentration, this would provide an effective means to regulate the function of these essential organelles.

How V-ATPase assembly is regulated is currently under intense investigation since the solution to this problem may itself provide some important clues as to the role of DCs in controlling immunity vs. tolerance. For example, if a given maturation signal exists that is able to activate the V-ATPase selectively — ie, without activating the transcription of costimulatory molecules — one would generate a mature DC that can efficiently generate peptide-MHC class II complexes in the absence of associated signals required to generate immunogenic T cells. Such DCs might well be "toleragenic" (Figure 1). As attractive an idea as this appears, one must also realize that the problem of tolerance is perhaps among the most complex in all of immunology. Whether that means it will or will not have a simple solution, even just at the level of DC biology, is a prediction best left to the likes of H.L. Mencken, as suggested above.

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10

HUMAN THYMIC STROMAL LYMPHOPOIETIN TRIGGERS DENDRITIC CELL-MEDIATED ALLERGIC INFLAMMATION AND CD4+ T CELL HOMEOSTATIC EXPANSION

Norihiko Watanabe, Vassili Soumelis, and Yong-Jun Liu*

1. INTRODUCTION

Thymic stromal lymphopoietin (TSLP) is a novel interleukin (IL)-7-like cytokine [1]. The functional receptor for TSLP is a heterodimer consisting of the IL-7Rα chain and a common γ chain-like receptor called TSLP receptor (TSLPR) [1,2]. In humans, IL-7Rα chain and TSLPR mRNA are coexpressed on CD11c+ immature myeloid dendritic cells (DCs), but not in other cell types [2]. Because human TSLP activates peripheral blood CD11c+ immature DCs, we investigated how TSLP-activated DCs (TSLP-DCs) regulate human naive CD4+ T cell activation and differentiation [3,4].

2. RESULTS AND DISCUSSION

2.1. Human TSLP Triggers DC-Mediated Allergic Inflammation

To examine how TSLP-DCs regulate CD4+ T cell activation, we cultured adult peripheral blood CD11c+ immature DCs isolated by cell sorting, for 24 h with various stimuli, and cocultured these DCs for 6 days with allogeneic naive CD4+ T cells. We found that TSLP-DCs induced much greater naive, CD4+ T cell proliferation in an allogeneic mixed-lymphocyte reaction than did CD40 ligand-activated DCs (CD40L-DCs), lipopolysaccharide-activated DCs (LPS-DCs), or IL-7-activated DCs (IL-7-DCs) (Fig. 1A). At a ratio of 1 DC per 150 T cells, the naive CD4+ T cell proliferation induced

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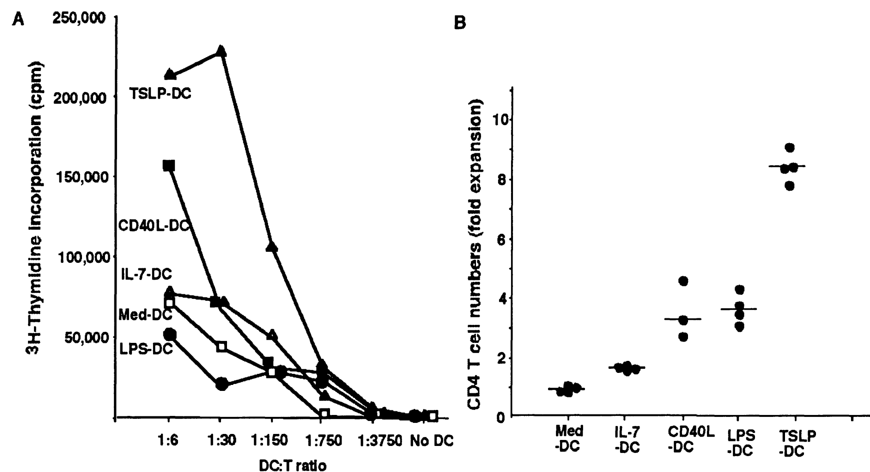

Figure 1. Human TSLP-activated DCs induce pronounced proliferation and expansion of allogeneic naive CD4+ T cells. (A) TSLP-DCs induced the greatest CD4+ T cell proliferation after 5 days of coculture, as assessed by [^{3}H]thymidine incorporation, both at high (1:6) and low (1:150) DC:T-cell ratios. (B) TSLP-DCs induced the highest CD4+ T cell expansion after 6 days of coculture. Results are expressed as the fold expansion compared with the initial T cell number. Results shown are from five independent experiments. Horizontal bars indicate the median.

by TSLP-DCs was about three times greater than that induced by CD40L-DCs (Fig. 1A). After 6 days of culture, TSLP-DCs induced a 7.5- to 9-fold increase in the total T cell numbers, which was more than that induced by CD40L-DCs, LPS-DCs, or IL-7-DCs (Fig. 1B).

Next, we compared the capacity of TSLP-DCs to polarize naive CD4+ T cells to that of DCs cultured with medium, IL-7, CD40L or LPS. Naive CD4+ T cells were cultured with DCs at a 1:5 ratio for 6 days; they were then washed to remove all cytokines, restimulated for 24 h with anti-CD3 and anti-CD28 and then cytokine production was measured in the culture supernatant by ELISA. TSLP-DCs induced naive CD4+ T cells to produce large amounts of IL-13, IL-5 and TNF and a moderate amount of IL-4 (Fig. 2). Compared to DCs cultured with medium alone or other activators, TSLP-DCs induced naive CD4+ T cells to produce the lowest amounts of the anti-inflammatory cytokine IL-10 and the TH1 cytokine interferon (IFN)-γ. Therefore, TSLP-DCs induced naive CD4+ T cells to produce a unique set of cytokines that was distinct from a TH1 profile (IFN-γ) or a classical TH2 profile (IL-4, IL-5 and IL-10). TSLP-DCs may induce robust TH2 allergic inflammation by inducing naive CD4 T cells to produce large amounts of IL-13 and IL-5 and a moderate amount of IL-4 in the presence of TNF and in the absence of two physiologic inhibitors of TH2 inflammation, IL-10 and IFN-γ.

To investigate whether TSLP expression is associated with TH2-type allergic inflammation *in vivo*, TSLP protein expression in skin lesion of atopic dermatitis patients was analyzed by TSLP specific mAb 12F3. Although TSLP was undetectable in normal skin [3], high expression of TSLP was found in the keratinocytes of skin lesion of atopic dermatitis patients (Fig. 3A). In addition, strong TSLP expression in atopic dermatitis

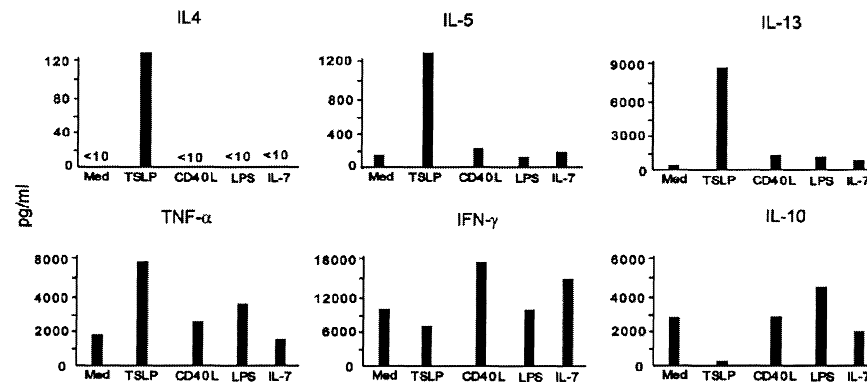

Figure 2. TSLP-DCs induce TH2 responses. TSLP-DCs prime CD4+ T cells to produce the highest amounts of IL-4, IL-5, IL-13, and TNF but lower amounts of IFN-γ and IL-10, compared with medium-activated DCs, CD40L-DCs, LPS-DCs, or IL-7-DCs. Data represent one of six independent experiments.

was associated with the concurrent appearance of many DC–lysosome-associated membrane protein (DC-LAMP, DC-associated activation marker)+ activated DCs within the dermis (Fig. 3A).

We also found that hTSLP was expressed by epithelial cells of the thymus and tonsils under normal physiological conditions. TSLP was found to be expressed by crypt epithelial cells of all human tonsils tested, regardless of age, degree of inflammation, or allergic history (Fig. 3B). In addition, TSLP was found to be expressed by epithelial cells of Hassal's corpuscules in the medulla of fetal and newborn thymus (Fig. 3C). TSLP expression was associated with the presence of many DC-LAMP+ DCs in the T cell-rich areas of human tonsils, and in the thymic medullar (Fig. 3B and 3C). These results suggest that hTSLP has normal regulatory roles on a process unrelated to allergic inflammation.

2.2. Human TSLP Promotes DC-Mediated CD4 T Cell Homeostatic Expansion

T cell homeostasis is a self-regulating process for maintaining the size of the peripheral T cell pool, which is critical for the adaptive immune system to respond to a variety of new pathogens and for maintaining immunological memory to previously encountered pathogens [5]. T cell homeostasis also contributes to the recovery of the peripheral T cell pool after T cell depletion caused by irradiation or viral infection. T cell homeostasis is maintained by T cell survival and homeostatic proliferation, triggered by self peptide-MHC ligands and cytokines, such as IL-7 and IL-15 [5]. Although DCs appear to play an important role in T cell homeostasis, the molecular regulation of DC-mediated T cell homeostasis is unknown.

To investigate whether TSLP promotes DC-mediated CD4+ T cell homeostatic proliferation, we used an autologous DC and CD4+ T cell coculture system. CD11c+ DCs purified from adult peripheral blood were cultured with various stimuli and then co-

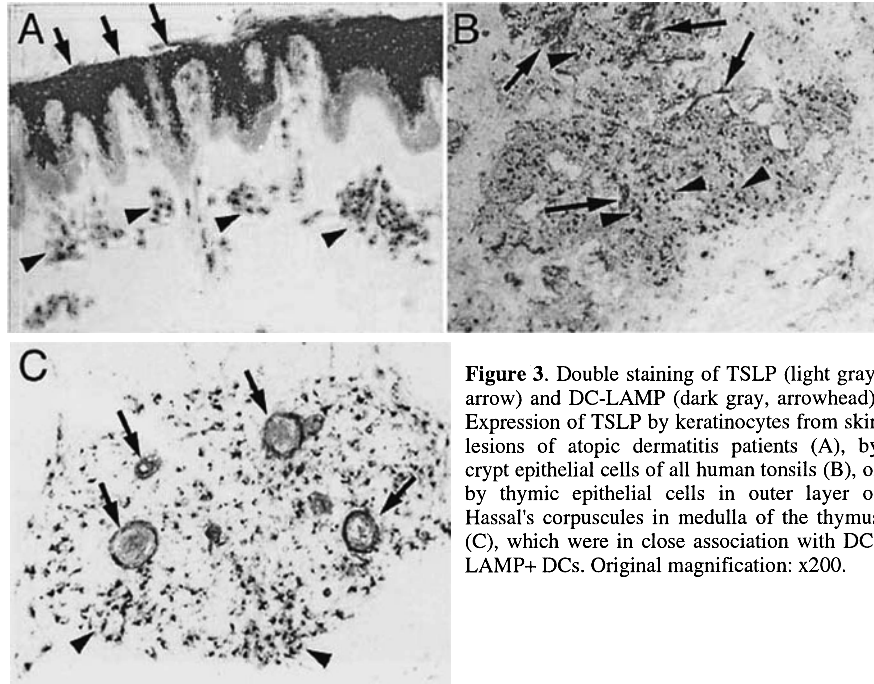

Figure 3. Double staining of TSLP (light gray, arrow) and DC-LAMP (dark gray, arrowhead). Expression of TSLP by keratinocytes from skin lesions of atopic dermatitis patients (A), by crypt epithelial cells of all human tonsils (B), or by thymic epithelial cells in outer layer of Hassal's corpuscules in medulla of the thymus (C), which were in close association with DC-LAMP+ DCs. Original magnification: x200.

cultured with autologous naive CD4+ T cells without any additional cytokines or fetal bovine serum. After 7 days of culture, TSLP-DCs induced strong proliferation of autologous naive CD4+ T cells, whereas IL-7–DCs, LPS-DCs, poly(I:C)-activated DCs, CD40L-DCs or DCs cultured with medium (Med-DCs) had only marginal effects on the proliferation of autologous naive CD4+ T cells (Fig. 4A). In addition, neither TSLP-DCs alone nor naive CD4+ T cells with TSLP alone proliferated. TSLP-DCs can induce strong allogeneic naive CD4+ T cell proliferation even at a very low DC:T cell ratios (Fig. 1A). However, TSLP-DCs induced a marked proliferation of autologous naive CD4+ T cells only at a high DC: T cell ratio (e.g. 1 DC per 2 T cells), but not at DC:T cell ratio below 1:3 (Fig. 4A). This is consistent with a previous report that T cell homeostatic proliferation in mice requires a high DC: T cell ratio [6].

After 7 days of culture, TSLP-DCs induced more than a 5-fold increase in total autologous CD4+ T cell numbers, which was greater than that induced by DCs activated by other stimuli (Fig. 4B). In addition, CD4+ T cell expansion induced by TSLP-DCs was sustained for at least 15 days after the onset of the coculture and resulted in more than a 10-fold increase in the total T cell numbers (Fig. 4B). In contrast, CD40L-DCs induced a marginal CD4+ T cell expansion at day 7, which was not sustained thereafter. Thus, TSLP-DCs induced a robust and long-lasting expansion of autologous naive CD4+ T cells at a high DC: T cell ratio.

Homeostatic proliferation of naive T cells results in polyclonal expansion, relevant to the positive selection of early immature T cells by self peptide-MHC complexes within the thymus [5]. If the proliferation of autologous naive CD4+ T cells induced by TSLP-DCs was homeostatic, the expanded T cells should exhibit polyclonal TCR V_β usage

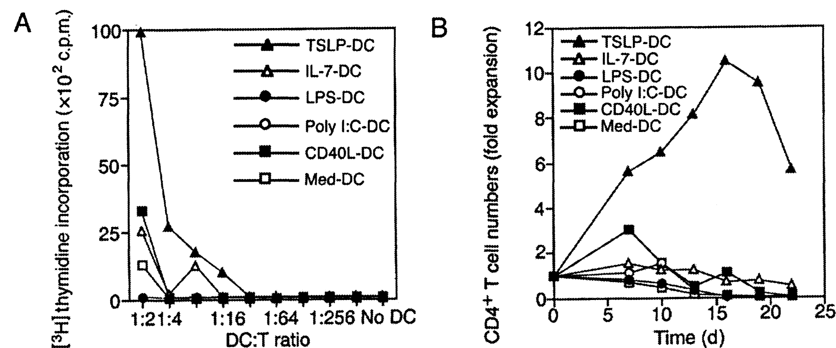

Figure 4. TSLP-DCs induce autologous naive CD4+ T cell proliferation and long-lasting expansion. (**A**) Naive CD4+ T cell proliferation after 7 days of culture with autologous DCs activated by indicated activators at various DC:T cell ratios. Proliferations were assessed by [3H]thymidine incorporation. (**B**) Naive CD4+ T cell expansion during the 22 days of culture with indicated autologous DCs at a 1:2 DC:T ratio. Data represent one of five independent experiments.

equivalent to naive CD4+ T cells before culture. Thus, we used flow cytometry to examine the TCR V_β usage of naive CD4+ T cells before and after 7 days of culture. Following culture, TSLP-DCs expanded autologous naive CD4+ T cells 5 times and these CD4+ T cells showed broad TCR V_β usage, similar to that of naive CD4+ T cells before culture, or following culture with IL-7 (Fig. 5). Stimulation of naive CD4+ T cells with autologous TSLP-DCs pulsed with the bacterial superantigen, staphylococcal enterotoxin B (SEB), greatly increased the frequency of $V_\beta3$+ cells and $V_\beta11$+ cells, as expected [7]. Taken together, these results suggest that autologous naive CD4+ T cell proliferation by TSLP-DCs represents homeostatic polyclonal proliferation and most likely requires the engagement of TCR with self peptide-MHC complexes.

When naive T cells are transferred into T cell-depleted rodents, peripheral T cells acquire a memory phenotype during homeostatic proliferation[5]. To determine the cell-surface surface markers characteristic of CD4+ T cells expanded by autologous TSLP-DCs, we used flow cytometry to analyze naive CD4+ T cells before culture and after culture for 10 days under several conditions. After the isolation, naive CD4+ T cells decreased their CD62L expression possibly due to the effect of cell isolation processes (Fig. 6A). Naive CD4+ T cells cultured with IL-7 for 10 days exhibited high CD62L expression, maintaining CD45RA+CD45RO+CD25-CD62L+CCR7+ naive CD4+ T cell phenotype [8]. Autologous TSLP-DC-expanded CD4 T cells downregulated CD45RA and upregulated CD45RO, acquiring a CD45RA-CD45RO+CD25lowCD62L+CCR7+, central memory T cell phenotype [8]. In contrast, stimulation of naive CD4+ T cells with autologous TSLP-DCs pulsed with the superantigen SEB markedly enhanced CD25 and a tissue-specific homing receptor, cutaneous lymphocyte antigen (CLA), but exhibited a slight reduction on expression of the homing receptor to secondary lymphoid organs, CD62L. In addition, the stimulation of naive CD4+ T cells with allogeneic Med-DCs plus IL-12 and anti-IL-4 (T_H1 polarizing condition) enhanced CD62L downregulation even more and decreased CCR7 expression. Thus, autologous TSLP-DCs without foreign antigens induce a central memory phenotype of expanded CD4+ T cells, whereas interactions with foreign antigens may change the central memory T cell phenotype to that of effector cells.

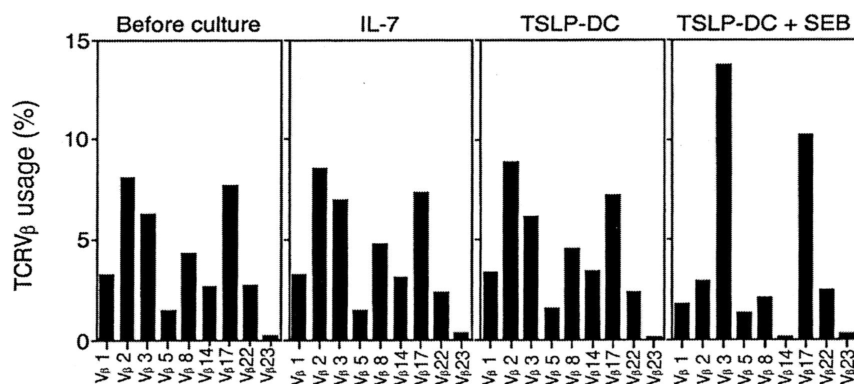

Figure 5. TSLP-DCs induced polyclonal expansion of autologous naive CD4+ T cells. TCR V_β usage of naive CD4+ T cells before and after culture with IL-7 or autologous TSLP-DCs pulsed with or without SEB for 7 days was assessed by flow cytometry. Closed bars represent the percentage of cells with indicated TCR V_β within the CD4+ T cell population.

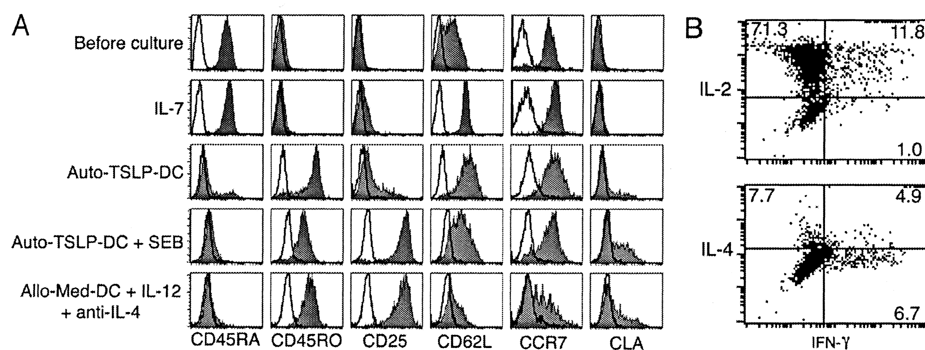

Figure 6. (A) Cell-surface markers characteristic of CD4+ T cells expanded by autologous TSLP-DCs. Naive CD4+ T cells cultured for 10 days with IL-7, autologous TSLP-DCs with or without SEB, or allogeneic medium-DCs with IL-12 and anti-IL-4, TH1 polarizing condition. Cell-surface marker phenotypes were determined by flow cytometry. Data shown are phenotypes of CD4+ T cells. (B) Cytokine-producing capacity of CD4+ T cells expanded by autologous TSLP-DCs. Naive CD4+ T cells cultured for 7 days with autologous TSLP-DCs were restimulated with phorbol 12-myristate 13-acetate + ionomycin for analysis by intracelluar cytokine staining.

Because central memory CD4+ T cells lack immediate effector functions, such as the ability to produce TH1 or TH2 cytokines [8], we examined the cytokine-producing capacity of CD4+ T cells expanded by autologous TSLP-DCs. Naive CD4+ T cells were cultured with DCs for 7 days; they were then washed to remove all cytokines and restimulated with phorbol 12-myristate 13-acetate and ionomycin. Naive CD4+ T cells cultured with autologous TSLP-DCs produced a large amount of IL-2, but not IL-4, or IFN-γ, indicating that expanded CD4+ T cells by autologous TSLP-DCs have the cytokine production profile of central memory T cells.

In conclusion, we demonstrated biological and pathophysiological functions of human TSLP-activated DCs. Human TSLP-DCs strongly induce allogeneic naive CD4+ T cell proliferation and expansion. Primed allogeneic CD4+ T cells produce pro-allergic cytokines such as IL-4, IL-5, IL-13 and proinflammatory cytokine, TNF. This, together with the finding of high TSLP expression by keratinocytes from skin lesions of atopic dermatitis patients, suggest that human TSLP plays a critical role in initiation of allergic inflammations. In addition, TSLP-DCs also induced a robust expansion of autologous naive CD4+ T cells. The proliferating T cells adopted and maintained a central memorypolyclonal phenotype. These, together with findings of TSLP expression in epithelial cells of mucosal lymphoid tissues and thymus, suggest that TSLP plays a role in DC-mediated CD4+ T cell homeostasis.

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11

ROLE OF TRAF6 IN THE IMMUNE SYSTEM

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1. INTRODUCTION

TRAF6 is a member of the TNF receptor associated factor (TRAF) family, members of which are important for signaling induced by a variety of the TNF receptor family members. TRAF6 was initially identified as a signaling adapter for CD40, but has subsequently been shown to be a critical factor for the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) family. Therefore, TRAF6 represents a central point of convergence for the signal transduction by the TNFR and the IL-1R/TLR superfamilies, and thus plays a critical role in the regulation of innate immune responses. Considering the importance of the TNFR and IL-R/TLR family members to the regulation of the innate immune system, the extent to which TRAF6 regulates the physiology of innate immunity, as well as the connection between the innate and adaptive immune responses, is of great interest. Here we have described the potential role of TRAF6 in regulating dendritic cell fates.

2. THE TNFR AND IL-R/TLR FAMILY

TNF and TNF receptor (TNFR) superfamily members both subserve and govern diverse cellular events during development, and following infectious insult or immunologic challenge [1,2]. Such varied outcomes arise from the selective activation of different signal transduction pathways: the caspase cascade, the NF-κB family of transcription factors, the mitogen-activated protein kinases (MAPKs), including both the c-Jun N-terminal protein kinase (JNK) and p38 subsets, and Src family PTKs [2]. Caspases are responsible for the proteolytic events leading to apoptosis [3] whereas NF-κB inhibits cell death in many different cell types [4,5]. The JNK and p38 kinases contribute to AP-1 activation which regulate growth signals or induce cytokines [6,7]. Once coordinately

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activated, these convergent signals may then regulate cell proliferation, differentiation or death [2].

It is believed that discrete signaling functions are initiated by recruiting different types of intracellular signal transducers to the TNFR superfamily complexes. Thus far, two major classes of signal transducers have been identified. The first is characterized by a conserved death domain which enables interaction with TNFR1, Fas(CD95), or TRAIL receptors [2,8-10].

A second class of signal transducing molecules that orchestrate the functions of the TNFR superfamily members encompass the TRAF proteins, which interact with the receptors TNFR2, CD40, CD30, 4-1BB or LT-βR, among others [2,11-14]. TRAF proteins interact with the cytoplasmic tails of the TNFR superfamily members and serve as adapter proteins to recruit downstream signal transducers like NIK and IKKs, which are responsible for the activation of NF-κB [2,5,15-17]. To date, six *bona fide* members of the TRAF family have been isolated [2]. None exhibit enzymatic activity, suggesting they operate solely as signal adapters. All contain a conserved C-terminal TRAF domain that is used for either homo- or hetero-oligomerization within the TRAF family and for interactions with the cytoplasmic regions of the TNFR superfamily [2,18]. In addition to the TRAF domain, most of the TRAF proteins contain an N-terminal RING finger, as well as several zinc finger structures which appear critical for their effector functions [2]. The *in vivo* roles of TRAF proteins are also emerging through the use of transgenic or knockout mice[2]. For example, TRAF2 provides anti-apoptotic signals during TNF-α-induced apoptosis[2,19,20]. TRAF1 appears to be a negative regulator of TNFR signaling during T cell activation[21]. Lack of TRAF3 results in defective T-dependent immune responses [22]. In addition, TRAF6 is required for osteoclast development and peripheral lymph node genesis [23,24].

An important role of the TNF family in DC biology has also emerged [25,26]. DCs have several special features that lead to the stimulation of naive T cells and play a role in the initiation of the immune response or tolerance[25,26]. Immature DCs, located in most potential sites of antigen entry, capture antigens and process them. Upon contact with antigens, DCs migrate to lymphoid organs where they encounter antigen-specific T cells. During this migration, DCs become fully mature and express high levels of various accessory molecules, including lymphocyte function antigens (LFA), intercellular adhesion molecules (ICAM) and costimulatory molecules CD80, CD86 and CD40[25-27]. Expression of these molecules allows DCs to be very potent antigen-presenting cells (APCs), capable of efficiently activating naive T cells. The small number of DCs required to present various antigens, including transplantation antigens, superantigens, and conventional protein antigens, provides evidence of the potency of DCs as APCs. For example, it was shown that one DC per 100-3000 naive T cells leads to an efficient mixed lymphocyte reaction (MLR) including proliferation, lymphokine production and the development of CTLs [25-27]. In addition, DCs have also been implicated as a major cell type responsible for T cell tolerance [25-27].

Some TNF family members regulate the differentiation, function and survival of DCs. For example, TNF-α and CD40L are molecules involved in the differentiation of DCs from CD34+ bone marrow or cord blood progenitors [25-30]. Moreover, TNF family members can regulate the T cell-DC dialogue via DCs responses to T cells using TNFR superfamily members [31-35]. For example, CD40L expressed by activated and memory T cells, interacts with CD40 on DCs, and subsequently increases DC survival, upregulates MHC and costimulatory molecule expression, and induces DC cytokine production (e.g.,

IL-12) [25-27,36,37]. TRANCE, also expressed on activated T cells [38], can similarly increase DC survival by upregulating Bcl-X_L expression, and induce IL-12 production in these cells[35,39]. Thus the CD40L/CD40 or TRANCE/TRANCE-R system indirectly enhances T cell stimulation by regulating APCs, such as DCs. In addition to their roles in immune responses, some TNF family members appear to regulate T cell tolerance by controlling the fate of CD4+CD25+ Treg cells [2,40].

TRANCE elicits signals by interacting with its receptor, TRANCE-R [2,35,41], which initiates its signaling by recruiting TRAF proteins. RANK was shown to interact with TRAF1, 2, 3, 5, and 6 by transient transfection assays [2,42-44]. However, further studies in primary cells suggest that TRAF6 might be a major signaling adapter for RANK in a physiological setting. It was shown that TRANCE or TRANCE-R KO mice exhibit grossly similar phenotypes to TRAF6 KO mice, including osteopetrosis and absence of peripheral lymph nodes [23,24,45-49]. No other TRAF KO mouse exhibit such defects [20-22,50].

In addition to its role for TNFR family members (CD40 and TRANCE-R), TRAF6 is a major signaling molecule for the IL-1R/Toll-like receptor (TLR) superfamily [51-53]. However, the biochemical nature of the interaction of TRAF6 with these superfamilies appears to be distinct. While TNFR superfamily members, such as CD40 and TRANCE-R, activate TRAF6 through direct physical association[2], the sequential recruitment of MyD88 and IRAK is required for TRAF6 to activate the IL-1R/TLR superfamily[51-53]. This IRAK-TRAF6 interaction appears to occur in the cytoplasm after the departure of IRAK from the receptor-signaling complex. Nevertheless, TRAF6 represents a central point of convergence for signal transduction via the TNFR superfamily and the IL-1R/TLR superfamily, both of which regulate DC maturation, activation and survival [14,23-27,52-54].

3. TRAF6 AS A REGULATOR OF DC FATES

3.1. Defects in DC Development in TRAF6 KO Mice

In mice, there are at least three distinct subsets of DC in spleens. Examination of these DC subsets in TRAF6 KO mice revealed a significant defect in the development of $CD4^+CD11b^+ CD11c^{high}$ DCs. Since RelB KO mice show a similarly specific defect in the development of CD4+ DCs, it is likely that a TRAF6-RelB pathway regulates the fates of CD4+ DC subset in vivo. However, it remains to be determined whether the absence of the $CD4^+$ DC subset is due to a defect in differentiation of this lineage or a selective survival defect in this population.

3.2. Defective DC Maturation and Activation in the Absence of TRAF6

Injection of LPS, one of the most potent DC maturation stimuli, upregulates the expression of costimulatory molecules on splenic DCs *in vivo* [55,56]. To test, whether LPS matures TRAF6 KO DCs *in vivo*, TRAF6 KO mice were intraperitoneally injected with LPS or PBS. Although LPS injection upregulated CD86 expression in splenic DCs in wild-type mice, it failed to induce the upregulation of MHC II and CD86 on CD11c+ DCs from TRAF6 KO mice. Similarly, injection of agonistic anti-CD40 Ab failed to induce the upregulation of costimulatory molecules in TRAF6-deficient DCs. Moreover, various bacterial products and CD40 ligand stimulation failed to induce the production of

proinflammatory cytokines in TRAF6 KO DCs. These results strongly suggest that TRAF6 is a key factor for mediating DC maturation and activation induced by multiple TLR ligands and by CD40 ligation.

3.3. Defects in the T Cell Activation by DCs Derived from TRAF6 KO Mice

Another hallmark of DC maturation is an increased capacity to stimulate T cells. To test T cell stimulatory activity, wild-type and TRAF6 KO DCs were prepared *in vitro*, and stimulated with LPS. As expected, T cell proliferation by LPS-stimulated wild-type DCs was dramatically increased when compared to that by untreated wild-type DCs. In contrast, LPS stimulation of TRAF6 KO DCs did not enhance their stimulatory capacity above background levels. A defect in the T cell stimulatory capacity of TRAF6 KO DCs was also observed by measuring IFN-γ secretion by all responsive T cells. These data demonstrate that defects in phenotypic maturation observed in TRAF6 KO DCs are reflected in their relative inability to augment T cell stimulatory capacity, a hallmark of DC maturation.

3.4. Defective Signal Transduction in DCs Derived from TRAF6 KO Mice

The absence of TRAF6, results in a severe defect in the early phase of NF-κB, JNK and p38 kinase activation induced by IL-1, CD40L or TRANCE. Among TLR ligands, CpG (for TLR9) stimulation demonstrated a requirement for TRAF6 to activate NF-κB, JNK and p38 kinase. However, LPS (for TLR4), and particularly dsRNA (for TLR3), were not dependent on TRAF6 to activate NF-κB, JNK, and p38 kinase. In the absence of TRAF6, there was only a slight reduction in the magnitude and delay in the kinetics of activation. It is most likely that TRIF can stimulate NF-κB and MAPKs independent of TRAF6.

4. CONCLUSION

TRAF6 appears to be required for maturation and activation of DCs induced by various microbial products that interact with TLRs. In addition, TRAF6 plays a central role in DC maturation and activation induced by the TNF receptor family member CD40. Thus it appears that TRAF6 is a converging point for signals required for DC maturation and activation induced by microbial and host products. However, it remains to be determined whether there are TRAF6-independent DC maturation signals when DCs encountered with other stimuli.

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12

THE INNATE FUNCTIONS OF DENDRITIC CELLS IN PERIPHERAL LYMPHOID TISSUES

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1. INTRODUCTION

The term "innate" has several functional connotations for dendritic cell (DC) biology (Table 1). DCs can mediate innate immunity directly; they also link innate and adaptive arms of the immune system during immune responses and in maintaining tolerance.

The traditional view of innate function is to provide rapid resistance to infection by mechanisms such as phagocytosis, which was the first example of innate immunity discovered by Metchnikoff. Phagocytosis takes place in certain DCs, but the function of microbial uptake in DCs may pertain more to antigen presentation rather than to large scale innate resistance. (In some cases, DCs sue endocytosis to sequester and transmit pathogens, which is the case for HIV-1 at least in culture.) More needs to be done to follow the fate of different organisms in these cells. For example, in a recent study, DCs could restrain the growth of *Legionella pneumophilia* and at the same time present *Legionella* antigens, whereas macrophages were permissive for growth and less active as presenting cells [1]. Several other functions in innate immunity are better established for DCs. For example, during virus infection, DCs or subsets of DCs can make particularly large amounts of protective cytokines, especially type I and II interferons [2, 3] in response to ligation of Toll Like Receptors (TLR's) [4, 5], stimulation of intracellular protein kinase R [6], and NKT cells [7]. A newly recognized function of DCs is to mobilize most types of innate lymphocytes, such as NK, NKT, and $\gamma\delta$ T cells. This field is just beginning to be studied in vivo [7, 8] but may represent a major way for DCs to provide innate immunity.

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Table 1. Innate Functions of Dendritic Cells

Innate resistance to infection
Phagocytosis,
Secretion especially cytokines,
Activation of innate lymphocytes
DC maturation, a link between innate to adaptive T cell mediated immunity
Toll like receptors
Heat shock proteins, necrotic cells, uric acid
Inflammatory cytokines
Immune complexes
CD40 ligand, e.g., on T cells, platelets and mast cells
Multiple pathways of peripheral tolerance as an innate function of DCs
Self tissues
Environmental proteins

DCs link innate and adaptive immunity by a terminal differentiation process called DC maturation. Maturation can be initiated by select microbial signals acting through TLRs. However, maturation can also occur with stimuli that are not clearly microbial, such as ligation of CD40 (Table 1). A major consequence of maturation is that the DCs are able to initiate adaptive T cell immunity to presented antigens. The type of immunity e.g., Th1 vs. Th2 and even regulatory T cells, may be influenced by the way that the pathogen differentiates or matures the DCs[9]. This article will not deal with the developing area of "pathogen tuning"[10], but it is an important topic since the way DCs sense the environment influences the subsequent host response. We will focus on maturation as it applies to strong cell mediated immune response to defined proteins. One of our goals is to learn to control DC maturation in vivo to develop vaccines composed of simple proteins and defined maturation stimuli.

DCs also bridge innate and adaptive immune responses by presenting antigens in the steady state to maintain peripheral tolerance. Many examples are now available whereby DCs capture self and environmental antigens in the steady state, and present these in lymphoid tissues for purposes of tolerance. Classically the view was that DCs in the steady state were simply waiting for a maturation stimulus to initiate immunity. However, it appears that DCs in lymphoid tissues actively mediate many of the known pathways to peripheral tolerance, both intrinsic to the tolerized T cell (deletion, anergy)[11-13] and extrinsic (regulatory and suppressor T cells)[14-16]. By tolerizing the T cell repertoire in the steady state, DCs obviate the induction of immunity to self and environmental antigens when these antigens are subsequently presented together with microbial antigens during infection.

We will begin this chapter by summarizing certain physiological features of DCs that are innate, i.e., built in and functional in the steady state. These features include: 1) the anatomic position of DCs in vivo, particularly within lymphoid tissues, 2) the capacity of DCs to take up and process antigens for presentation, and 3) the ability of DCs to respond quickly to a panel of differentiation or maturation stimuli that change the cells in several

pertinent ways to initiate and control adaptive immunity. We will then consider how these features allow DCs to control peripheral tolerance and immunity and emphasize a new DC targeting approach to vaccination.

2. DENDRITIC CELL FUNCTIONS IN LYMPHOID TISSUES

2.1. Anatomic Position

A hallmark of the distribution of DCs in vivo is their abundance in the T cell areas of lymphoid tissues. In the T areas, DCs form a maze of processes through which lymphocytes continually recirculate[17,18]. DCs are therefore in an ideal position to select rare clones of antigen-specific lymphocytes for purposes of either immunity or tolerance[19-21]. In the same way, DCs sustain T cell viability, since recognition of MHC products on DCs is sufficient for keeping naive lymphocytes alive [22].

For example, epidermal Langerhans cells move via afferent lymphatics to lymph nodes that drain the skin, and these migrants are identified in the node by strong expression of two endocytosis receptors, Langerin/CD207 and DEC-205/CD205 (as well as low levels of CD8α in mice)[24]. Additional lymph-derived DCs originate from the dermis and from the interstitial spaces of other organs, in the case of nonskin draining lymph nodes. The dermal or interstitial DCs may in turn derive from blood precursors, frequently termed myeloid DCs. These express PSGL, glycans recognized by P and E selectins on blood vessels[25]. DCs also can originate from blood monocytes. In the steady state there may be a subset of monocytes destined to become DCs and marked by high levels of CXCR3 or by CD16[26,27]. Additionally, there are inflammatory situations where monocytes differentiate along a DC rather than macrophage pathway. Active cytokines like GM-CSF, flt-3L, IL-4, IL-13 have been identified in various in vivo and in vitro models of monocyte to DC differentiation. However, there is little direct in vivo data on the conversion of monocytes to DCs during infection, and in fact, the conversion can sometimes be inhibited by bacteria[28].

In sum, the origin of DCs in the T cell areas is an intricate topic, even without considering the chemokines and other molecules required to position DCs. One must consider possible distinctions between the steady state and infection, as well as differences that relate to DC subsets such as plasmacytoid DCs, Langerhans cells, and others.

2.2. Dendritic Cell Maturation

The term maturation denotes the extensive differentiation that allows DCs to become strong stimulators of T cell mediated immunity (Table 2). Maturation was first encountered in cultured Langerhans cells[29], where it was proposed to be a critical process for initiating immunity[30-32]. As part of maturation, there was extensive remodeling of the LC surface. Many markers increased in expression (e.g., MHC II, CD86, DEC-205) and others decreased (e.g., FcγR and F4/80). The markers changed in a similar fashion when LCs were undergoing enhanced migration from intact pieces of skin, as in transplantation[33] or simple explantation into culture[33,34]. Antigen capture and presentation occurred in immature DCs[30,35,36], but surprisingly, mature DCs were unable to take up several antigens. Instead mature DCs had acquired the capacity to act as the most potent accessories for T cell immunity, initially assessed by T cell proliferation and T-dependent

Table 2. Some Functions that Change Substantially during DC Maturation

Production of cytokines like IL-1, TNF, IL-6, interferons, IL-12, IL-23
Production of chemokines like IL-8, MIP-1α,β, MCP-1, RANTES
Alterations of chemokine receptors e.g., increased CCR7
Decreased pinocytosis and phagocytosis, and several endocytic receptors
Increased antigen processing and expression of MHC peptide complexes
Increased expression of B7 and TNF costimulatory family members
Increased CD40

antibody formation[30,31]. This was the first dissection of immunization into two sets of functions: antigen capture and presentation vs. accessory (later "costimulatory") functions for T cell stimulation. This separation later proved vital to understand tolerance, since immature DCs in lymphoid tissues capture and process antigens in the steady state but induce peripheral tolerance rather than immunity (below).

DC maturation has also been studied in tissue culture experiments where immature cells, derived from marrow and blood precursors, are induced to differentiate by the addition of cytokines such as GM-CSF and TNF-α. Differentiation of these DC precursors to more mature forms greatly increases the processing of previously acquired antigens[37], the export of processed antigen as MHC peptide complexes to the cell surface[38], and the lifespan of the complexes[39]. The findings of Mellman and colleagues are outlining the unusual features of the endocytic system of these maturing DCs, particularly the extensive regulation at the levels of antigen uptake[40], transport of MHC class II molecules[38,41], lysosomal pH and hydrolytic activity[42].

Most DCs in vivo in lymphoid tissues have the functional features of immature cells even though the DCs express co-stimulatory molecules such as CD86. DCs in the T area of lymphoid organs are continually and efficiently processing antigens to form MHC peptide complexes in the steady state[11-13,43-46]. This presentation leads to tolerance and not immunity[11-13,44,45]. Maturation is required to activate immunity.

Many stimuli have been identified that trigger some or all of the above changes in DCs (Table 1). It is not clear if each stimulus results in functionally similar mature cells. Also, it needs to be kept in mind that different maturation stimuli may interact to control immunogenicity. For example, if a microbial ligand activates a DC through a TLR, cytokines (IL-1β, TNF-α, IFN-α and IFN-γ) are then produced which could act back on the DCs to drive further differentiation. Also, newly produced MHC peptide complexes might activate helper cells and expression of CD40L, which also acts back on the DCs. We have recently begun to dissect the components of DC maturation in vivo in response to innate NKT lymphocytes[47]. We find that inflammatory cytokines are responsible for the upregulation of CD80 and CD86, two frequently used surrogate markers of DC maturation, but that full maturation of immunogenicity to a coadministered protein requires CD40 ligation even in DCs that are already expressing high levels of MHC-peptide complexes and CD80/86 costimulators.

In summary, DC maturation should be assessed in vivo in terms of immunogenicity and not by expression of surrogate markers like MHC or co-stimulatory molecules. A

major innate feature of DCs is to act as sentinels and adjuvants for the immune system by rapidly maturing and becoming powerful inducers of adaptive immunity in response to an array of microbial and other stimuli.

2.3. Antigen Uptake and Processing by DCs onto Multiple Classes of Antigen Presenting Molecules

An essential innate feature of DCs is their expression of a broad range of receptors for endocytosis and antigen presentation. Some of these are shared with other leukocytes, particularly monocytes and macrophages. These would include Fcγ and complement receptors for immune complexes and antibody coated tumor cells, and receptors involved in the uptake of dying cells. Other receptors are more restricted to DCs and often subsets of DCs. These include orphan receptors with yet to be determined ligands; but their amino acid sequences suggest recognition of carbohydrates. Many are type II transmembrane proteins with a single external C-type lectin domain, while others are type I proteins with multiple external and contiguous carbohydrate recognition domains. DC-SIGN/CD209 is an example of the type II variety, but it has primarily been studied on cultured monocyte-derived DCs. (A spectrum of pathogens is recognized by DC-SIGN incoludingn several viruses (HIV-1, CMV, HCV, Dengue, Ebola), fungi (candida), protozoan parasites (certain Leishmania), and mycobacteria.) DEC-205/CD205 is a type I transmembrane protein, and it has been studied on both cultured DCs and DCs in lymphoid tissues. While natural ligands are not known, antibodies to DEC-205 have been used as surrogate ligands, as will be discussed below. In retrospect, the uptake of antigens by DCs in vivo has in the past largely been left to "chance". The identification of DC receptors for antigen uptake should change this situation and make it possible to target antigens to DCs more efficiently and selectively.

An important feature of antigen uptake in DCs relates to the efficient formation of peptide complexes with both MHC class I and II products. Processing to form ligands for $CD4^+$ T cells can be very efficient[37], but more intriguing is that DCs can present nonreplicating forms of antigens on MHC class I products[48,49](reviewed in [50,51]). DCs process immune complexes, dying cells, and DEC-205 ligands to form MHC class I (and MHC II) peptide complexes. For class I, this "exogenous pathway" can utilize transporters for antigenic peptides or TAPs[12,44,52,53]. It is now thought that TAP-dependence takes place in the endocytic vacuole, following fusion of elements of the rough endoplasmic reticulum[54,55]. DCs also present antigens on other types of molecules such as the glycolipid binding CD1 family. In sum, the capacity of DCs to efficiently present nonreplicating antigens on many types of antigen presenting molecules allows them to control tolerance and immunity in several different classes of lymphocytes.

2.4. Summary

DCs are far from quiescent in the steady state. Instead, they have a number of innate functions (Table 1) that depend on such properties as their positioning in lymphoid organs, environmental sensing through numerous pathways for maturation, and expression of efficient antigen uptake and processing pathways. Together, these features allow DCs to play pivotal roles in innate resistance as well as adaptive tolerance and immunity.

3. EXAMPLES OF THE INNATE ROLES OF DENDRITIC CELLS IN LYMPHOID TISSUES IN PERIPHERAL TOLERANCE

3.1. Presentation of Antigens Targeted to DCs

The most detailed and direct studies of antigen presentation by DCs in lymphoid tissues in the steady state has involved a new approach. The approach targets the DEC-205/CD205 endocytois receptor on DCs. DEC-205 originally came to attention when Breel and Kraal immunized rats with lymphoid tissue "stroma" and screened hybridomas for reactivity to the "interdigitating" DCs of the T cell areas. One of the hybridomas, termed NLDC-145, reacted strongly with DCs in the T cell areas of all peripheral lymphoid tissues (spleen, lymph nodes, Peyer's patch); the antibody also stained Langerhans cells (though weakly) and thymic cortical epithelium (very strongly)[18]. The corresponding antigen was unknown for some time until it was isolated as a 205 kD glycoprotein from the thymus by Swiggard et al[56] and cloned by Jiang et al[57]. The cloned molecule had considerable homology to the macrophage mannose receptor (MMR/CD206)[57]. The amino termini of both receptors had two domains, one cysteine rich and the next fibronectin like, followed by 10 external contiguous carbohydrate recognition domains in DEC-205 and 8 in the MMR. Both the MMR and DEC-205 had cytosolic domains with motifs for localization to coated pits and rapid entry into the cell[57].

However additional cell biological studies showed that DEC-205 had an intracellular traffic pattern that was different from the MMR and other recycling endocytic receptors. DEC-205 did not recycle through peripheral endosomes but instead moved through deeper MHC II rich compartments[58]. This traffic was attributed to a triad of acidic amino acids in the cytosolic domain, which also was associated with much more efficient antigen presentation on MHC class II products relative to ligands for the MMR. Another major difference between DEC-205 and its MMR cousin was that DEC-205 was abundant on a large fraction of DCs in lymph nodes, whereas the MMR was primarily found elsewhere, e.g., on sinusoidal lining endothelium and macrophages[59,60]. These features of DEC-205, as well as the availability of the Kraal rat anti-mouse DEC-205 antibody, set the stage for an examination of the consequences of DEC-205 mediated antigen uptake in situ. (Although DCs express many different receptors with the potential for endocytosis, these have been studied mainly in human, and antibodies to mouse counterparts are not yet available except for anti-mouse DEC-205/CD205.)

First we needed methods to deliver antigens via DEC-205, since natural ligands have been difficult to identify. Therefore the antibody to DEC 205 was used as a surrogate but specific and high affinity ligand, following modification to deliver antigens. This was done in two ways. In one, the cDNA of the heavy chain was engineered to introduce sequences for antigens, such as Hen Egg Lysozyme, at the carboxyl terminus[11]. In the other, OVA protein was chemically linked to inter-heavy chain thiol groups generated by mild reduction of the antibody[12,46]. In both instances, the modified antibody selectively targeted the antigen to DCs in vivo. This could be demonstrated in several ways. 1) In tissue sections, the DEC-205 antibody localized selectively to MHC class II and CD11c positive cells in the T cell areas; 2) in FACS analyses, the injected rat Ig and associated OVA were found selectively in DCs; and 3) in functional studies, CD11c$^+$ DCs selectively presented antigen to antigen-specific TCR transgenic T cells.

As in the uptake of dying cells and environmental proteins (see below), there was no change in the surface markers of DCs that had interacted with anti-DEC-205 antibodies. Nonetheless, under these steady state conditions, there was vigorous antigen presentation

to T cells. A single injection of submicrogram doses of antigen lead to extensive proliferation of a bolus of 1 million or more TCR transgenic, OVA-specific, $CD8^+$ and $CD4^+$ T cells, with the former exogenous pathway being TAP dependent. Therefore, an innate property of DCs in lymphoid organs was to capture and process antigens with considerable efficiency.

When the consequences of DEC-205 delivery were examined, different forms of tolerance developed in the steady state. We observed deletional tolerance with two high affinity peptides from OVA and hen egg lysozyme[11,12], and a form of functional inactivation with a self MOG peptide[13]. More work is needed to understand these different outcomes, but the unifying theme is that peripheral tolerance is sustained by the innate properties of DCs (or at least some subsets of DCs) in lymphoid tissues.

3.2. Presentation of Antigens from Dying Cells

Cell death occurs frequently in vivo, and this can have significant consequences for the presentation of self and foreign antigens. Cell death takes place in the so-called "turnover" of many tissues in the steady state, and cell death accompanies injury as well as infection. DCs in vivo can capture dying cells in these circumstances, i.e., in the steady state and following various perturbations. DCs are likely to be important in determining the immunologic consequences of this cell death. For example, peripheral tolerance may be induced to self antigens captured in the steady state and possibly during most examples of injury and trauma, while immunity develops in the context of cell death occurring during infection.

To directly investigate the handling of dying cells by the immune system, we followed the uptake and fate of syngeneic "osmotically shocked" spleen cells loaded with a marker protein, ovalbumin (OVA). This system was identified by Bevan and colleagues (who bathed cells in a high concentration of OVA and then briefly exposed the cells to hypertonic followed by hypotonic medium; this osmotic shock would rupture endocytic vesicles, release OVA into the cytoplasm, and allow presentation on MHC class I) to study the direct presentation of cell associated antigens[61]. It later became apparent that osmotic shock was leading to cell death, and that the dying cells could be captured efficiently by the $CD8\alpha^+$ subset of DCs following i.v. injection into mice[44,53]. Presentation by host DCs in this indirect or cross presentation pathway was detected by the proliferation of OVA-specific $CD8^+$ and $CD4^+$ TCR transgenic T cells. However, the DCs taking up dying cells did not show evidence for maturation, at least at the level of several cell surface markers. When the fate of the OVA-specific, $CD8^+$ T cells was followed, they disappeared over the course of 2 weeks, and importantly, the animal became profoundly tolerant to rechallenge with OVA in CFA. Therefore we concluded that DCs have an important innate function with regard to dying cells in the steady state: the latter can be taken up, processed and presented for purposes of tolerance.

To prove that most DCs could process the phagocytosed cells, and to follow what could happen when cell death was induced in vivo (rather than ex vivo by osmotic shock above), we used another system in which we could monitor the formation of MHC class II-peptide complexes directly. The system was to inject allogeneic B cells (from BALB/c mice), which in turn were rapidly killed in vivo by recipient NK cells (in C57BL/6 mice). The MHC class II disparate ($I\text{-}A^b$) host DCs efficiently took up the allogeneic B cells killed by host NK cells, i.e., within 12 hrs, 30% of $CD8^+$ splenic DCs took up B cell fragments. The successful processing of the dying cells, which expressed a distinct I-E MHC class II molecule, led to the formation of an epitope recognized by the Y-Ae

monoclonal antibody, an epitope formed by the complex of I-A^b and an I-E peptide. All the DCs that took up the injected allogeneic B cells quickly became Y-Ae positive[53]. In addition, the DCs could stimulate naive, $CD4^+$ TCR transgenic T cells specific for the same MHC peptide complex. We think that this may be an important pathway for DCs to capture and process targets that are killed by NK cells, including class I deficient tumor cells and infected cells (e.g., targets made class I deficient by herpes virus infection). Reciprocally, NK cells are activated and expanded by DCs, and NK cells have the capacity to mature DCs (Table 2). This may change the outcome from tolerance to immunity, as we shall discuss for other maturation stimuli in the next section.

There is an additional pathway for presentation that needs to be kept in mind. Incoming DCs typically do not leave lymphoid organs via efferent lymph but instead die within a short period after arrival. This was visualized by following the fate of BALB/C DCs injected s.c. into C57BL/6 recipients. Using the Y-Ae antibody described above, we noted that many host H-2^b DCs in the lymph node became Y-Ae positive within a day, indicating that they had captured and successfully processed the injected, I-E bearing BALB/C DCs[37]. This pathway is relevant to transplantation, since it shows how donor DCs can initiate the indirect pathway of rejection by delivering donor MHC and other antigens to recipient DCs. The death of incoming DCs from the periphery could thereby distribute a bolus of peripheral self antigen to a larger number of DCs in the lymph node. This could increase the efficiency of peripheral tolerance, e.g., other DCs or DC subsets in the lymph node might be better at forming MHC peptide complexes and may be longer lived than DCs entering from peripheral tissues.

3.3. Presentation of Antigens from the Environment

Not only are self tissues presented by DCs in draining lymph nodes in the steady state, but harmless environmental proteins can be handled similarly. We set out to test this with OVA as a model airway protein. Mice were allowed to inhale endotoxin free OVA. This did not change the phenotype of the DCs in the draining lymph nodes in the chest (mediastinum). However, the mediastinal lymph node DCs were efficiently presenting OVA peptides on MHC class I and II products, as indicated by robust proliferation of $CD4^+$ and $CD8^+$, OVA specific, TCR transgenic T cells[45]. These T cells however, produced very little IL-2 and IFN-γ, as detected with intracellular cytokine staining assays. When we examined cells from the mediastinal lymph nodes for antigen presenting activity, only the $CD11c^+$ DCs could present OVA to $CD8^+$, OVA specific, TCR transgenic T cells. Therefore it seems that DCs can acquire and present environmental proteins continually in the steady state, but immune effector T cells do not develop.

We did not determine the origin of the DCs presenting environmental protein, in our case OVA as a surrogate airway protein. Prior work suggested the OVA was captured in the lung followed by migration to the node[67]. Alternatively, OVA might have entered the afferent lymph to be captured by DCs already in the lymph node, or peripheral OVA capturing DCs could come to the lymph node, die and then undergo reprocessing by DCs in the lymph node.

We did ask if tolerance resulted from the presentation of inhaled OVA in the steady state. In fact, tolerance was apparent. If mice inhaled OVA for 30 minutes on 3 successive days, OVA specific $CD8^+$ killer T cells did not develop when the mice were challenged with OVA under conditions that would normally lead to immunity (see below). We did not study the mechanism for this peripheral tolerance. One possibility is

that there are IL-10 producing DCs in the lung and/or draining lymph nodes, and these DCs induce regulatory T cells for extrinsic tolerance[14,15].

3.4. Summary

In this section, we have used the word innate to signify DC functions that exist naturally rather than being acquired, and we distinguish "innate function" from "innate immunity," where the latter implies a protective role against infection. The reason for this distinction in terminology is that a major innate function of DCs in lymphoid tissues is specific immune tolerance rather than resistance. This function reflects the features of DCs in lymphoid tissues that were summarized above. DCs are positioned in the T cell areas to access the recirculating pool of lymphocytes, and they have efficient pathways for antigen uptake and processing such that small amounts of captured antigens (self, environmental, foreign) can be recognized by large numbers of T cells. The consequence is peripheral tolerance. A major unknown relates to the tolerizing mechanisms of these DCs, and their state of activation or maturation in the lymph node. Is tolerance simply a matter of abundant "signal one" presentation by DCs, or are other accessory molecules required? Are the immature DCs in a lymph node distinct from immature DCs from other sources, i.e., do lymph node DCs have additional specializations for tolerance?

4. DENDRITIC CELL MATURATION AS THE LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

4.1. T Cell Immunity to Antigens Targeted in Vivo via the DEC-205 Receptor

We have recently evaluated the selective targeting of antigens to maturing DCs in vivo in naive mice with a polyclonal repertoire[46]. We found that the combination of anti-DEC-205:OVA plus an agonistic CD40 antibody to mature the DCs led to strong $CD4^+$ and $CD8^+$ T cell immunity[46]. In the absence of anti-CD40, no primary immune response occurred, whereas in the presence of anti-CD40, the immune response was robust and durable, such that cytolytic and IFN-γ producing $CD8^+$ T cells were observed for months after a single subcutaneous injection of this simple protein vaccine. This new vaccination approach was associated with greater protection relative to standard approaches using CFA as an adjuvant. For example, mice immunized with anti-DEC-205:OVA and anti-CD40 could resist a lethal intransal challenge with vaccinia OVA virus, and also could exert resistance against an established B16-OVA melanoma tumor.

When we analyzed some of the underlying mechanisms, some expected and some unexpected findings were made. The use of receptor mediated targeting, as expected, greatly enhanced the efficiency with which OVA was presented to T cells, several hundred fold or more relative to soluble unconjugated OVA. Even so, the combination of 1000 fold higher doses of soluble OVA and anti-CD40 only induced a fraction of the $CD4^+$ and $CD8^+$ T cell priming observed with anti-DEC:OVA and anti-CD40. This encouraged us to probe more deeply and identify two other findings that we think contributed to the stronger and more durable immunity. When we checked for the longevity of antigen presentation in vivo, using TCR transgenic T cells as reporters, we found that class I MHC peptide complexes were readily detected for 2 weeks after a single intracutaneous injection of anti-DEC-205: OVA. This may indicate that DEC-205 was expressed by a subset of long lived DCs, or perhaps the DCs that initially took up the

OVA were dying and being reprocessed. A second unexpected finding was that the injected anti-DEC-205 antibody gained access to DCs systemically in distal lymphoid tissues. If the antibody was injected into the skin of the paws, within 30 minutes it had targeted (and was presented by) DEC-205 expressing splenic DCs, and within a few hrs, the antibody had labeled DCs in distal mediastinal and mesenteric lymph nodes. This indicates that an antibody in the afferent lymph exploits the normal protein retrieval function of the lymphatic system, and gains access to the efferent lymph and blood stream. The DEC-205 targeting function of the antibody leads to systemic loading of DCs with a single injection.

4.2. T Cell Immunity Initiated by Mature DCs Presenting Antigens from Dying Cells

We have examined the consequences of providing DCs with a maturation stimulus in each of the above models of antigen uptake in vivo, where tolerance was observed in the steady state. We initially used agonistic anti-CD40 antibodies for maturation in the case of DCs that had captured DEC-205 targeted antigens[11] or OVA loaded dying splenocytes[44]. Now, instead of being deleted, the responding T cells were retained and exhibited an enhanced secondary response. Furthermore, during the primary response, active effector function was noted when anti-CD40 antibodies were injected[11,44]. The TCR transgenic T cells made large amounts of IFN-γ and were cytolytic to peptide loaded targets given i.v.

To directly prove that the maturing DCs were responsible for immunogenicity, we removed DCs from animals that had been given antigen together with α-GalCer glycolipid 4 hrs earlier. When these DCs were injected into naive animals, they were able to prime $CD4^+$and $CD8^+$ T cells in the recipients, but now there was no further need for antigen, α-GalCer, or NKT cells[7]. DCs from $CD40^{-/-}$ mice were inactive in this adoptive transfer approach, even though the DCs were presenting antigens and expressing high levels of CD80/86, comparably to wild type mice[47]. This was the first direct evidence showing that DCs matured in vivo, in this case via CD40 and innate lymphocytes, switch their innate tolerizing function to the induction of adaptive immunity.

4.3. T Cell Immunity to Environmental Proteins

Our interest in antigens in the environment, e.g., proteins in the airway and intestinal tract, related to a dilemma inherent in the concept that maturation was the critical switch for DCs to induce immunity rather than specific tolerance. When DCs present microbial antigens, the DCs are likely to be simultaneously capturing and presenting self antigens from dying cells and harmless environmental proteins. We postulated that DCs avoid this dilemma by capturing many of these self and environmental antigens in the steady state, and silence the T cell repertoire. This would obviate the potential for the DCs to generate autoimmunity or chronic inflammation during a subsequent infection[76,77].

We tested this hypothesis in the system described above wherein DCs capture and present inhaled OVA in the steady state[45]. We asked what happened if OVA were inhaled during the course of an influenza infection. This infection led to DC maturation in the draining lymph node, as assessed by increased expression of MHC II, CD80 and CD86. Interestingly, macrophages and B cells in the node did not show these changes. As mentioned above, $CD11c^+$ DCs were the only cell type in which we could detect presenting activity for OVA-specific (and also influenza specific) T cells. When OVA

was inhaled in association with infectious influenza, the immunologic outcome was different. OVA-specific T cells started making large amounts of IL-2 and IFN-γ. When we next studied naive mice with a polyclonal repertoire, rather than adoptively transferred transgenic T cells, OVA immunity developed following the combination of OVA and influenza, whereas tolerance took place if the OVA were given for 3 days prior to the influenza. These findings indicate that an innate function of DCs in lymph nodes is to induce tolerance to environmental proteins, to avoid the dilemma of subsequently inducing immunity to these proteins in the setting of infection.

4.4. Summary

It becomes more feasible to link innate with adaptive immunity if one pays attention to the need to direct the antigen to DCs and mature these cells. Antigen uptake can be harnessed through receptor-mediated mechanisms, while maturation can be controlled in vivo with stimuli such as innate lymphocytes, microbial infection, and CD40 ligation. Much remains to be explored in terms of the stimuli for, and consequences of, maturation in vivo. Nevertheless, DC maturation is required to induce immunity, and to avoid tolerance. The mechanisms that underlie DC functions in tolerance and immunity are significant areas for future work.

5. DISCUSSION

This chapter did not consider certain areas of innate DC function. We have focused on T cells, but DCs also influence B cells and all classes of innate lymphocytes. For example, type I interferon production during the innate response of plasmacytoid DCs plays an important accessory role in the human antibody response to influenza in culture[78], and DCs in the marginal zone of mouse spleen can present bacterial antigens to antibody forming B cells[79]. Nor have we considered the functions of DC subsets, which may be very different in terms of their antigen capturing pathways and their capacity to respond to maturation stimuli. For example, the uptake of dying cells in several instances is limited to the CD8α subset of splenic DCs, while the lectins Langerin and BDCA-2 are likewise expressed by select DC subsets.

Instead, we have concentrated here on a change in one of the longstanding emphases in DC physiology. Prior emphasis has been on the initiation of adaptive T cell mediated immunity to specific proteins, be they of microbial, tumor or self origin. Now it is apparent that DCs have innate functions that lead to peripheral tolerance (Table 1). A critical switch is postulated to be the maturation status of the tolerizing and immunizing DCs, but underlying mechanisms need to be pinpointed.

The newly recognized roles of DCs reflect a shift in experimental approach. Much of the initial work on DC biology was carried out with DCs in culture, often derived from precursors such as blood monocytes and proliferating bone marrow progenitors. Now it is becoming more feasible to study the functions of DCs in vivo, particularly DCs in lymphoid tissues. By using genetic approaches to selectively express antigens in these cells[80], or by delivering antigens directly to DCs in lymphoid tissues[11-13,44,46], one can begin to harness both the tolerizing and immunizing functions of DCs in vivo.

The material in this chapter could provide new opportunities for vaccine design. Antigen targeting to DCs together with appropriate maturation stimuli represents a potentially exciting new route to vaccination. Chemically defined, relatively simple

agents, i.e., antigens engineered into antibodies and defined maturation stimuli, should provide a feasible means to harness the innate functions of DCs and induce strong, durable and protective adaptive immunity directed to specific microbial and tumor proteins. This approach would make use of DC targeting for the first time in vaccine biology allowing delivery of multiple antigenic epitopes on multiple presenting molecules, along with DC accessory functions.

The standard explanation for the generation of tolerance and immunity is "signal one-signal two" theory. The theory is that antigen alone ("signal one") leads to tolerance by deletion or anergy, while antigen plus a second costimulatory signal ("signal two") leads to immunity. Instead, we would suggest that antigen delivery to DCs in the steady state leads to tolerance (by both intrinsic and extrinsic mechanisms), and antigen delivery to maturing DCs leads to different forms of T cell differentiation and immunity. In this model tolerance and immunity are sets of functions carried out by different functional states of DCs in vivo in lymphoid tissues. To gain control of tolerance and immunity in an antigen-specific manner, we suggest that it will be valuable to expand experiments to include a direct analysis of DCs.

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13

THE MANNOSE-BINDING LECTIN: AN INFECTION SUSCEPTIBILITY GENE

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1. INTRODUCTION

A critical but unanswered question is what defines each individual's pre-morbid susceptibility to infection? We propose that individuals must have an "immune haplotype" that shapes their response to infectious agents. Infection is a balance between the intrinsic virulence of the infectious agent and the host defenses. Recent viral outbreaks of SARS and influenza serve to illustrate this point as these viruses cause severe disease in certain individuals, yet there are others in whom the same infectious challenge results in minimal symptoms. On the other hand it might be that those self same people who are resistance to one particular viral infection might be susceptible to other infection challenges. Similar rules can apply to susceptibility to bacterial infections.

We hypothesize that individual variations in a set number of genes that regulate both innate and adaptive immune responses might explain this individual variation in response to an infectious challenge. The mannose-binding lectin (MBL) serves as a broad first line host defense molecule and presents an interesting opportunity to explore this hypothesis further. MBL appears to be a prototypic pattern recognition molecule that is able to recognize the molecular patterns that decorate a wide range of microorganisms. Infectious agents that are recognized by MBL include certain Gram positive and Gram negative bacteria, yeast, parasites, mycobacteria, and viruses [1-3]. The idea that a relative lack of MBL might predispose the host to infection was based on the description of an MBL-dependent opsonic defect in human serum that correlated with a phenotype of recurrent infection [4]. These patients were found to have one of three substitution single nucleotide polymorphisms (SNPs) in exon 1 of the MBL gene that disrupt the collagen helix [5]. It appears that the disordered collagen chain acts a dominant negative fasion, resulting in a decrease in circulating levels of MBL that do not activate complement. More detailed analysis of the MBL gene has revealed at least ten distinct MBL

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haplotypes in humans, four of which (LYPB, LYQC, HYPD and LXPA) dictate low serum levels [6]. Interestingly, there is a high rate of haplotype variation in various human populations with a range of heterozygosity from 15% in Caucasians to 30% in certain African populations [7,8].

A basic function of innate immunity is a concerted response by numerous molecules and effector cells that conspire to restrict the initial spread of an infectious focus. First line host defense molecules include antimicrobial peptides, natural antibodies, complement proteins, lipopolysaccharide binding protein (LBP), soluble receptors and collectins [9-11]. The collectins are multimeric lectin-containing molecules with collagen stalks that include the pulmonary surfactant proteins-A and -D, conglutinin, CL-43, CL-46, and MBL [1,12-14].

Importantly, MBL seems to be able to distinguish species self or altered self from non-self though it is able to recognize dying cells [15]. The specificity that allows the distinction of surfaces of virally infected cells and transformed cells from normal host cells depends on both fine recognition of molecular micro patterns, and on the spatial geometry of macro pattern of these molecules on the surface of the cell. The cognate ligands that are recognized by MBL appears to be dictated by the spatial orientation of the carbohydrate binding domains and the differences in geometry of the sugars that adorn microorganisms versus host glycoproteins exposed on viable cells. MBL is able to activate complement via a novel mechanism that co-opts the mannose-binding lectin associated serine protease (MASP) [16,17]. There is a family of three related MASP genes, but it is MASP-2 that utilizes the classical pathway convertase to cleave the third complement component (C3) [17]. MBL therefore activates complement in an antibody independent manner. The analogy of MBL to antibodies extends MBL's function as an opsonin [18-20].

2. MBL NULL MICE ARE HIGHLY SUSCEPTIBLE TO INFECTION WITH *STAPHLYOCOCCUS AUREUS*

In order to provide formal proof that MBL is indeed important in host defense *in vivo*, we set out to create a mouse model of MBL deficiency. Humans and new world monkeys have a single MBL gene, whereas rodents have two homologous forms of MBL that are designated MBL-A and MBL-C, the respective gene products of the *mbl1* and *mbl2* genes, and are 50% homologous [21-23]. These two homologues proteins have distinct and overlapping binding specificities, are found predominantly in serum, and are able to bind MASPs to activate complement [23-25]. The relative physiological role of these two proteins *in vivo* has not been clearly defined. In order to address some of these questions, we created MBL-A and MBL-C double KO (MBL null) mice. We verified that the MBL null mice lack MBL in serum and therefore have a nonfunctional MBL complement pathway. We chose to infect these mice with *S. aureus*, as this organism is a significant cause of human infection worldwide. The emergence of widespread antibiotic resistance to *S. aureus* poses new therapeutic challenges and so identification of host factors that play a role in resistance to infection with this Gram-positive infection is of great interest. We found that (1) all MBL null mice died two days after i.v. inoculation of *S. aureus* compared with 55% survival of wild type mice; (2) pretreatment of the mice with recombinant MBL reversed the phenotype; (3) there were significantly more bacteria in the blood of MBL mice compared to wild type mice at 24 hours; (4) the viscera of MBL null mice accumulated significantly more bacteria than wild type mice

24 hours post infection; (5) there was a decrease in phagocytosis of bacteria in blood and peritoneal cavity in MBL null mice. In contrast to intravenous infection, i.p. inoculation of *S. aureus* did not result in enhanced infectious complications in MBL null mice compared with wild type mice. However, when the MBL mice were rendered neutropenic, these neutropenic MBL null mice displayed enhanced bacterial accumulation in organs and had persistent bacteremia 10 days post inoculation.

3. CONCLUSIONS

MBL appears to fulfill the criteria as an important host defense molecule against initial infection with *S. aureus*. The animal data indicate that MBL acts in serum as an opsonin. The effector mechanism appears to be mediated in part by MBL-dependent complement lysis of bacteria and in part, via MBL-dependent phagocytosis by leukocytes. Based on these studies, it is not clear whether MBL dependent clearance of *S. aureus* is mediated via complement receptors or MBL (collectin) receptors. Unpublished observations from our laboratory indicate that there is indeed an MBL-dependent, complement independent clearance mechanism. What remains an open question is the consequence of MBL dependent clearance versus clearance via complement receptors.

It thus appears that MBL is part of the initial response to infection, which is a complex interaction between a variety of pattern recognition molecules that trigger the downstream physiological cascades of complement, clotting, cytokine, and chemokine release and interface with effectors cells such as neutrophils [26,27]. Furthermore, the effector action of MBL appears intimately tied to circulating phagocytes. Neutrophils and monocytes express complement receptors, MBL receptors (collectin receptors) [28,29] and the receptor for lipopolysaccharide binding protein (LBP) [30]. Wright and colleagues linked humoral and cellular interactions and drew attention to the importance of co-operative interactions between neutrophils and opsonins in combating infection [31,32]. More recent examples have exploited the use of null animals to explore such interactions and are germane to this present study, including the interaction of LBP and neutrophils in resistance to intraperitoneal *Salmonella* infection [33,34]. A similar synergistic interaction between neutrophils and MBL is suggested by clinical observations that chemotherapy-induced neutropenic patients with haplotypes that specify low serum MBL levels [14,35,36] appear more susceptible to infection [37]. These clinical observations together with *in vitro* studies suggest that MBL plays a key role as an ante-antibody in first line host defense [38,39] and supports a role for MBL in combating infection *in vivo*.

What has not been clearly determined is the role of MBL against a variety of pathogens. Does MBL play a role against other Gram-positive and Gram-negative bacteria, mycobacteria and viruses *in vivo*? What is the relative role of MBL, complement and antibody in first line host defense? Finally, what is the real selective pressure for MBL haplotypes that specify low levels of MBL in humans? One speculation is that low levels of MBL might be protective against infection with intracellular pathogens like tuberculosis and malaria. While there might be some merit in this suggestion, it seems that it might well be that low MBL levels decrease the activity of the MBL complement pathway, resulting in a response that is less proinflammatory, and therefore less injurious to the host. Accordingly, low MBL levels might be protective against reperfusion injury. Overall, we are entering an exciting new chapter in this saga.

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14

IMMUNOTHERAPY VIA DENDRITIC CELLS

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1. SUMMARY

The immune system evolved to protect us from microbes. The antigen (Ag)-nonspecific innate immunity and Ag-specific adaptive immunity synergize to eradicate the invading pathogen through cells, such as dendritic cells (DCZ7) and lymphocytes, and through their effector proteins including antimicrobial peptides, complement, and antibodies. Its intrinsic complexity renders the immune system prone to dysfunction including cancer, autoimmunity, chronic inflammation and allergy. DCs are unique in their capacity to induce and regulate immune responses and are therefore attractive candidates for immunotherapy. However, DCs consist of distinct subsets with common as well as unique functions that lead to distinct types of immune responses. Therefore, understanding DC heterogeneity and their role in immunopathology is critical to design better strategies for immunotherapy. Indeed, what we learn from studying autoimmunity will help us induce strong vaccine specific immunity, either protective, as in the case of microbes, or therapeutic, as in the case of tumors.

2. DENDRITIC CELLS

T and B cells are under the control of DCs [1-3] which thereby control immunity and tolerance (reviewed in [4-6]). The first 25 years of DC research mostly focused on how they turn on immunity particularly following microbial encounter. Immature, antigen-capturing mDCs sitting in peripheral tissues sense pathogens, tissue necrosis, and local inflammation. These signals induce DCs to undergo a maturation process while migrating through the afferent lymphatics into the T cell areas of draining lymph nodes. There, they present processed Ags to T cells via both classical (MHC class I and class II) and non-classical (CD1 family) antigen presenting molecules [1]. This results in T cell

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proliferation and differentiation into helper and effector cells with unique function and cytokine profiles. DCs also activate B cells, NK cells and NK T cells. Mature, antigen-loaded DCs are geared towards the launching of antigen-specific immunity [7, 8] though recent data indicate that mature DCs also activate regulatory T cells. Immature (non-activated) DCs capture and present self-antigens (e.g. apoptotic cells) to T cells [9, 10], which in the absence of appropriate costimulation leads to tolerance [11, 12]. How this complex balance is maintained in health and broken in autoimmunity is now starting to be understood.

Paul Langerhans first saw DCs in 1868, within the skin epithelium. Ralph Steinman, identified in 1973 a rare cell type from mouse spleen that is involved in the induction of immune responses. For nearly 20 years, DCs had to be painstakingly isolated from tissues and the progress was slow. In 1992, culture systems were discovered that produced large amounts of mouse [13] and human DCs [14, 15] thereby accelerating the study of DCs. Besides their rarity, the complexity of DCs lies in two other aspects: different subsets and different stages of maturation. Two major DC pathways are thought to exist [1, 16] (Fig. 1). A myeloid pathway, which generates two subsets, Langerhans cells (LCs), found in stratified epithelia such as skin and interstitial DCs (intDCs), found in all other tissues [17, 18]. These subsets can produce large amounts of IL-12. Another pathway includes plasmacytoid DCs (pDCs), which secrete, upon viral encounter, within a few hours large amounts of Type I Interferon, an antiviral cytokine [19]. Therefore, pDCs represent a first barrier to the expansion of intruding viruses thus acting as member of the innate immunity. Importantly these cells subsequently differentiate into DCs able to induce immune responses thus acting as members of adaptive immunity.

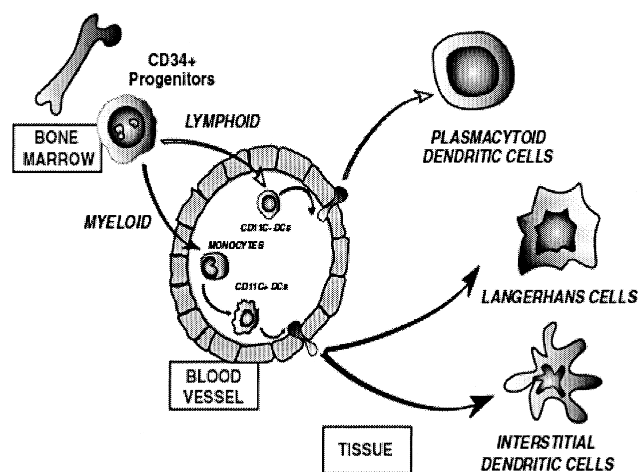

Figure 1: Subsets of human dendritic cells.

Circulating DCs precursors represent less than 1% of white blood cells [1, 16]. These precursors replenish the immature DCs that sit within tissues and are endowed with mechanisms to capture invading microbes such as receptor mediated endocytosis (lectins, Fc receptors), macropinocytosis and phagocytosis. Minute amounts of captured antigens are processed into small peptides while DCs move towards the draining secondary lymphoid organs. There, the DCs present the peptides to T cells and complete their

maturation after receiving signals from the antigen-specific T cells [20]. Each of the three DC subsets express a unique lectin. LCs express Langerin, critical to the formation of Birbeck granules [21]. The intDCs express DC-SIGN that binds 1) ICAM-1 on T cells facilitating MHC-peptide complex recognition 2) ICAM-3 expressed on endothelial cells therefore allowing transmigration of DCs into tissues and 3) HIV protein (GP120) among other microbial antigen/molecules [22, 23]. pDCs express another lectin called BDCA2 [24]. Toll receptors are also differentially expressed. For instance Toll 9 (a receptor for demethylated DNA) is expressed only by pDCs [25]. Such differential expression of molecules that are efficient anchors for pathogens, may have a capital influence on the type of immune response generated against a given microbe.

3. ENHANCING IMMUNITY VIA DCs

The concept of cancer immunotherapy has evolved in the past decade owing to the molecular identification of human cancer antigens [26, 27]. These therapeutic approaches were further facilitated through identification of in vitro culture methods allowing generation of large numbers of DCs on which the cancer antigens can be presented to T cells. Studies in mice have shown that injection of DCs loaded with tumor associated antigens (TAAs) leads to antitumor immune responses resulting in tumor rejection. Early trials in humans have shown the safety of TAA-loaded DCs as well as some clinical and immune responses. Recent studies concentrated on establishing maximal immune responses to control antigens and tumor antigens. Many issues remain to be addressed before DC therapy becomes an integral part of active immunotherapy (Fig. 2). These include the choice of the DC subset to be administered and the way to generate it. We have vaccinated 18 HLA A*0201$^+$ patients with metastatic melanoma with autologous CD34+ HPC derived DCs, that contain two subsets i.e. Langerhans cells and Interstitial DCs. DCs were pulsed with MelanA/MART-1, tyrosinase, MAGE-3 and gp100 peptides, as well as Flu-MP peptide and KLH as control Ags [28]. We found that vaccination with peptide-loaded CD34-DCs leads to expansion of i) melanoma-specific IFN-gamma producing CD8$^+$ T cells, and ii) melanoma-specific cytolytic CD8$^+$ T cell precursors that yield, upon single restimulation with peptide-pulsed DCs, cytotoxic T lymphocytes (CTLs) able to kill melanoma cells. The present results therefore justify the design of larger follow up studies to assess the immunological and clinical response to peptide-pulsed CD34-DC vaccines.

Another important parameter to establish is the dose and frequency of DC administration. Unlike traditional chemotherapy, the highest dose may not be yielding the best clinical response. Likewise, too frequent administration may result in activation induced cell death, resulting in elimination of T cells able to kill cancer cells. It is believed that optimal anti-tumor effects will be obtained with many vaccinations possibly over a lifelong schedule. A considerable object of research is the antigen loading. At present, DCs are mostly loaded with peptides from defined (tumor) antigens that bind to MHC Class I and II antigens. This presents numerous limitations such as i) the restriction to a given MHC type; ii) the limited number of TAAs, which restricts vaccination therapy to tumors for which many TAAs have been identified, for example melanoma, and iii) the limited repertoire of elicited immune effectors which may not allow eradication of the multiple tumor variants. Therefore, alternative strategies that provide both MHC class I and class II epitopes and lead to a diverse immune response involving many clones of CD4+ T cells and CTL are needed. These include: recombinant proteins, exosomes [29],

viral vectors [30], plasmid DNA, RNA transfection [31, 32], immune complexes [33] and, more recently, antibodies against DC surface molecules [34, 35]. Yet another way is to exploit the capacity of DC to present peptides from phagocytosed dead tumor cells on both MHC class I and II molecules, so called cross-priming [36, 10, 37, 38]. Indeed, we have demonstrated, using both prostate carcinoma and melanoma as model systems, that DCs loaded with killed allogeneic tumor cell lines can induce CD8+ T cells to differentiate into CTLs specific for shared tumor antigens [37, 38].

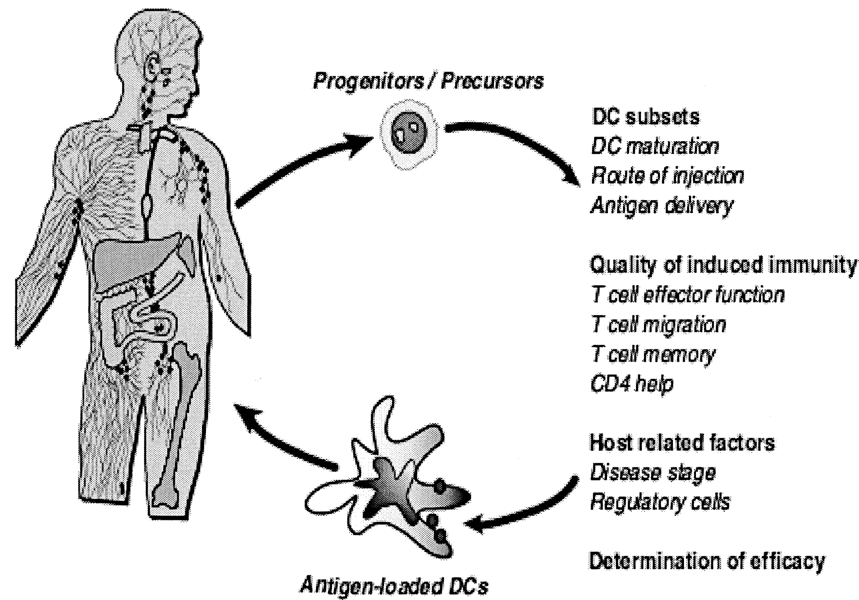

Figure 2: Parameters of dendritic cell vaccines.

4. TOLERANCE VIA DCs

Our body has evolved means to avoid the immune system attack on the components of self. Two mechanisms were created, central and peripheral tolerance both of which are controlled and maintained by DCs. Central tolerance occurs in thymus where newly generated T cells with a receptor that recognizes components exposed by mature thymic DCs are deleted [39-41]. There is evidence that both thymic epithelial cells as well as mature DCs in the thymus may be involved in this process [42, 43]. However, many self antigens may not access the thymus while other are expressed later in life. Upon activation, these autoreactive cells may lead to autoimmunity. Hence, the need for peripheral tolerance, which occurs in lymphoid organs by induction of T cell anergy, i.e. unresponsiveness, rather than deletion. The development of peripheral tolerance involves immature DCs [5]. These cells sitting within tissues capture the remains of cells that die in the process of physiological tissue turn-over. As there is no inflammation accompanying this process, the DCs remain immature and migrate towards the draining lymph nodes. These immature DCs, which lack costimulatory molecules, present the tissue antigens to autoreactive T cells, which in absence of costimulation, enter into a

state of anergy. Immature DCs may also control peripheral tolerance through induction and maintenance of regulatory T cells [44-48].

5. IFN-α BREAKS TOLERANCE: A NOVEL DC VACCINE

Breaking this anergic state, e.g. through increased availability of mature DCs, may result in autoimmunity. This concept is illustrated by our studies in patients suffering from the systemic lupus erythematosus (SLE) [49]. IFN-alpha/beta plays a major role in SLE, a prototype autoimmune disease characterized by a break of tolerance to nuclear components. Until recently SLE has been viewed mainly as a B cell disease resulting from altered T/B cell interactions. The recognition of the fundamental role of immature DCs in the control of peripheral tolerance led to the hypothesis that SLE may be driven through unabated DC activation. CD14+ monocytes isolated from SLE patients blood, but not those from healthy individuals, act as DCs. Their activation is driven by circulating IFN-alpha that may come from one of the DC subsets, i.e., plasmacytoid DCs that infiltrate SLE skin lesions. The importance of IFN-alpha in SLE is further shown by the presence of IFN- alpha signature in the blood of all SLE patients and its extinction upon therapy with high dose steroids given to control disease flares. The excess IFN-alpha may also explain the hyperglobulinemia. Indeed, pDCs triggered with virus induce activated B cells to differentiate into plasma cells. Two pDCs cytokines act sequentially, with IFN- alpha/beta generating non-Ig secreting plasma blasts and IL-6 inducing their differentiation into Ig-secreting plasma cells. These plasma cells display the high levels of CD38 found on tissue plasma cells. Thus, pDCs are critical in generation of plasma cells and antibody responses (Fig. 3).

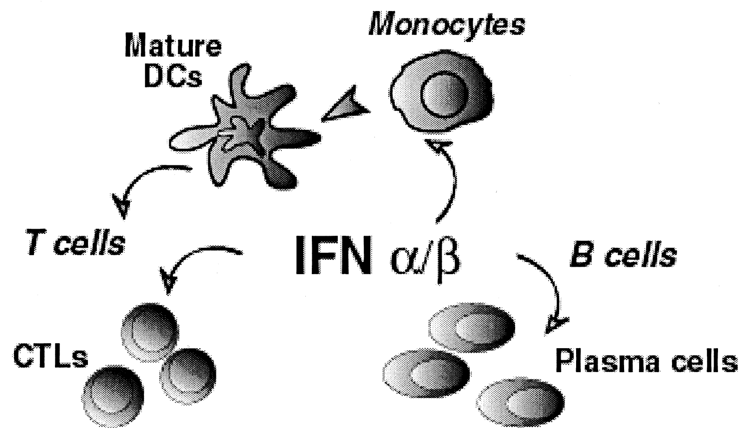

Figure 3: IFN alpha drives differentiation of immune effector cells including CTLs and plasma cells.

Importantly, SLE CD8⁺T cells appear to express higher levels of Granzymes and their numbers correlates with disease severity [Blanco, personal communication and our unpublished data]. Overexpression of effector molecules involved in cell lysis may not be restricted to CD8⁺T cells, as CD4⁺T cells from patients with active SLE show perforin overexpression as well [50]. Accordingly, IFN-DCs skew T cell differentiation into cytolytic phenotype with high levels of Granzyme and perforin expression. Thus, these DCs may be very efficient as vectors to enhance immunity either in cancer or infectious diseases. Indeed, our preliminary results suggest that IFN-DCs are more efficient than IL-4-DCs in cross-priming naive T cells against breast cancer antigens.

6. HuMOUSE: AN IN VIVO MODEL OF THE HUMAN IMMUNE SYSTEM

Understanding the specific functions of DC subsets and their interplay *in vivo* will be critical to understand the launching and modulation of immune responses. Hence, the need for pre-clinical models of the human immune system. Indeed, conclusions from studies in mice cannot be directly extrapolated to humans because of biological differences between species [51, 52]. SCID mice reconstituted with human cells represent interesting candidates for human disease models [53-55] and have been used for example in the evaluation of tumor metastasis [56], mechanisms of progression [57] as well as anti-tumor therapies (reviewed in [58]). However, many difficulties have been encountered. For example, grafting of PBMCs leads to rather limited immune reconstitution that may not allow potent priming of T cells. The grafting of human hematopoietic progenitor cells (HPC) [59, 60] improved reconstitution, particularly when fetal tissues co-engrafted with human thymus [61] or lymph node were used [62]. Yet, the complexity of the system limits its general applicability. The introduction of the NOD/SCID mice [63], improved engraftment of human cells [64, 65]. However, residual NK cell activity could interfere with the efficiency of engraftment [66] leading to development of mice with deletion of β2- macroglobulin [67, 68] or γ chain genes [69].

We surmised that many of the difficulties encountered in these models could be due to insufficient reconstitution of human DCs, a parameter that has not been extensively studied. Given the key role that DCs play in T cell homeostasis [42], DC reconstitution might facilitate human T lymphocyte reconstitution. Indeed, recent studies with ex vivo generated monocyte-derived DCs suggest their capacity to support CD4 T cell differentiation as well as humoral responses in vivo [70, 71]. However, these adoptive transfer models do not permit the evaluation of the interplay between DC subsets. We found that NOD/SCID mice engrafted with human CD34⁺ hematopoietic progenitors develop human myeloid and plasmacytoid DCs. Skin display immature DCs expressing Langerin while other tissues display interstitial DCs. Myeloid DCs from these mice induce proliferation of allogeneic CD4 T cells in vitro, and bone marrow human cells containing plasmacytoid DCs release IFN-α upon influenza virus exposure. Injection of influenza virus into reconstituted mice triggers IFN-α release and maturation of mDCs. Thus, these mice may provide a model to study the pathophysiology of human DC subsets in the context of microbial infection or cancer. Indeed, our preliminary studies demonstrate that these mice can be transplanted with tumors (the OncoHumouse) allowing in vivo analysis of tumor-DC interactions. Furthermore, a most recently published study shows the establishment of the full human immune system.

7. CONCLUSIONS

DCs are an attractive target for therapeutic manipulation of the immune system to enhance insufficient immune responses, in infectious diseases and cancer, or attenuate excessive immune responses, in allergy and autoimmunity. However, the complexity of the DC system brings about the necessity for their rational manipulation to achieve protective or therapeutic immunity.

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15

ONTOGENY OF LAGERHANS CELLS AND GRAFT VERSUS HOST DISEASE

Miriam Merad

1. INTRODUCTION

Langerhans cells (LCs) through their stimulation of donor T cells likely play a key role in skin graft-versus-host-disease (GVHD), a serious complication that limits the use of allogeneic BM transplantation (1-5). LCs belong to a family of highly specialized antigen presenting cells called dendritic cells (DCs) (6, 7) and represent the only DCs of the epidermis (8). In common with all DCs, LCs are well equipped to capture environmental antigens, migrate to lymph nodes (LNs), and initiate specific T cell immune responses playing a critical role in skin immunity (6). Despite their importance, little is known about the life cycle of LCs, their precursor cell in the blood, the mechanism of LC replenishment after skin injury, and their homeostasis after allogeneic bone marrow (BM) transplantation. In this paper, we will discuss recent advances in our understanding of LC homeostasis during steady state and inflammatory conditions and the potential role of LCs in transplant immune reactions.

2. LCs AND IMMUNITY

Multiple leukocyte populations are found in the skin, including LCs in the epidermis, and macrophages, DCs and mast cells in the dermis. LCs are localized in the basal and suprabasal layers of the epidermis, where they represent the first hematopoietic barrier with the environment (7, 9). The presence of unique intracellular organelles, known as Birbeck granules (9) and expression of Langerin a lectine binding molecule, which constitutively bind to Birbeck granule (10) distinguish these cells from dermal DCs. They also differ in the factors that drive their differentiation. LC development seems to be critically dependent on transforming growth factor (TGF)-β because TGF-β knockout

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mice are devoid of LCs, but not of their precursors (11, 12). LCs are well equipped to ingest foreign antigens that breach the skin mucosa. Upon activation, LCs increase their expression of MHC class II and costimulatory molecules, migrate to the draining lymph nodes (LNs) and initiate specific T cell immune responses playing a key role as sentinels of skin immunity (6). LCs are also found in skin lymphatics in steady state conditions in both animals and humans (13, 14). It is postulated that LCs migrate during steady state conditions to maintain or induce peripheral tolerance to skin antigens which may be critical for the prevention of skin autoimmune disease (15).

3. ORIGIN AND HOMEOSTASIS OF LCs

Given the importance of LCs in skin immunity, their mobilization to regional LNs, as well as the recruitment of LC precursors from the circulation into the skin, must be tightly regulated events. The presence of LCs in lymphatics in the incidence or absence of skin injury suggests that both in steady state and inflammatory conditions, migratory LCs are replaced (13, 14, 16). Although, the mechanisms that regulate the migration of LCs from the skin to the draining LNs are beginning to be understood, far less is known about the mechanisms that regulate the recruitment of LCs from the blood to the skin.

3.1. Murine Studies

Earlier studies in mice have shown that after allogeneic BM transplants, LCs are completely replaced by donor cells within a few weeks, providing the basis for the concept that LCs are derived from a mobile pool of BM-derived precursors that are constantly recruited to the skin (17, 18). In contrast to these results, we have recently discovered that under steady state conditions and after injuries that are only weakly inflammatory, BM precursors are not recruited to the skin and LCs are maintained by a stable renewable population present in the skin (19). However, as a consequence of UV-light induced skin inflammation, blood-borne LC precursors are actively recruited to the skin and replace resident LCs (19). The remarkable stability of LCs in the skin during steady state conditions contrasts with DC populations in other organs that undergo constant replacement by circulating BM derived precursors. A major difference between earlier studies and ours is that we transplanted mice with syngeneic rather than allogeneic BM. Importantly, we found that LCs remained of host origin for at least 18 months after transplantation of a purified population of allogeneic hematopoietic stem cells or T cell depleted (TCD) BM, but are replaced by donor-derived LCs if donor T cells are administered together with BM (Merad et al. Manuscript submitted). By contrast, dermal DCs as well as DCs in the blood, liver and lymphoid organs were replaced whether or not donor T cells were added to the graft. Given that allogenic T cells induce graft versus host disease (GVHD) it seems likely that, similar to the effect of UV-irradiation, GVHD associated skin inflammation promotes recruitment of BM derived LC precursors.

3.2. Human Studies

The study of LC chimerism in humans are difficult due to the lack of identifiable markers. Nonetheless, in an elegant study Emile et al. analyzed LC chimerism in 8 children with MHC class II deficiency (Bare Lymphocyte Syndrome) transplanted with allogeneic BM, and searched for donor MHC II$^+$ LCs in the skin after transplantation

(20). Consistent with our findings, donor LCs were not detected in the 2 children reconstituted with TCD allogeneic BM despite unequivocal engraftment of donor cells in the peripheral blood and dermis, while donor LCs were found in children who received whole allogeneic BM grafts. Although these results must be confirmed in a larger number of patients, they suggest that in humans as well as mice LC chimerism after allogeneic BM transplantation is induced only in the presence of alloreactive T cells. Moreover, persistence of host LCs more than 1 year after transplantation of allogeneic BM has been reported in patients (21). These results suggest that in humans, like mice, LC are remarkably stable in the skin and their replacement by circulating precursors is an active phenomenon dependent on the degree of the inflammatory injury.

3.3. LCs: Two Populations with Separate Ontogeny

Our finding that LCs self-renew throughout life in quiescent skin, and are replaced by circulating precursors only in the presence of inflammatory injuries, suggest that LCs with separate ontogeny exist. Interestingly, similar ontogeny has been described for macrophages, a population of antigen presenting cells found in most tissues. Two populations of macrophages are present in the body and include exudate and inflammatory macrophages. Exudate macrophages are found only in inflamed tissues, where they are recruited through a chemokine gradient induced by monocyte-chemokine protein-1 (MCP-1), they do not proliferate in situ and are thought to derive from a monocyte that originate in adult BM. Resident macrophages are present in non-inflamed tissues independently of MCP-1 (22), can proliferate in situ (22) and are thought to derive from primitive/fetal macrophages that originates in the yolk sac and in the fetal liver preceding that of BM derivation of typical monocytic cells (23-27). Similarly, LCs are already found in human embryos at 8.5 weeks of gestation and in mice and rats embryos at fetal day 15. LCs are found in non-inflamed and inflamed skin, and can proliferate in the skin (19, 28-30). Although, LCs are found in normal numbers in MCP-$1^{-/-}$ mice (31), their recruitment to injured skin is dependent on MCP chemokines (19). Based on these studies and our results, we hypothesize that 2 populations of LCs with separate ontogeny exist. One population of LCs proliferates in situ, resides in the epidermis under steady state conditions and originates from fetal hematopoietic progenitors that seed the skin during embryonic life independently of MCP chemokines. The other population of LCs seeds only inflamed skin in response to MCP chemokines and originates in adult BM.

Other murine skin hematopoietic cell populations share a similar life cycle with LCs. These include epidermal δ T cells and dermal mast cells. Mast cells represent a heterogenous population of hematopoietic cells that express high affinity IgE receptor (32). Mast cells are present in most tissues including the skin where they localize mainly in the dermis (32). Similarly to LCs, dermal mast cells were shown to self-renew in the skin and to persist in quiescent skin more than six months after congenic BM transplantation (33, 34). By contrast, after exposure to UV light, host mast cells were depleted and replaced by donor mast cells in 2 to 3 weeks (35). Epidermal T cells also called "dendritic epidermal T cells" express a δ T-cell receptor with minor diversity (Vβ3/Vβ1) (36). They derive only from fetal hematopoietic stem cells (HSC) precursors that seed the skin during fetal life and renew in the skin throughout life (37). These results suggest that the skin provide an environment suitable for self-renewal of leukocytes population.

4. UV SKIN INFLAMMATION MODEL TO STUDY THE HEMATOPOIETIC LINEAGE OF LCs

Although LCs are hematopoietic cells, their hematopoietic lineage as well as their immediate precursor in the blood are still unclear. This was due to the lack of pre-clinical model allowing to explore the recruitment of LC precursors to the skin and their differentiation into LCs. Our finding that circulating BM-derived cells can give rise to LCs in inflamed skin provides a model to explore this question. Clonogenic common myeloid (CMP) (38) and lymphoid precursors (CLP) (39) have been identified in murine BM and shown to give rise exclusively to all myeloid and lymphoid cells, respectively. We and others have found that both CMP and CLP can differentiate into DCs in lymphoid organs including spleen, LNs and thymus (40, 41). We have also shown that CMP are more efficient on a per cell basis to give rise to DCs in the spleen and LNs, compared to CLP (40, 42). Although the lineage of DCs in lymphoid organs is starting to be unraveled, the origin of DCs in non-lymphoid tissue including LCs in the skin, is still unclear. One study found that a lymphoid progenitor able to give rise to T cells, NK and DCs in lymphoid organs also gives rise to LCs in mice exposed to UV (43). In contrast, myeloid cells including circulating monocytes and $CD14^+$ dermal macrophages have been shown to give rise to LCs in vitro (44, 45) but the capacity of these cells to give rise to LCs was not tested in vivo. Human monocytes were also shown to give rise to DCs in vitro (46) while murine monocytes were shown to differentiate into DCs after migration from the periphery to the draining LNs (47, 48). Recently, two populations of circulating monocytes with different capacity to give rise to DCs were identified (49). The results of this study shows that $CD11b^+Gr.1/Ly6G^+$ expressing the MCP receptor CCR2 but not $CD11b^+$ $Gr.1/Ly6G^-CCR2^-$ monocytes differentiate into DCs in inflammatory sites (49), and it will be interesting to analyze the capacity of each of these populations to give rise to LCs in vivo using a model of UV-skin inflammation model.

5. CHEMOKINES AND LCs

The migration of leukocytes to inflammatory sites depends on a cascade of discrete events mediated, in part, by chemokines and their receptors (50-52). During skin inflammation, numerous chemokines are secreted in the skin, including CCL5 (also known as RANTES), a ligand for CCR1 and CCR5 (53); CCL2 (MCP-1), a ligand for CCR2(54); CCL22 (MDC) and CCL17 (TARC), ligands for CCR4(55, 56); CCL20 (MIP-3α), the ligand for CCR6 (57, 58) and CCL9 (MIG), CCL10 (IP-10) and CCL11 (ITAC), ligands for CXCR3 (59, 60). In addition, several chemokines are made constitutively in normal skin, including CXCL12 (SDF) (61) and CCL27 (CTACK) (62). We have recently found that transplantation of $CCR2^{-/-}$ BM cells into mice exposed to UV light resulted in delayed LC reconstitution, suggesting that CCR2 chemokine ligands play an important role in the recruitment of LCs to inflamed skin. Importantly, CCR2 chemokine ligands do not play a role in LC recruitment to the skin in steady state conditions as $CCR2^{-/-}$ mice have normal numbers of LCs in the skin. However the ability of $CCR2^{-/-}$ cells, after a 4 to 8 week delay, to give rise to normal numbers of LCs indicates that other chemokines may also contribute to LC precursor recruitment (19). CCL20/MIP-3α is another chemokine that has been shown to play a role in the recruitment of human $CD34^+$ derived LCs in vitro (63). In contrast, $CCR6^{-/-}$ mice that lack the receptor for CCL20 have normal numbers of LCs in the skin. These results suggest

that similarly to CCR2 chemokines ligands, CCL20 does not play a role in the seeding of LC precursors to the skin in early life, but does not preclude CCL20 from playing a role in the recruitment of LCs to inflamed skin.

6. LCs AND GVHD

Allogeneic BM transplantation is the treatment of choice for a variety of malignant and non-malignant disorders. Transplantation of allogeneic BM is usually administered after myelo-ablative therapy, to rescue hematopoiesis and to administer allogeneic T lymphocytes that are able to recognize and eradicate tumor cells (5). This effect is called the graft-versus-tumor effect. The counterpart of the graft-versus-tumor effect, is the development of GVHD which occurs when donor-derived T cells recognize and react to histo-incompatible recipient antigens leading to a variety of host tissue injuries (1-5, 64). GVHD is the major cause of morbidity and mortality after allogeneic BM transplantation, even when siblings are matched at the human leukocyte antigen (HLA) locus (65-67). GVHD occurs in both acute and chronic forms, each with different kinetics and distinctive pathology (3). The skin is the organ the most affected by GVHD and clinical symptoms range from a simple rash to a dramatic epidermolysis (3). Other affected organs are the gut, the liver, the lung and lymphoid organs (3). Chronic GVHD occurs less than 100 days after transplantation and affects the same tissues, in addition to the joints and the mucosal surfaces, with an incidence of 40 to 60% in transplant recipients surviving more than 100 days (3, 68). The principal strategies to prevent GVHD center around the depletion of donor T cells (69). However, this may lead to the loss of the graft-versus-tumor-effect, and to an increased risk of infections and graft failure.

More recently, advances in our understanding of basic immunology have underlined the central role of host DCs as key stimulators of donor T cells, inducing GVHD. In a pioneer study, Schlomchik et al. showed that mice lacking host DCs at the time of BM transplant do not develop GVHD upon transplantation of donor CD8 T cells (70). In a similar model, depletion of host liver DCs prior to transplantation of donor allo-reactive T cells, was shown to prevent liver GVHD (71). We have recently found, that by contrast to other DC population including spleen, liver, kidney and blood DCs, achievement of LC chimerism in the skin depends on the presence of donor T cells in the graft (Merad et al. manuscript submitted).

The observation that high levels of host LCs in the skin persist in animals after TCD BM transplantation has important implications for the development of GVHD in patients who undergo this procedure. Our results suggest that after allogeneic transplantation, DC chimerism in the blood does not correlate with DC chimerism in skin, suggesting that host DCs may persist in patients after allogeneic BM transplantation even if they are found to be fully chimeric in the blood. Previous studies have shown that host DCs are essential for both the activation (70-72) and effector phases of acute GVHD(73). Interestingly, in the latter study donor T cells induced GVHD only if host hematopoietic cells were present in target tissues (73). In this regard, host LCs persisting in skin may be responsible for the continued activation of donor T cells and for the production of cytokines that were found to be critical for the tissue damage observed in GVHD(73) .

In conclusion, we have recently showed that epidermal LCs self-renew in quiescent skin during adult life and are replaced by BM-derived hematopoietic precursors only during inflammatory injuries. In addition, we have found that this unique cycle of

homeostasis play an important role in transplant immune reactions. These findings may lead to new clinical strategies for the prevention of GVHD.

7. REFERENCES

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ROLE OF THE CD19 AND CD21/35 RECEPTOR COMPLEX IN INNATE IMMUNITY, HOST DEFENSE AND AUTOIMMUNITY

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1. INTRODUCTION

Humoral immune responses to foreign and self-antigens must be tightly regulated to facilitate protective immunity to pathogens while avoiding autoimmune responses. The outcome of these responses is determined in part by signals generated through the B lymphocyte antigen receptor (BCR). These signals are further supplemented and fine-tuned by other cell-surface molecules that modify and provide a context for BCR signal transduction[1]. Such molecules, or "response regulators", influence these events by positively or negatively biasing the context of BCR signaling, thus establishing appropriate signaling thresholds. Response regulators amplify or dampen BCR signaling by regulating the activity of intracellular kinases, phosphatases, and other effector proteins. Included among the list of BCR signal transduction response regulators is CD19, which integrates multiple intracellular signaling pathways. On the B cell surface, CD19 interacts directly with CD21 (complement receptor 2, CR2), a receptor for the C3d complement cleavage product that forms covalent bonds with foreign Ags or immune complexes to effectively link innate and acquired immunity. This review summarizes recent findings that have clarified how the CD19/CD21 receptor complex functions to regulate B cell responses in host defense and autoimmunity.

2. CD19

2.1. CD19 Structure and Expression

CD19 is a 95,000 M_r transmembrane glycoprotein of the Ig superfamily expressed by the B cell lineage from the early pre-B stage until its loss during plasma cell

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differentiation, as well as on the surface of follicular dendritic cells (FDCs)[2,3]. The extracellular domain of CD19 contains two C2-type Ig-like domains separated by a smaller, potentially disulfide-linked domain, as well as an extensive and highly conserved cytoplasmic domain[4, 5]. CD19 density on the cell surface is highly regulated during development, with similar expression levels by all mature conventional B cells from different peripheral lymphoid tissues[2, 6]. Although mature mouse B cells express ~3-fold higher CD19 levels than immature B cells, B-1 cells express slightly higher CD19 levels than conventional B cells[3]. CD19 expression levels are not significantly influenced by treatment of B cells with anti-IgM antibodies, lipopolysaccharide, or IL-4[2, 7].

CD19 interacts on the cell surface with CD21, as well as CD81, a broadly expressed member of the tetraspans family of cell-surface molecules[8]. Tetraspans family members are involved in multiple diverse signaling pathways[9]. Although CD81-deficient ($CD81^{-/-}$) mice are surprisingly normal, CD19 expression is halved in the absence of CD81 expression, and B cell signaling is reduced[10-13]. CD81 physically associates with another broadly-expressed cell surface molecule, CD225 (Leu-13), that has unknown function[14-16]. Thus, CD19 provides a B lineage-specific component for a receptor complex containing ubiquitously expressed molecules.

2.2. CD19 Signaling Function

CD19 function intersects with multiple signaling pathways crucial for modulating intrinsic and antigen receptor-induced signals. There are nine highly conserved tyrosine residues within the ~240 amino acid CD19 cytoplasmic domain[5]. Most of these tyrosines provide functionally active SH2 domain-recognition motifs that mediate recruitment of regulatory molecules to the cell surface[17-19]. Most significant is that CD19 functions as a specialized adapter protein for the amplification of Src-family protein tyrosine kinase (PTK) activity through a mechanism termed "processive amplification"[20-23]. Lyn activated endogenously or following BCR engagement is the primary kinase that phosphorylates CD19[21]. Following BCR or CD19 ligation, Lyn binding to phosphorylated CD19 results in amplified Lyn kinase activity[20-22]. The recent observation that phosphorylated CD19 preferentially localizes within detergent-insoluble lipid raft microsignaling domains suggests that CD19 may also influence the spatial distribution of Lyn at the membrane/cytoplasm interface[24, 25]. CD19 phosphorylation and amplification of Lyn kinase activity facilitates CD19 interactions with Vav and the p85 subunit of phosphatidylinositol 3 (PI3)-kinase, and initiates downstream events including the augmentation of $[Ca^{2+}]_i$ responses[20, 26-31]. In addition, CD19 interacts with other signaling molecules through its phosphorylated tyrosine residues, including Grb2, SOS, PLC-γ2 and the c-Abl PTKs[21, 24, 32]. The functional significance of these interactions is yet to be defined.

An important function of CD19 is regulating CD22 signaling. CD19 amplification of Lyn kinase activity is required for optimal CD22 phosphorylation and activation of the CD22/SHP1 regulatory pathway[26]. CD22 phosphorylation also induces formation of a CD22/Shc/Grb-2 ternary complex that may downregulate $[Ca^{2+}]_i$ responses through SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) recruitment[33]. As a consequence of these events, CD19 and BCR co-ligation dramatically lowers the threshold for B cell activation in vitro,[34, 35] and regulates mitogen-activated protein kinase

activation and proliferation[21, 34, 36]. CD19 thereby functions as a key regulator of B cell transmembrane signaling through its regulation of multiple signaling pathways.

2.3. CD19-Deficient and CD19-Transgenic Mice

That CD19 is an intrinsic response regulator[37] has been revealed in studies of mice that lack or overexpress CD19[38-40]. B cells develop normally in CD19-deficient ($CD19^{-/-}$) mice, but are reduced in number by ~50% in the periphery. B cells from these mice are hyporesponsive to most transmembrane signals, including BCR ligation and mitogens, leading to deficiencies in proliferation, clonal expansion and differentiation[2, 3, 38, 41, 42]. In addition, although $CD19^{-/-}$ B cells express cytoplasmic signaling molecules at normal levels, BCR ligation-induced phosphorylation of most downstream signaling molecules is reduced[20]. $CD19^{-/-}$ mice generate modest humoral immune responses and have reduced germinal center formation[2, 3, 38, 40-42]. By contrast, B cells from transgenic mice that overexpress human CD19 ($hCD19TG^{+/+}$) are hyperresponsive to transmembrane signals, proliferate at elevated levels, and generate elevated humoral immune responses[2, 3, 38, 39, 41, 42]. The number of B cells exiting the bone marrow and entering the circulating B cell pool is reduced by >95% in $hCD19TG^{+/+}$ mice, presumably the result of enhanced negative selection. Peritoneal B1 B cell numbers correlate positively with CD19 expression levels since B1 cell development is severely decreased in $CD19^{-/-}$ mice, while the frequency of peritoneal and spleen B1 cells in $hCD19TG^{+/+}$ mice is increased[3]. Thus, CD19 expression levels define signaling thresholds critical for expansion of the peripheral B cell pool[1, 23].

2.4. Models of CD19 Function

2.4.1. Costimulatory Molecule Model

CD19 was initially regarded as a negative regulator of BCR signal transduction. This notion resulted from antibody crosslinking studies which demonstrated that anti-CD19 monoclonal antibody binding inhibited proliferative responses, $[Ca^{++}]_i$ mobilization and differentiation subsequent to BCR crosslinking[43-45]. By contrast, crosslinking CD19 with the BCR can also synergistically augment B cell proliferation in vitro[37, 46]. Moreover, coligating CD19 with the BCR lowers the number of surface IgM molecules required for inducing B cell $[Ca^{++}]_i$ mobilization and proliferation[34]. This costimulatory effect led to the suggestion that C3d fragments covalently bound to antigen may coligate the CD19/CD21 complex with the BCR in vivo[47]. In this context, CD19 and BCR signaling would be upregulated when antigen-specific B cells encounter antigen-C3d complexes in vivo such as at sites of inflammation and complement activation. Although this hypothesis has not been formally proven, it provides a concept that generally explains CD19 function during immune responses. However, one concern with this concept is that CD21-deficient mice demonstrate a modest phenotype relative to CD19-deficient mice[48, 49]. At a minimum, this demonstrates that CD19 serves functions in addition to mediating CD21-initiated signals as we have recently demonstrated[50].

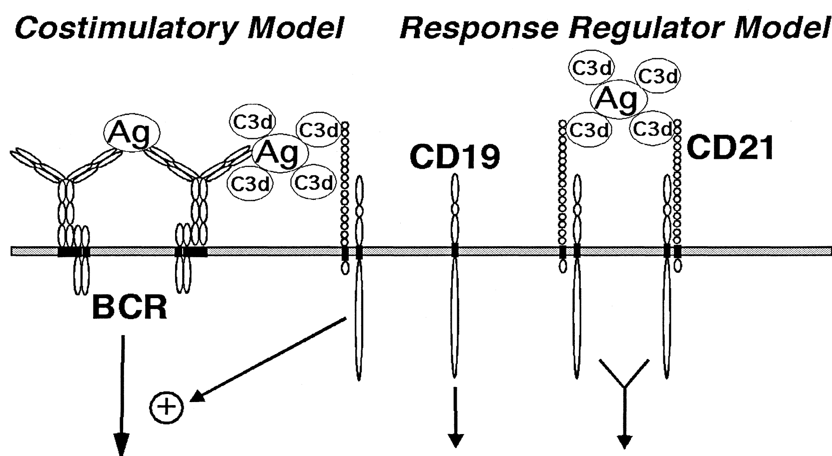

Figure 1. Models for CD19 function in vivo. In the response regulator model, CD21 associated with CD19 binds antigen complexes covalently modified with the C3d fragment of complement (C3d-Ag-C3d) independent of BCR specificity. In the costimulatory model, Ag-C3d complexes crosslink Ag-specific BCRs and CD19 by engaging both CD21 and the BCR.

2.4.2. *Response Regulator Model for CD19 Function*

Studies of mice that lack or overexpress CD19 confirm that CD19 functions as an intrinsic response-regulator[37]. That the majority of mature B cells are uniformly affected by loss or overexpression of CD19 suggests that CD19 regulates B cell function independent of BCR engagement[38-40]. Thus, CD19 functions as a general rheostat to adjust B cell signal transduction independent of B cell antigen-specificity. Thereby, intrinsic CD19 expression levels could regulate B cell activity autonomously, without a need for BCR engagement and independent of CD21 expression or engagement. CD19 may itself possess ligand-binding activity, although this remains to be demonstrated. Alternatively or in addition, C3d-antigen complexes may crosslink CD21 molecules and thereby utilize CD19 to generate signals (Fig. 1). In both cases, CD19/CD21 complex engagement would generate transmembrane signals that could synergize with BCR-induced signals and thereby result in augmented $[Ca^{++}]_i$ and proliferative responses. This is supported by the observation that simultaneous CD19 engagement and BCR engagement generates augmented responses without the need to physically crosslink the BCR and CD19 complexes with each other. Regardless of which model is operable, CD19 synergistically functions as both a co-stimulatory molecule and a response-regulator in the modulation of both basal and BCR-induced signaling in B cells.

3. CD21

3.1. CD21 Structure, Expression, and Function

CD21 is expressed both on B cells and FDCs. CD21 is expressed first by $IgM^{hi}IgD^{lo}$ transitional B cells and is expressed by most mature B cells, where CD19 is usually

expressed in molar excess of CD21[51, 52]. Uniquely, the highest levels of CD21 expression are found on marginal zone B lymphocytes. While CD21 and CD35 (CR1, complement receptor 1) are encoded by different genes in humans, these two receptors are alternative splice products of the same *Cr2* gene in mice[53]. CD21 contains an extracellular domain of 15 or 16 repeating structural elements called short consensus repeat (SCR) domains, a membrane-spanning region, and a 34 amino acid cytoplasmic domain[54, 55]. The short cytoplasmic tail is devoid of known signaling motifs, but is required for CD21 internalization upon ligand binding[56]. In mice, CD35 is generated by the addition of six SCRs to the amino-terminal end of the CD21 protein[53]. The iC3b/C3d,g cleavage fragments of complement component C3 form covalent bonds with foreign antigens or immune complexes to generate C3d(g)-antigen complexes that bind to CD21[57] and signal through the CD19 complex (Fig. 1). Mouse CD35 binds both C3b and C3d, and also associates with CD19[6]. CD35 serves as a cofactor for the hydrolysis of C3b-Ag complexes into C3d,g-Ag, which allows CD21 binding[58]. This process is important for the processing of Ag-antibody complexes and the final deposition of C3d-Ag complexes on the surface of B cells and follicular dendritic cells through CD21. Thus, C3d functions as a ligand for the CD19/21 complex, thereby linking complement activation and B cell signal transduction.

3.2. CD21/35 Deficient Mice

Three independent lines of CD21/35 deficient (CD21/35$^{-/-}$) mice have been generated[48, 49, 59]. However, the CD21/35$^{-/-}$ mice generated by Ahearn et al.[48] expresses a hypomorphic cell-surface CD21/35 protein[50, 59]. In these mice, splicing-out of the gene-targeted exon in the *Cr2* locus results in a smaller cell-surface CD21/35 protein (CD21/35^{hypo}) expressed at ~40% of wild type levels that retains ligand-binding activity. Regardless, B cell development is relatively normal in CD21/35$^{-/-}$ mice[49, 59]. Although B1a cell development was reported to be reduced in CD21/35^{hypo} mice,[48] we have been unable to detect such a reduction in these particular mice[50] or in our more recently generated CD21/35$^{-/-}$ mouse[59]. All three lines of mice with a genetically disrupted *Cr2* locus exhibit decreased antibody responses to challenge with low-dose T cell dependent (TD) antigens,[48, 49, 59] but generate near normal primary responses to high-dose TD antigens, antigen/adjuvant challenge, or during secondary responses[49, 50, 59-61]. CD21/35$^{-/-}$ mice generated in our lab also exhibit significantly reduced germinal center formation, reduced levels of natural serum antibody, and impaired antibody responses to TI-1 and TI-2 antigens. Most strikingly, IgG3 production is markedly impaired in these mice[59].

Many TI and TD antigens activate complement via the alternative pathway or activate the classical pathway after binding natural antibodies that are reactive with pathogenic microorganisms[62-64]. The covalent attachment of C3 cleavage products thereby provides a mechanism by which antigens can be directly targeted to complement receptor-expressing cells, such as marginal zone B cells which express high levels of CD21. TI antigens such as pneumococcal polysaccharide and Group B streptococcus (GBS) capsular polysaccharide preferentially localize on marginal zone B cells and FDCs along with C3[65, 66]. In the absence of C3 or with decreased levels of CD21/35 expression, marginal zone B cells do not bind the TI antigens TNP-Ficoll or GBS PS efficiently in vivo[65, 67]. CD21/35 is required for the localization of C3d-antigen complexes in vivo since CD21/35$^{-/-}$ mice are unable to focus tetrameric C3dg complexes to the marginal zones of splenic follicles or on the surface of B cells in vivo[59]. However, C3dg tetramers effectively reveal CD21 ligand binding in wild type mice and exhibit functional activity

on normal, but not $CD21^{-/-}$ B cells, including augmentation of anti-IgM mediated intracellular Ca^{++} flux and activation of p38 MAP kinase[59, 68]. Consistent with this finding, poor responsiveness of neonates to TI-2 antigens is attributed to their insufficient CD21 expression and lack of marginal zone B cells[69]. Thus, CD21/35 may regulate the production of TI antigen-elicited antibodies by targeting C3d-antigen complexes to marginal zone B cell populations, which are produced in normal to augmented numbers even when CD21/35 expression is reduced[59, 70]. Based on the high level of C3dg-tetramer binding observed for naïve B cells in vivo, C3-tagged complexes may augment CD19 function and thereby enhance B cell transmembrane signaling in a large fraction of B cells. This indicates a critical role for CD21/35 not only in the rapid trapping of C3dg-Ag-complexes in marginal zones, but in the localization of C3dg-decorated antigen to B cells in a manner independent of their antigen receptor specificity.

4. CD21/35 REGULATES PROTECTIVE IMMUNITY TO BACTERIA

4.1. Complement Is Required For Protection Against Encapsulated Bacteria

Protection against extracellular bacterial infections relies on complex and overlapping interactions between innate and adaptive immune responses. Innate protection requires complement activation since complement-deficient patients are susceptible to infections with encapsulated bacteria such as *Streptococcus pneumoniae* (*S. pneumoniae*), the predominant cause of community-acquired pneumonias, septicemia, otitis media and meningitis[71-73]. The C3 complement component is particularly important for the efficient opsonization, lysis, and clearance of bacteria[74-78]. Administration of cobra venom factor (CVF), a convertase analog that depletes C3, leads to impaired opsonization and clearance of *S. pneumoniae* by anti-capsular antibody[79]. Furthermore, C3-deficient ($C3^{-/-}$) mice have impaired clearance of *S. pneumoniae*[80]. The decoration of pathogen-derived antigens with C3 breakdown fragments, C3b, iC3b, and C3d provides signals important for the opsonization or destruction of bacteria by phagocytic and nonphagocytic cells bearing appropriate receptors. Furthermore, C3-decorated antigen complexes promote the development of protective humoral immunity. Thus, complement bridges innate and acquired immune responses and is important for complete protection against encapsulated bacteria.

4.2. CD21/35 Is Required For Protection Against Encapsulated Bacteria

We recently investigated the role that CD21/35 plays in the protective immune response to *S. pneumoniae* infection[59]. An important role for CD21/35 during innate and adaptive immune responses was revealed by the dramatic susceptibility of $CD21/35^{-/-}$ mice to acute lethal *S. pneumoniae* infection, despite immunization[59]. CD21/35 expression significantly enhanced the generation of protective humoral immune responses to low dose live bacterial challenge as well as to immunization with heat-killed bacteria. Complete protection of $CD21/35^{-/-}$ mice during bacterial challenge was achieved when mice were given either multiple immunizations or 100-fold higher doses of immunogenic heat-killed bacteria than wild type littermates. These results indicate an

impaired ability of low-concentration antigens to rapidly generate protective responses in $CD21/35^{-/-}$ mice,' despite the intrinsic adjuvanticity of intact bacteria. Since CD21/35 selectively targets antibody-antigen-C3d complexes to B cells and follicular dendritic cells (FDCs),[61, 81] CD21/35 is likely to also selectively focus bacterial antigens to these cells during acute infections. Thereby, CD21/35 expression may facilitate the processing and presentation of antigens at low concentrations, particularly in the absence of pre-formed or natural antibodies where Fc receptor interactions may also facilitate immune complex processing[82-86].

The role of CD21/35 in localization of blood-borne antigens on marginal zone B cells may be central to the susceptibility observed for $CD21/35^{-/-}$ mice. Using model antigens, others have proposed that marginal zone B cell trapping of antigens has a critical role in host defense against bacterial pathogens since the proximity of marginal zone B cells to marginal sinuses insures that they are amongst the first population of cells to encounter blood-borne antigens[87]. Consistent with this, marginal zone B cells generate rapid activation, proliferative and Ig secretory responses[88]. Thus, TI and TD bacterial antigens that activate complement are likely to become focused onto marginal zone B cells by virtue of their high level CD21/35 receptor expression and proximal association with the splenic microvasculature. Thereby, CD21 crosslinking and its activation of the CD19 regulatory pathway may make B cells more responsive to transmembrane signals[89]. This provides yet another molecular example of how the innate and adaptive immune responses cooperatively interact to hasten antigen recognition and enhance the generation of nascent humoral immune responses during life-threatening encounters with virulent pathogens.

The explanation for the impaired generation of protective immunity to encapsulated bacteria in $CD21/35^{-/-}$ mice may also be due to impaired IgG3 antibody responses in the absence of CD21/35 expression. IgG3 is the major mouse IgG isotype produced in response to TI-2 antigens[90]. Mice unable to produce IgG3 in response to TI-2 antigens, such as *xid* and γ3 gene-disrupted mice are more susceptible to pneumococcal infection since IgG3 anti-PS antibodies may be the major opsonin[91, 92]. In humans, IgG2 is the major IgG isotype produced in response to most TI antigens during late ontogeny[93]. Human IgG2-deficiency is associated with increased susceptibility to chronic sinopulmonary infections, highlighting its importance in resistance to encapsulated bacteria[94]. Mouse IgG3 demonstrates superior binding to polysaccharide antigens which results in enhanced activation of effector function, including complement activation and Fc receptor binding[95]. Therefore, deficient IgG3 production by $CD21/35^{-/-}$ mice may be the primary explanation for their increased susceptibility to bacterial infection. Given the essential role of B cells in protection from encapsulated bacteria[96] and the importance of marginal zone and B1a B cell populations in IgG3 responses,[67, 97] localization of C3d complexes to these B cell subsets may be central to the generation of protective antibody responses during acute infections.

5. C3d AS A MOLECULAR ADJUVANT

Covalently linking C3d fragments to antigens results in augmented humoral responses. In the first demonstration of this, immunization of transgenic mice expressing BCR specific for hen egg lysozyme (HEL) with recombinant lysozyme fused to multiple copies of C3d lowered the dose of antigen required for antibody responses comparable to

HEL alone by at least 1,000-fold[35]. Likewise, immunization of mice with DNA-based vaccines encoding HIV-1 gp120 fused to multiple copies of C3d results in higher antibody responses with enhanced avidity maturation when compared to gp120 immunization alone[98, 99]. Immunization of mice with DNA-based vaccines consisting of either influenza or measles virus hemagglutinin fused to multiple copies of C3d also results in more rapid antibody responses and higher neutralizing titers than immunization with antigen alone[100-102]. C3d also functions as a mucosal adjuvant for influenza virus hemagglutinin administered intranasally[103]. Finally, antibody titers and isotype switching in response to pneumococcal capsular polysaccharide type 14 are enhanced when it is conjugated to C3d[104]. C3d is therefore under consideration as an effective molecular adjuvant that may be safe and acceptable for use in vaccines.

C3d is postulated to augment humoral responses by targeting antigen complexes to B cells and FDCs that express CD21/35. In one model, coligation of the BCR and CD19/CD21 by C3d-antigen complexes is proposed to lower the signaling threshold required for B cell activation and expansion (Fig. 1)[34, 47, 105, 106]. Alternatively, C3d-antigen complexes may crosslink cell surface CD21, which can generate transmembrane signals through CD19 regardless of B cell antigen receptor specificity (Fig. 1)[89]. However, a direct role for CD21/35 in this process had never been investigated. Therefore, we assessed the importance of CD21/35 receptor engagement in mediating the immunostimulatory effects of C3d by immunizing CD21/35$^{-/-}$ mice with two antigens, streptavidin (SA) and recombinant HIV-1 envelope glycoprotein gp120$_{IIIB}$ (gp120), either alone or complexed to multimers of C3dg or C3d, respectively[107]. Humoral responses to soluble SA and gp120 were impaired in CD21/35$^{-/-}$ mice, indicative of the critical role that CD21/35 expression plays in antibody responses to antigens administered in the absence of adjuvants[59]. Unexpectedly, IgG antibody responses to SA-C3dg and gp120-C3d were significantly augmented in CD21/35$^{-/-}$ mice in comparison to these antigens given without C3d(g). These effects were also reflected in the markedly enhanced frequency of SA-specific antibody producing cells in both CD21/35$^{-/-}$ and wild type mice immunized with SA-C3dg. Remarkably, this study demonstrates that C3d can function as a molecular adjuvant through CD21/35 receptor-independent pathways.

Adjuvants function in multiple ways, which may include enhancing the in vivo half-life of antigens, augmenting antigen processing and presentation, and inducing cytokine production[108]. Although the precise mechanisms through which C3d functions as a molecular adjuvant remain to be elucidated, several hypotheses can be offered. First, C3d could function as a simple protein carrier. In support of this, OVA functions as an adjuvant for pneumococcal polysaccharide in a manner similar to C3d[104]. Similarly, coupling biotinylated chicken gamma globulin to SA significantly augmented SA antibody responses in both wild type and CD21/35$^{-/-}$ mice to levels similar to that elicited by SA-C3d tetramers (unpublished observations). Alternatively, attachment of C3d to antigens could prolong the in vivo half-life of antigen, perhaps by forming molecular aggregates or facilitating molecular interactions. Finally, C3d interacts with numerous serum proteins, cell surface receptors, and membrane-associated regulatory proteins[109]. Thus, C3d aggregates may bind antigen complexes to proteins other than CD21/35, which also enhance humoral responses. Given the unexpected finding that C3d augments humoral immune responses through CD21/35-independent pathways, understanding the mechanisms of C3d action may provide important insight into the identity of other molecules with adjuvant activity that will allow the design of even more potent vaccines.

6. CD19 AND CD21/35 EXPRESSION REGULATE AUTOIMMUNITY

6.1. CD19 Expression Levels Regulate the Development of Autoimmunity

The level of CD19 expressed by B cells influences the development of autoimmunity. For example, lines of hCD19TG$^{+/+}$ mice that overexpress CD19 by as little as 20% produce autoantibodies in a genetic background not normally associated with autoimmunity[110]. Additional autoantibody specificities and titers are manifest with further increases in CD19 expression[3]. Antinuclear antibodies, especially anti-spindle pole antibodies, as well as anti-single-stranded DNA, anti-double-stranded DNA, and anti-histone antibodies, and rheumatoid factor were induced in hCD19TG$^{+/+}$ mice, but not wild type littermate controls. Although multiple molecules involved in a common CD19 signal transduction pathway influence autoimmunity in mice, similar examples in humans have only recently become available. Systemic sclerosis (SSc) is a multisystem disorder of connective tissue characterized by sclerotic changes in the skin and internal organs. Autoantibodies are detected in more than 90% of SSc patients and are considered to play a critical role in the pathogenesis of SSc[111]. Surprisingly, CD19 and CD21 expression levels are 20% higher on B cells from SSc patients compared with healthy individuals, while the expression of other cell surface markers such as CD20, CD22, and CD40 is normal[110]. Like mice that overexpress CD19, the tight-skin mouse, a genetic model for human SSc, also contains spontaneously activated B cells and autoantibodies against SSc-specific target autoantigens[112]. Tight-skin mice also develop cutaneous fibrosis, like in SSc patients. In contrast to mice that overexpress CD19, mice that are CD19$^{-/-}$ are hyporesponsive to transmembrane signals as compared to wild type mice. CD19-deficiency in tight-skin mice results in quiescent B cells, with significantly reduced autoantibody production and skin fibrosis[112]. Thus, modest alterations in CD19 expression could contribute to the development of autoantibodies in humans. Moreover, subtle alterations in the expression or function of other regulatory molecules involved in the CD19 signal transduction pathway may also predetermine autoimmune susceptibility in other syndromes. Although speculative, it is possible that graded alterations in expression or function in these "response-regulators" may result in the spectra of autoantibody specificities that characterize different autoimmune diseases.

6.2. CD21 Regulates CD19 Expression: Implications For Autoimmunity

Altered CD21 function correlates with autoimmunity in mouse models[113-115]. In addition, self-reactive B cells with 60% reduced CD21 expression are not anergized by soluble self-antigen in mouse models of tolerance[50, 114]. Recent studies using CD21/35$^{-/-}$ and C4$^{-/-}$ mice suggest that complement and CD21 also regulate the elimination of self-reactive B cells, since lupus-prone *lpr* mice lacking CD21 or C4 have exacerbated disease, presumably due to increased autoantibodies[114]. Spontaneous autoimmunity due to the impaired clearance of immune complexes has also been found in C4$^{-/-}$ mice, but not in CD21/35$^{-/-}$ mice[116]. Although these studies suggest a direct role for CD21 in regulating B cell function and autoantibody production, this may actually reflect a role for CD21 in regulating cell surface CD19 expression.

The phenotypes of CD19$^{-/-}$, CD21/35$^{-/-}$ and C3$^{-/-}$ mice have demonstrated that CD19, CD21 and C3 expression are interrelated and may form a regulatory loop that influences B cell function. Specifically, CD21 expression is increased by >30% on peripheral B cells from C3$^{-/-}$ littermates[50]. This suggests that ongoing C3d,g generation may

chronically engage CD21, resulting in receptor internalization as occurs during inflammatory responses or in patients with systemic autoimmune disease[117]. CD21 engagement may result in increased CD19 turnover, which could limit signal transduction or partially desensitize B cells chronically stimulated through the CD19-CD21 complex. Consistent with this concept, CD19 expression levels are >20% higher on peripheral B cells of CD21/35^{hypo} mice[50] and ~50% higher on B cells from mice completely deficient in CD21/35 expression[59]. Increased CD19 expression on CD21/35$^{-/-}$ B cells may be functionally significant as similar increases in CD19 expression predispose mice to autoimmunity[3, 110]. Therefore, increased CD19 expression may explain why CD21/35-deficiency contributes to autoimmunity, since CD21 expression influences CD19 function indirectly by regulating its cell surface expression. Alternatively, or in addition, the CD19/CD21 complex may downregulate BCR signaling in B cells following CD21 hypercrosslinking by immune complexes[118, 119]. In this context, CD19/CD21 hypercrosslinking appears to sequester the available pool of intracellular Lyn away from other signaling molecules and thereby downregulates B cell responses to BCR ligation. In CD21/35$^{-/-}$ mice, C3d-bearing immune complexes may augment autoantibody production and predispose these mice to autoimmunity. Thus, tightly regulated CD19 and CD21/35 expression levels balance intrinsic signal transduction thresholds and B cell responsiveness to transmembrane signals.

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17

ROLE OF COMPLEMENT RECEPTOR 2 IN THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

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1. INTRODUCTION

A number of recent studies have suggested that complement receptor type 2 (CR2, CD21) may play a role in the development of systemic autoimmunity. This receptor, located primarily on B cells and follicular dendritic cells in mice with a broader distribution in humans, binds C3 degradation products that have become covalently bound to antigen or immune complexes in the process of complement activation. Its role in both normal immune responses as well as systemic autoimmune disease has been supported by studies of mice in which the gene has been knocked out by homologous recombination. Furthermore, it is structurally and functionally altered in the NZM2410 mouse model of lupus, and is a strong candidate gene for lupus susceptibility in this model. Based on its known functions, several mechanisms can be hypothesized to explain its potential role in the pathogenesis of systemic lupus erythematosus.

2. COMPLEMENT RECEPTOR 2

2.1. Structure, Expression, and Regulation of CR2

CR2 is a surface glycoprotein composed of 15 repeating 60-70 amino acid extracellular subunits termed short consensus repeats (SCRs), a transmembrane domain, and a short cytoplasmic tail. In humans, a 16 SCR form of CR2 has been identified that is generated by alternative splicing of a single exon [1, 2]. CR2 is located primarily on mature B cells and follicular dendritic cells (FDC) in mice, although it has also been identified on peritoneal mast cells [3] and on a subset of activated T cells [4]. In humans, it is expressed more broadly, and has been found on mature B cells [5], thymocytes [6, 7], a subset

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of CD4 and CD8 peripheral T cells [8, 9], FDC [10], basophils [11], keratinocytes [12], astrocytes[13], and epithelial cells [14]. Regulatory elements in the proximal promoter control the level of CR2 expressed on cells [15-19], and an intronic silencing mechanism controls cell and lineage specificity [20-23]. In mice, CR1 and CR2 are transcribed from a single gene by alternative mRNA splicing [24], while in humans they are derived from two distinct but closely linked genes on chromosome 1. A soluble form of CR2 has been identified in human serum and supernatants of human B cell lines. Soluble CR2 is likely generated by proteolysis and is believed to be shed from activated B cells [25, 26].

2.2. CR2 Ligands

All three pathways of complement activation, classical, alternative, and lectin, converge in the cleavage of C3 by convertase enzymes specific to each pathway. Cleavage of C3 results in the generation of C3b fragments that are covalently attached to the activating substrate and serve as ligands, along with C4b-bound antigens, for complement receptor type 1 (CR1/CD35). C3b is then inactivated to iC3b and C3d, which are the specific ligands for complement receptor type 2 (CR2/CD21) [27, 28]. Human CR2 interacts with its C3d ligand primarily via SCR2 [29]. In addition, human CR2 also binds Epstein Barr virus [30], CD23 [31], and interferon-α [32].

2.3. Functions of CR2

CR2 has been shown to have multiple effects on B and T cell responses, including the amplification of antigen-induced B cell activation through surface IgM (sIgM) [33-35], the rescue of peripheral B cells from sIgM-mediated apoptosis [36], the promotion of antigen processing and presentation of C3d-bound targets [37-41], the modulation of the expression of costimulatory molecules [42], the stabilization of the B cell receptor in lipid rafts [43], and the targeting of immune complexes to germinal centers in secondary lymphoid organs [44, 45]. Since CR2 has only a short cytoplasmic tail, it is likely that many of these functions occur through interactions of CR2 with CD19 and CD81 on the B cell surface, where these receptors form a multimolecular signal transduction complex [46, 47]. CR2 has also recently been shown to participate in the transfer of immune complexes [48], and thus may play a role in immune complex clearance. Mice deficient in CR2 by homologous recombination have defects in antibody responses to T-dependent and T-independent antigens, formation of germinal centers, generation of memory B cells, and the development of a normal natural antibody repertoire [49-54]. CR2 on both B cells and FDC are required for the generation of normal T-dependent antibody responses [55, 56].

3. COMPLEMENT RECEPTOR 2 IN AUTOIMMUNE DISEASE

3.1. Decreased CR2 Expression in Patients with SLE

Patients with lupus express levels of CR2 on their B cells that are approximately 50-60% lower than control patients [9, 57, 58]. Expression of CR2 may be decreased as a result of decreased gene transcription, increased receptor shedding, ligand-mediated downregu-

lation, or terminal differentiation. Since longitudinal studies have not yet been performed in patients with lupus, it is not clear whether decreased CR2 expression is a cause or a result of increased disease activity. Certainly, relative deficiency of CR2 in lupus may alter tolerance induction of B or T cells or impair immune complex clearance (see section 4), and may have important functional consequences irrespective of its mechanism.

3.2. Effects of CR1/CR2 Deficiency on Animals Models of Autoimmunity and Tolerance

In the MRL/*lpr* mouse model of lupus, levels of CR2 decrease prior to the development of clinically apparent disease [59], suggesting that a relative deficiency of CR2 may indeed contribute to the initiation or progression of autoimmune disease. In further support of this, CR1/CR2 deficiency has been shown to exacerbate or accelerate several animal models of autoimmunity and tolerance. On a mixed B6/129 background, CR1/CR2-knockout *lpr* mice develop marked splenomegaly and lymphadenopathy, increased anti-nuclear and anti-dsDNA antibodies, and increased glomerular deposits of immune complexes [60]. After more extensive backcrossing onto a B6 background, however, the main manifestation of CR1/CR2 deficiency in *lpr* mice appears to be on autoantibody production [61]. CR1/CR2 deficiency in the hen egg lysozyme (HEL) double transgenic model of B cell tolerance results in a break in tolerance, albeit incomplete, with normalization of B cell numbers and responsiveness [60], but continued impairment in production of serum anti-HEL. However, since both CR1 and CR2 are deficient in the knockout mice used in these studies, the phenotypes identified in these models cannot be attributed definitively to CR2.

3.3. Identification of *Cr2* as a Strong Candidate Gene for Lupus Susceptibility in Mice

Cr2, which encodes CR1 and CR2 in mice, has recently been identified to be a strong candidate gene for lupus susceptibility in the NZM2410 mouse model of lupus [62]. B6 mice have been generated that are congenic for the NZM2410 *Sle1c* lupus susceptibility interval, which includes *Cr2*. These congenic mice develop autoantibodies to chromatin but do not develop glomerulonephritis. CR1 and CR2 in these mice are increased in molecular weight because of a single-nucleotide polymorphism in the ligand binding domain of CR2 which introduces a novel N-linked glycosylation site and results in differential glycosylation. The altered amino acid was found by molecular modeling of the mouse gene sequence to be located at a site in the human receptor that is critical for receptor dimerization, and glycosylation at this site would be expected to alter dimerization. B cells from B6.*Sle1c* congenic mice were found to exhibit defects in C3d-ligand binding as well as CR2-mediated signaling. These data provide support for *Cr2* as a strong candidate gene for lupus susceptibility in the NZM2410 *Sle1c* interval. Furthermore, since the functions of CR2 were specifically affected in this model, these data suggest that CR2 rather than CR1 is responsible for the autoimmune phenotypes observed.

Nonetheless, although *Cr2* is a strong candidate gene for lupus susceptibility in this model, its role has not yet been proven. This awaits further narrowing of the *Sle1c* congenic interval as well as replacement of the altered *Cr2* gene products with normal proteins using transgenic technology. Although initial *in vitro* studies suggest that the polymorphism in the ligand binding domain is indeed responsible for the impairment in ligand binding, we have not yet ruled out a functional effect of the other 11 polymorphisms identified in this gene. However, if *Cr2* does prove to be the *Sle1c* lupus susceptibility gene, this model will provide a powerful tool for understanding the role of CR2 in the pathogenesis of lupus. Using knockin mice that express the *Sle1c* allele of CR2, the specific effects of this altered protein on tolerance induction and autoimmune disease can be isolated and studied.

4. COMPLEMENT RECEPTOR 2 IN PATHOGENESIS OF SLE

4.1. Effects of CR2 on B Cell Tolerance

CR2 may play a direct role in induction or maintenance of B cell tolerance. For example, coligation of CR2 with sIg may lower the threshold for B cell tolerance to autoantigens, just as it lowers the threshold for B cell activation. Thus, B cells that express lower levels of CR2 (as in human lupus) or an altered form of CR2 (as in NZM2410 mice) may not be tolerized appropriately. In addition, FDC in secondary lymphoid organs that express lower levels or altered forms of CR2 may not be able to target complement-coated autoantigen effectively. If this reservoir of self-antigen is important for the maintenance of B cell tolerance, then autoreactive B cells would be expected to develop under these conditions. Since expression of CR2 on both B cells and FDC is important in the generation of normal immune responses [55, 63], ligand binding to both cell types is likely to be important in the regulation of B cell tolerance.

4.2. Effects of CR2 on T Cell Tolerance

CR2 may also affect induction and maintenance of T cell tolerance. Although CR2 binds and internalizes antigen for presentation to T cells [38, 39], in the absence of sIg coligation, it does not induce upregulation of costimulatory molecules [39] and thus may be critical in the regulation of T cell tolerance. It is also possible that coligation of CR2 with sIg may alter the levels or types of costimulatory molecules upregulated and skew the T cell cytokine profile to create an environment that favors loss of tolerance to self-antigen[4]. Finally, CR2 may influence the development of regulatory T cells, such as $CD4^+CD25^+$ cells, which are decreased in the periphery in B cell-deficient mice [64], or NK-T cells (reviewed in [65]), which recognize antigen presented by $CD1d^+CR2^{high}$ marginal zone B cells.

4.3. Effects of CR2 on Immune Complex Clearance

Finally, CR2 may influence lupus pathogenesis by participating in immune complex clearance. Altered function or expression of CR2 in lupus may contribute to excessive deposition of immune complexes in tissues, resulting in tissue damage.

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18

COMPLEMENT REGULATION DURING PREGNANCY

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1. INTRODUCTION

The complement system has a significant role in innate immunity, in the inflammatory process, and in the adaptive immune response. Activated complement fragments also have the capacity to bind and damage tissues, especially in areas of inflammation. Cells must be sheltered from the harmful consequences of complement activation. To investigate the role of these molecules *in vivo*, we generated mice deficient in the expression of one of the molecules involved in complement regulation. The mouse Crry protein belongs to a family of molecules that regulates complement activation, protecting tissues from complement-mediated damage *Crry*$^{-/-}$ mice do not survive pregnancy due to abnormal complement deposition in the placenta. The Crry-deficient mouse, therefore, is a valuable model to study the role of complement and complement regulators during pregnancy. Herein we describe studies that analyze further the mechanisms by which the Crry deficiency affects fetal survival, clarifying our understanding of the role of innate immune responses in pregnancy.

2. THE COMPLEMENT SYSTEM

The complement system consists of plasma proteins that, once activated, are involved in many of the functions of the immune response and of inflammation.[1,2] Activation of complement promotes chemotaxis of inflammatory cells, generates proteolytic fragments that enhance phagocytosis by neutrophils and monocytes, and facilitates the clearance of immune complexes[3]. Lysis of cells and foreign organisms is mediated by the formation of the membrane attack complex. Activation occurs by three main mechanisms.[1] The classical pathway is initiated by the interaction of complement

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proteins with antigen-antibody complexes. The alternative pathway is initiated by the binding of complement fragments directly to the target such as microbial surfaces. The lectin pathway is initiated by plasma proteins, known as mannan-binding lectins, which bind to carbohydrate moieties on the surface of pathogens. These pathways share a common essential purpose consisting of the cleavage and subsequent activation of C3.

Activation of C3 plays a vital role in the biology of the system.[2] Cleavage of C3 generates several biologically active products that are responsible for most of the complement functions described above. One fragment, known as C3a, is a potent anaphylatoxin that binds receptors (C3aR) on leukocytes, endothelial cells, and other cells, resulting in the activation and secretion of soluble inflammatory mediators.[4,5] This peptide also induces chemotaxis and degranulation of eosinophils and mast cells resulting in the secretion of potent vasoactive and proinflammatory substances. Another fragment, C3b, and its related cleavage fragments known as iC3b and C3d, are ligands for complement receptor 1 and 2, and the ß2-integrins CD11b/CD18 and CD11c/CD18.[2] Furthermore, C3b can attach covalently to targets forming an essential part of the alternative pathway C3 convertase, and the C5 convertase enzyme complex, responsible for the amplification and adequate activation of C3 and C5.[2] A cleavage product derived from C5, C5a, is a potent inflammatory anaphylatoxin and chemotactic peptide that interacts with receptors (C5aR) on the surface of leukocytes and endothelial cells.[3] C5a also interacts with granulocytes and monocytes/macrophages causing increased chemotaxis, degranulation, adhesion to endothelial cells, and production of reactive oxygen intermediates. In mast cells it shares a comparable function with C3a inducing degranulation, resulting in the secretion of potent vasoactive and proinflammatory mediators. Another fragment, C5b, interacts with C6 and C7, binds to the target, and initiates the assembly of the C5b-9 membrane attack complex responsible for abnormalities in cell membrane function.[2]

3. REGULATION OF COMPLEMENT ACTIVATION

An important stage in complement regulation is at the level of C3.[6] Inhibition of C3 activation effectively suppresses both the classical, alternative, and lectin pathways of complement activation. Furthermore, inhibition of C3 activation avoids the formation of most of the complement mediators involved in humoral immunity, inflammation, and tissue destruction. This regulation is achieved by two main mechanisms.[6,7] First, certain proteins prevent the formation, or accelerate the dissociation, of the C3 convertase enzyme complex by a process known as decay accelerating activity. Second, degradation of activated C3 is mediated by Factor I, a serine esterase that cleaves C3b, but only in the presence of protein cofactors.

There are three membrane-bound proteins that regulate activation of C3 on the surface of host cells.[6,7] Decay accelerating factor (DAF) is a 70 kd protein present on most human and murine blood cells, endothelial cells, and epithelial cells, and is responsible for the inactivation of the C3 convertases. Membrane cofactor protein (MCP) is a ~45-70 Kd membrane-bound glycoprotein present on most human blood cells (except erythrocytes), endothelial, and epithelial cells. In mice, however, MCP expression is restricted to the testis.[8] MCP serves as a cofactor for Factor I cleavage of activated C4b and C3b. Finally, Crry is a widely distributed murine protein with MCP-like and DAF-like activities, exhibiting decay accelerating activity against the C3 and C5 convertase,

and also serving as cofactor for Factor I mediated cleavage of C3b and C4b.[9-12] Thus, Crry is a mouse functional homologue of human MCP and human DAF.

The importance of these regulators of C3 activation in protecting host cells from complement-mediated damage is highlighted by several experimental observations. *In vitro*, antibody-induced functional blockade of human DAF and MCP, or mouse Crry, increases C3 deposition on the surface of autologous cells and sensitizes them to complement lysis.[13-17] In addition, expression of human DAF and MCP on the surface of mouse cells protects them from human complement damage.[18] *In vivo*, antibody-mediated interference of rat Crry induces deposition of C3 in the vascular endothelium with a concomitant inflammatory reaction and cell injury.[16,17] Mice injected with a soluble form of Crry are resistant to complement-mediated organ damage in a murine model of antibody-induced glomerulonephritis.[19] Finally, blocking antibodies reacting against Crry are important to the pathogenesis of active Heymann nephritis, a murine model of human membranous nephropathy.[20] Thus, expression of these molecules is essential to protect cells from complement-mediated injury.

4. REGULATORS OF COMPLEMENT ACTIVATION DURING PREGNANCY

Since the mammalian embryo expresses antigens that are foreign to the maternal environment, biologic paradigms dictate that recognition and rejection by the immune system should result in fetal damage.[21] The reasons why the maternal immune system does not view the paternal component of the embryo as alien and rejects it is still poorly understood. Fetal survival during pregnancy depends on the ability of embryonic tissue to avoid rejection by the maternal immune system.[22] Recent studies indicate that abnormal complement activation during pregnancy affects fetomaternal tolerance.[23-26]

The complement system is important in the reproductive system and in pregnancy. Placental tissue and fetal trophoblast cells are in direct contact with maternal blood and could induce complement activation by either maternal-derived anti-trophoblast antibodies or by the alternative pathway.[23] Complement activation does occur as shown by the deposition of activated complement components on the trophoblast surface of normal and pathological pregnancies.[27,28] Pre-eclamptic placentas have increased complement activation on their surface and this may be responsible for placental failure. Immunostaining of human placental tissue has shown the presence of DAF and MCP on the surface of trophoblast cells.[24] Furthermore, up to twenty percent of first trimester pregnancy losses can be characterized by the onset of hypocomplementemia and with complement deposition in the placenta.[29] This hypocomplementemia is associated with reduced DAF expression on the placenta and increased complement consumption at the fetomaternal interface.[29] Moreover, a human MCP polymorphism is associated with recurrent spontaneous miscarriages.[30] Complement activation is also required in a mouse model of recurrent fetal loss associated with anti-phospholipid antibodies, a condition characterized by increased miscarriages and spontaneous abortion.[25] In this context, it is tempting to hypothesize that some cases of unexplained recurrent human fetal losses may be secondary to loss-of-function mutations in DAF and/or MCP. Based on this observation it has been proposed that these molecules play major roles *in vivo* in the protection of trophoblast cells from the deleterious effects of complement activation.

5. STUDIES USING CRRY-DEFICIENT MICE

To investigate the role of these regulators *in vivo*, mice deficient in Crry were generated using gene targeting techniques.[24] The Crry deficiency leads to embryonic lethality that occurs at 10.5 day post coitus (d.p.c.). In addition, most *Crry*$^{-/-}$ embryos at 9.5 d.p.c. had signs of developmental arrest, such as the smaller deciduas resembling those of earlier stages. To determine the role of Crry on this developmental defect, its expression pattern in wild-type early embryos was analyzed. Immunohistochemical detection of Crry in cryosectioned embryos indicated that Crry is highly expressed in trophoblasts as early as 7.5 d.p.c., with little expression in the embryo proper. Trophoblasts are cells derived from the single-cell outer layer of the blastocyst and eventually form the parenchyma cells of the placenta. In addition, Crry is also expressed in the maternally derived decidual tissues. This expression pattern persists in later stages of embryonic development (examined up to 16 d.p.c). As expected, there is no Crry expression in *Crry*$^{-/-}$ trophoblast and embryos. Interestingly, trophoblasts and the embryo proper lack expression of DAF and MCP.[24] By Northern blot analysis and immunohistochemistry, MCP and DAF are not expressed in the early 10.5 d.p.c. and 14.5 d.p.c. embryo and placenta.

The 7.5 d.p.c. *Crry*$^{-/-}$ embryos exhibit positive staining for activated C3b on their trophoblasts and in the early placenta (ectoplacental cone) as compared to minimal or no staining in wild type trophoblasts[24] This observation indicates that the lack of Crry promotes abnormal activation and deposition of complement. In addition, 7.5 d.p.c. *Crry*$^{-/-}$ embryos exhibit an inflammatory reaction as shown by the presence of neutrophils around the ectoplacental cone and the associated trophoblast cells.[24] The embryonic lethality present in the Crry-deficient animals is completely rescued if these mice are bred to C3-deficient animals.

On the trophoblast, C3 activation is maximal at 7.5 d.p.c. At 8.5 d.p.c., minimal C3 deposition is noted, and no difference in C3 deposition is noted at 9.5 and 10.5 d.p.c. as compared to wild type controls.[31] In addition, most of the inflammation occurs at 7.5 d.p.c., with minimal infiltration of inflammatory cells at day 8.5 d.p.c., and no inflammation at 9.5 d.p.c. Although growth is retarded, embryos are still alive and growing at 9.5 d.p.c. These observations suggest that the critical time for C3 activation and inflammation is at 7.5 d.p.c. Interestingly, histological findings at 10.5 d.p.c. demonstrate the absence of normal vascular development suggesting the effect of complement is to compromise early placenta vascular development.[31] Thus, C3 activation and inflammation at 7.5 d.p.c. affect placental developmental events important for growth, and for survival beyond 10.5 d.p.c.

6. ACTIVATION OF COMPLEMENT IN CRRY-DEFICIENT MICE

Breeding of the Crry mutation to animal models with selective deficiencies in complement components was performed to determine the mechanism by which complement is activated in *Crry*$^{-/-}$ embryos.[31] C4 deficiency[32] does not rescue this embryonic lethality indicating that classical pathway components (C1, C4, and C2) are not involved in the activation of complement and the death of the embryos. Further supporting the dispensable role of the classical pathway is the observation that the absence of antibodies in B cell-deficient mice[33] ($\mu MT^{-/-}Crry^{+/-}$mating) does not rescue this embryonic lethality since antibodies are needed for the activation of the classical

pathway. This observation also provided evidence for a expendable role of antibodies and Fc receptors, not only as primary precipitating events, but also as effector mechanisms in the *Crry*$^{-/-}$ embryonic lethality, and a potential negligible role for the lectin pathway since C4 is needed for its activation.[2] Maternal C3 is mostly responsible for the *Crry*$^{-/-}$ embryonic death.[31] Thus, of the complement components with biological functions directly involved in tissue damage, only activation of maternal C3 is causing the embryonic death in the *Crry*$^{-/-}$ mice.

To examine if the alternative pathway provided the main mechanism of complement activation and fetal rejection, breeding the Crry mutation to factor B-deficient mice (*fB*$^{-/-}$) was performed.[34] Genotype analysis revealed that 27% (13/49) of the resulting 3-week old pups were *fB*$^{-/-}$*Crry*$^{-/-}$.[31] No increased C3 deposition and no neutrophil inflammation was also noted in 7.5 d.p.c. *fB*$^{-/-}$*Crry*$^{-/-}$ embryos. These results indicate that the alternative pathway mediate the impaired fetomaternal tolerance related with the Crry deficiency.[2]

7. EFFECTOR MECHANISMS RELATED TO EMBRYONIC LOSS

The specific functions of complement that could been involved in this embryonic lethality have been examined. Complement components with biological functions directly involved in tissue damage include C5b-9 and fragments derived from C1, C3, C4, and C5.[2] C1 and C4 are not necessary to cause the Crry embryonic lethality[31] since C3 deficient mice should have no compromise in the activation of C1 and C4, and are still able to activate these complement components, but *C3*$^{-/-}$*Crry*$^{-/-}$ mice are viable. Although C3 deficiency rescues the embryonic lethality, C5 deficiency does not . Hence, by genetic analysis we have determined that C5 and the C5b-9 membrane attack complex may not be needed to cause the death of the embryos.[31]

One way that fragments derived from the activation of C3 mediate their biological functions is by interacting with complement receptors (CR) on the surface of cells.[2] No expression of CR1 and CR2 is detected in placental and embryonic tissues from wild type and *Crry*$^{-/-}$ mice as determined by immunohistochemistry using specific monoclonal antibodies. These receptors, therefore, are probably not involved in causing this embryonic lethality. CR3 and CR4 staining is comparable between the wild type and Crry-deficient mice. Thus, the possibility exits that either iC3b (CR3 and CR4) or C3a (C3aR) are the main effector fragments conducive to the demise of the Crry-deficient embryos.

Another way complement induces tissue damage is by attracting inflammatory cells.[2] The main cellular component involved in the inflammatory reaction observed at 7.5 d.p.c. are neutrophils, as determined by H/E staining and Ly-6G expression.[26] No evidence of increased tissue infiltration by macrophages, T cells, or B cells is noted in the Crry-deficient animals as determined by immunohistochemistry with specific antibodies, or by analysis of H/E sections. Surprisingly, neutrophil infiltration is not needed for the *Crry*$^{-/-}$ embryonic demise.

To investigate the role of neutrophils in the *Crry*$^{-/-}$ embryonic lethality, antibody-mediated depletion of these cells using monoclonal antibodies was performed. Injection of anti-Ly-6G antibodies causes severe neutropenia.[35] *Crry*$^{+/-}$ pregnant females were injected with 250 μg dose of anti-Ly-6G at 3.5 and 4.5 d.p.c. (two days pre-implantation to assure the presence of neutropenia at the time of implantation).

At day 7.5 d.p.c., mice were sacrificed and the number of *Crry*$^{-/-}$ embryos assessed by immunohistochemistry and PCR analysis. Of 20 embryos collected, three were Crry-deficient. These embryos failed to develop placental inflammation as compared to the inflammation generated in non-injected *Crry*$^{-/-}$ mice. Histology revealed a normal appearing placenta and embryo. This finding correlated with undetectable numbers of blood neutrophils in maternal blood as assessed by FACS analysis. No gross abnormalities were observed in the Crry-sufficient embryos suggesting that the induction of neutropenia did not affect pregnancy. In similar experiments, pregnant *Crry*$^{+/-}$ females, injected with 250 μg dose of anti-Ly-6G at 3.5, 4.5, and 7.5 d.p.c. (to achieve a prolonged neutropenia), were sacrificed at 9.5 d.p.c. Of 23 embryos analyzed, six were *Crry*$^{-/-}$. Nevertheless, these embryos were small, exhibiting growth retardation as compared to Crry-sufficient embryos from the same mother. Although histological analysis revealed the absence of neutrophils within the tissue, and FACS analysis from maternal blood revealed no neutrophils, abnormalities in placental development, reminiscent of those seen in untreated *Crry*$^{-/-}$ embryos, were observed. This result suggested that, even without an inflammatory component at 7.5 d.p.c., abnormalities in embryonic development still occurred in 9.5 d.p.c. *Crry*$^{-/-}$ embryos. In similar experiments, mice were injected with a 250 μg dose of anti-Ly-6G at 3.5, 4.5, and 7.5 d.p.c. Pregnant *Crry*$^{+/-}$ females were then sacrificed at 11.5 d.p.c.. Out of 21 embryos, no *Crry*$^{-/-}$ embryos were recovered, indicating that abnormalities in embryonic development still occurred in *Crry*$^{-/-}$ embryos derived from neutropenic mothers. It is important to emphasize that the critical time point for abnormal complement activation and inflammation is at 7.5 d.p.c. Elimination of neutrophils from maternal blood and the absence of inflammation at this critical time point does not inhibited embryonic lethality, indicating that neutrophils are not absolutely necessary for the embryonic lethality seen in Crry-deficient fetuses to occur.

8. CONCLUSION

Abnormalities in the regulation of the maternal immune response against the fetus are likely a significant cause for the conditions associated with pregnancy failure. Unfortunately, factors involved in pregnancy failure due to abnormal immunological fetomaternal tolerance are poorly understood. Recent experimental observations implicate the complement system as an important contributor to this type of immunoregulation.[23-26] Several mechanisms may be involved in complement-dependent spontaneous otherwise unexplained pregnancy loss.[1,2] Defining the relative contribution of these mechanisms to fetal loss will facilitate the understanding of the innate immune response during pregnancy and provide potential therapeutic options in the treatment of recurrent miscarriages. To this purpose, we have used the *Crry*$^{-/-}$ mouse model to further investigate the role of complement and complement regulators in recurrent fetal loss.[26,31] We have identified the pathways related to the activation of complement in the *Crry*$^{-/-}$ maternal-to-fetal rejection process, some of the complement components needed to cause the fetal demise, and the pathological consequences of this activation. Surprisingly, sole activation of the maternal third component of complement through the alternative pathway is enough to mediate defects in placenta formation and subsequent pregnancy loss. This effect is independent of other complement activation pathways and complement components, or the presence of B cells and antibodies. Thus, abnormal fetomaternal tolerance may be established using exclusively C3 as both, the inductive and effector element, without the contribution of downstream complement components or

mobilization of adaptive immune response components. However, it is still unclear from these studies the relative contribution of the numerous activation and effector mechanisms that may still be involved in this complement-dependent pregnancy loss.[2]

These results may also apply to human pregnancy loss in which anomalies in complement regulators within the placenta could increase the rate of fetal miscarriages.[24,29,30] The rate of human miscarriages related to abnormal complement regulation may be underappreciated. Our data also implies potential therapeutic targeting areas based on complement regulation. Specific regulation of the alternative pathway may provide a practical way of controlling tissue inflammation. Therefore, our studies indicate that further analysis of this pathogenic mechanism of fetomaternal intolerance is now needed in human pregnancy loss.

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