MyD88 Signaling in B-Cell Development and Differentiation

by

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Department of Immunology
Duke University

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Garnett Kelsoe, Supervisor

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You-Wen He

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Immunology in the Graduate School
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ABSTRACT

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Abstract

Toll-like receptor (TLR) signaling pathways have been demonstrated to be important in many aspects of innate and adaptive immunity. Binding of TLRs by their respective ligands initiates signaling cascades which promote a number of cellular responses including, but not limited to, pathogen recognition, co-stimulation, cell maturation and activation and initiation of adaptive immune responses. While many roles for TLRs have been rigorously tested, the exact contributions of these receptors to the quality of B-cell immune responses remain unclear. Specifically, a role for intracellular TLR signaling in B-cell development is one area that remains largely uncharacterized. Endogenous TLRs that recognize viral and microbial DNAs and RNAs are also capable of recognizing self-nucleic acids. B cells, which express TLRs, undergo tolerizing mechanisms in the bone marrow (BM) and periphery to eliminate self-reactive clones. I hypothesize that endogenous TLR recognition of self-nucleic acids during B cell development promotes the elimination of these autoreactive cells from the B cell repertoire.

TLRs are also known to be integral in the subsequent triggering of the adaptive immune response. A series of controversial studies has attempted to determine whether TLR signaling is required for antibody responses to thymus-dependent protein antigens. Initial reports indicated that MyD88 was required for antibody responses to a native protein antigen. However, later studies using haptenated protein antigens did not confirm a requirement for TLR signaling in adjuvant-enhanced antibody responses. In an effort to resolve these discordant results, it was suggested that haptenated protein antigens, unlike native proteins, are fundamentally distinct in that they do not require MyD88
signaling. I hypothesize that antibody and germinal center (GC) responses to native and haptenated proteins are independent of MyD88 signaling. I further propose that the unique immunogenicity ascribed to haptenated proteins is simply immunodominance of hapten, a phenomena observed and described almost 100 years ago.

Deficiency in the global adaptor protein for TLR signaling, myeloid differentiation primary response protein 88 (MyD88), through which all TLRs signal (with the exception of TLR 3), effectively silences the TLR pathway. Utilizing mice deficient in MyD88, I show that TLR signaling through MyD88 mediates central tolerance of B cells. Specifically, crossing the MyD88 deficiency onto a 3H9 autoreactive heavy-chain knock-in rescues the immature (imm) and transitional 1(T1) B cell compartments in the BM. This finding demonstrates the requirement for MyD88 signaling in the removal of autoreactive B cell clones at the first tolerance checkpoint.

I also find that MyD88 signaling is not required for antibody, GC or memory responses to native or haptenated proteins. Additionally, affinity maturation, determined by BCR mutation frequency in GC was comparable between MyD88 deficient and sufficient mice. Both MyD88 deficient and sufficient mice were able to elicit secondary immune responses to native and haptenated proteins. Furthermore, I demonstrate that the MyD88 independent immunogenicity attributed to haptenated protein is a misinterpretation of the established concept of immunodominance- haptenated proteins elicited hapten-specific responses that were approx. 20- to 100-fold greater than to the carrier. Regardless of MyD88 signaling, native proteins elicited significantly less serum Ab than their haptenated forms.
I conclude that TLR signaling through MyD88 mediates tolerance during B cell development in the BM, but is not required for B cell immune responses.
Dedication

To those whose prayers, encouragement and constructive criticism drive me to persevere.
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List of Abbreviations

Ag  Antigen
AID  Activation induced cytidine deaminase
AM  Affinity maturation
ANA  Anti-nuclear antibodies
APC  Antigen-presenting cell
AP-1  Activating protein-1
BAFF  B-cell activating factor
BM  Bone marrow
CGG  Chicken gamma globulin
CpG  C phosphate G
DAMPs  Damage-associated molecular patterns
DC  Dendritic cell
DNP-HSA  (Dinitropheno) human serum albumin
dsDNA  Double stranded DNA
GC  Germinal center
HC  Heavy chain
HEL  Hen egg-white lysozyme
HSA  Human serum albumin
hsp70  Heat shock protein
IL  Interleukin
Ig  Immunoglobulin
IKK  Inhibitor of nuclear factor-κB (IκB)-kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Imm</td>
<td>Immature</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeats</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>NFκB essential modulator</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NP-CGG</td>
<td>(4-hydroxy-3-nitrophenyl) acetyl-chicken gamma globulin</td>
</tr>
<tr>
<td>NP-Ova</td>
<td>(4-hydroxy-3-nitrophenyl) acetyl-ovalbumin</td>
</tr>
<tr>
<td>Ova</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>T1</td>
<td>Transitional 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TAB</td>
<td>TAK1-binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming-growth-factor-β-activated kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T-dependent</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNP-Hy</td>
<td>Trinitrphenol-hemocyanin</td>
</tr>
<tr>
<td>T&lt;sub&gt;r&lt;/sub&gt;</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor-necrosis-factor-receptor-associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>Trif-related adaptor molecule</td>
</tr>
<tr>
<td>Trif</td>
<td>TIR domain-containing adaptor inducing IFNβ</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable (V), Diversity (D), and Joining (J)</td>
</tr>
</tbody>
</table>
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In all thy ways acknowledge Him, and He shall direct thy paths. - Proverbs 3:6.

To my husband for his many sacrifices during this endeavor
To my parents and siblings for their continued love and support
To my Doktorvater, Dr. Garnett Kelsoe, without whom I would not have learned how to engage in hard thinking
To my classmates who began this journey with me and became my family along the way
To my lab family for their support and love
1. Overview

The immune system generates protection against invading pathogens through the detection and subsequent elimination of these microorganisms. A key component of immune recognition is the ability of the immune system to discriminate microbial products from self-components (self-nonself recognition) (Janeway and Medzhitov, 2002). The immune system can be divided into two inter-dependent branches: the innate immune system and the adaptive immune system. The differences in innate and adaptive immunity are based on how each branch recognizes and responds to pathogens. Invading microorganisms first encounter the innate immune system which recognizes a conserved set of microbe-specific molecular structures (pathogen associated molecular patterns, PAMPS) by germ-line encoded pattern recognition receptors (PRRs) (Iwasaki and Medzhitov, 2010). The adaptive immune system provides a second, more specific line of defense against invading pathogens. Composed largely of T and B lymphocytes and antibodies, the adaptive immune system’s specificity and diversity is generated as a result of mechanisms that introduce genetic recombination (variable (V), diversity (D), and joining (J) recombination, V(D)J recombination) and mutations (somatic hypermutation, SHM) in antigen receptors (Xu, 2006).

The efficiency of the immune system in eliminating invading pathogens is achieved by interactions between innate and adaptive immunity. One class of PRRs that link these two systems is the Toll-like receptor (TLR) family. In innate immunity, TLR-mediated recognition of PAMPs on pathogens activates signaling pathways that
result in the production of inflammatory cytokines and chemokines and the recruitment of innate effector cells to kill foreign invaders. Studies examining the contribution of TLRs to adaptive immunity show a role for these receptors in dendritic cell (DC)-mediated activation of naïve T cells and T regulatory (Tr)-mediated suppression of T cells (Pasare and Medzhitov, 2003; Pasare and Medzhitov, 2004).

Many studies have elucidated the contribution of TLR signaling to innate immunity (Lemaitre et al., 1996; Medzhitov et al., 1997; Poltorak et al., 1998). The role of TLR signaling in the adaptive immune system, however, has not been as rigorously tested. While the contribution of TLR signaling to the activation of T-cell mediated adaptive responses has been described (Pasare and Medzhitov, 2004), the augmentation of B-cell development and activation by the TLR pathway remains untested and controversial, respectively (Gavin et al., 2006; Meyer-Bahlburg et al., 2007; Palm and Medzhitov, 2009a; Pasare and Medzhitov, 2005a).

1.1 TLRs in innate immunity and inflammation

Following pathogen invasion, the immune system must perform two functions to eliminate the invader: sense or recognize the pathogen and respond to the pathogen through the activation of a system of signaling pathways. Innate immune cells comprise the first line of defense against pathogen and include phagocytic neutrophils and macrophages (antigen-presenting cells, APC), lytic natural killer cells (NK) and DC. Dendritic cells are also phagocytic APCs that have been shown to be a crucial link between innate and adaptive immunity (McKenna et al., 2005). The initial recognition of pathogen by these cell types occurs via binding of PAMPs to PRRs expressed by innate
(cells. One class of innate immune receptors is the TLR family. The *Toll* gene was first described as an essential molecule in embryogenesis in *Drosophila* (Anderson et al., 1985). Following this discovery, the *Toll* gene was found to encode a transmembrane protein with a cytoplasmic domain and a large extracytoplasmic domain containing leucine rich amino acids that are conserved in various yeast and human membrane proteins (Hashimoto et al., 1988). Additionally, a conserved signaling pathway in *Drosophila* homologous to the mammalian nuclear factor-κB (NFκB) pathway (known to mediate signaling mechanisms in the initiation of the innate immune response) (Kopp and Ghosh, 1995), was identified (Wasserman, 1993). This finding guided seminal work showing that the *Toll* gene functions in immunity through the control of anti-fungal responses in *Drosophila* (Lemaitre et al., 1996). Subsequent findings, the discovery of an NFκB-regulating mammalian Toll orthologue (Medzhitov et al., 1997) and the identification of TLR4 as the lipopolysaccharide (LPS) receptor (Poltorak et al., 1998), demonstrated unequivocally that TLRs play an essential role in the induction of mammalian immunity.

TLRs recognize a broad range of PAMPs, including highly conserved microbial proteins, fungal and bacterial cell wall components, and bacterial lipoproteins (Akira and Takeda, 2004). Another category of molecular patterns recognized by TLRs, damage-associated molecular patterns (DAMPs), recognize mainly nuclear and cytosolic proteins (Matzinger, 1994). While a key concept of innate immunity is self-nonself discrimination, the recognition of nucleic acids (a component of both foreign microorganisms and self) by TLRs highlights a unique state whereby TLR activation by self-nucleic acids can be initiated to the detriment of the host (Blasius and Beutler, 2010).
The TLR family consists of 10 receptors in humans and 12 in mice that have distinct ligands and specific functions (Table 1) (Casanova et al., 2011). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are found at the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are located within endolysosomal compartments (Akira and Takeda, 2004). Pathogen recognition by TLRs is initiated by the binding of extracellular leucine rich repeats (LRR) to the PAMP(s) of pathogen. While LRR domains are highly conserved between TLRs, TLRs are capable of recognizing multiple structurally unrelated ligands. For example, TLR2 can recognize the unrelated ligands zymosan, heat shock protein 70 (hsp70) and peptidoglycan (Akira and Takeda, 2004). TLRs are thought to dimerize and signal as homodimers upon ligand engagement (with the exception of TLR1/TLR2 and TLR2/TLR6, which signal as heterodimers). All TLRs carry transmembrane and cytoplasmic Toll/interleukin-1 receptor (TIR) domains which are necessary for initiating intracellular signaling. The TIR domains in turn, interact with the TIR-domain-containing cytosolic adaptors, myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor inducing IFNβ (Trif), TIR domain-containing adaptor protein (TIRAP)/Mal, and Trif-related adaptor molecule (TRAM) (O'Neill and Bowie, 2007). Of these adaptor proteins, MyD88 is utilized by all TLRs except for TLR3, which signals through Trif. TLR4 signals through both MyD88 and Trif. MyD88, after association with the TLR TIR domain, recruits IRAK4 (IL-1R-associated kinase 4), allowing for association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1 and phosphorylated IRAK1 recruits TRAF6 (tumor-necrosis-factor-receptor-
<table>
<thead>
<tr>
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<th>Location</th>
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<th>Origin of Ligand</th>
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<td>cell surface</td>
<td>Tri-acyl lipopeptides</td>
<td>bacteria, mycobacteria</td>
<td>MyD88/TIRAP</td>
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<td>cell surface</td>
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<td>a variety of pathogens, Gram-positive bacteria, Gram-positive bacteria, mycobacteria, Staphylococcus epidermidis, Trypanosoma, Cruzi, Treponema, malphphilum, Neisseria, Fungi, Leptospira, interrogens, Porphyromonas, gingivallis, host</td>
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<td>N/A</td>
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Information within table compiled from (Beutler, 2009; Pone et al., 2010; Takeda et al., 2003)
associated factor 6) to the receptor complex. IRAK1 and TRAF6 dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-β-activated kinase 1), TAB1 (TAK1-binding protein 1) and TAB2, inducing the phosphorylation of TAB2 and TAK1. Following this phosphorylation event, IRAK1 is degraded at the plasma membrane and the TRAF6-TAK1-TAB1-TAB2 complex translocates to the cytosol (Takeda and Akira, 2004).

Ubiquitination of TRAF6 and the subsequent activation of TAK1 results in the phosphorylation of both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex). The IKK complex consists of IKK-α, IKK-β and IKK-γ (also known as NFκB essential modulator, NEMO). The IKK complex phosphorylates IκB, which in turn leads to its ubiquitination and degradation. The TAK1 complex facilitates the translocation to the nucleus of NFκB and activating protein-1 (AP-1), transcription factors which induce the production of a number of inflammatory cytokines such as tumor necrosis factor (TNF α), IL-1β, IL-6 and IL-12 (Barton and Kagan, 2009) (Fig.1).

The inflammatory cascade initiated by TLR engagement regulates the activation and recruitment of leukocytes to sites of invasion or injury. Several cell subsets are involved in eliminating pathogens including tissue stromal cells, monocytes, neutrophils, DC and NK (Mogensen, 2009).
Figure 1. Toll-like receptor signal transduction: MyD88 Signaling Pathway.

Following engagement of the TLR by its cognate ligand, TLR signal transduction is initiated by the recruitment of adaptor proteins. The MyD88 adaptor protein, utilized by all TLRs except for TLR3, recruits IRAK4 allowing for association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1 and phosphorylated IRAK1 recruits TRAF6 to the receptor complex. IRAK1 and TRAF6 dissociate from the receptor and form a complex with TAK1, TAB1 and TAB2, inducing the phosphorylation of TAB2 and TAK1. Following this phosphorylation event, IRAK1 is degraded at the plasma membrane and the TRAF6-TAK1-TAB1-TAB2 complex translocates to the cytosol. Ubiquitylation of TRAF6 and the subsequent activation of TAK1 results in the phosphorylation of the IKK complex. The IKK complex consists of IKK-α, IKK-β and IKK-γ (also known as NFkB) essential modulator, NEMO). The IKK complex phosphorylates IκB, which in turn leads to its ubiquitylation and degradation. The TAK1 complex facilitates the translocation to the nucleus of NFkB, a transcription factor which induces the production of a number of inflammatory cytokines such as tumor necrosis factor (TNFα), IL-1β, IL-6 and IL-12. (Adapted from (MacKichan, 2005; Takeda and Akira, 2004)
1.2 TLRs in adaptive immunity

Unlike innate immunity, a collection of processes regulated by PRR interaction with a broad array of conserved PAMPs, adaptive immunity is characterized by the recognition of unique antigenic determinants, or epitopes (Beutler, 2009; Pasare and Medzhitov, 2005b). The specificity and diversity needed for this type of recognition is generated by two mechanisms: V(D)J recombination and SHM. Both B and T cells undergo V(D)J recombination during development in the bone marrow and thymus, respectively. The random recombination of variable (V), diversity (D) and joining (J) gene segments results in the diversity inherent in B-cell immunoglobulins (Ig) and T-cell receptors (TCR) (Jung and Alt, 2004). Additionally, mutations introduced by SHM vary the specificity and diversity of B-cell receptors and antibody (Kelsoe, 1994; Kim et al., 1981). In addition to diversity generated in B and T cells, adaptive immunity is characterized by the formation of memory following immunologic challenge, a property not shared by innate immunity. After encounter with antigen, B and T cells are able to differentiate into memory cells, which upon rechallenge with cognate antigen, are able to initiate response against the antigen much more rapidly and efficiently (Lanzavecchia and Sallusto, 2009; Wherry et al., 2003). The diversification and differentiation of T and B lymphocytes allow this branch of the immune system to readily recognize and eliminate immunologic challenge.

It has been known for almost 50 years that effective adaptive immune responses depend on innate effector cell mechanisms (Dutton, 1967; Unanue and Askonas, 1968). It has been demonstrated that presentation of antigen by APCs of the innate immune system facilitates the initiation of adaptive immunity (Palm and Medzhitov, 2009b). The
most important APC of the innate immune system is the DC (Liu, 2001). One type of PRR through which DCs initiate signaling and facilitate the activation of adaptive responses is the TLR (Mellman and Steinman, 2001).

1.2.1 TLRs and DC

DCs are innate cells that are able to not only bind to pathogenic PAMPs by TLRs, but also, through interaction with these pathogenic ligands, are able to mediate adaptive immune responses (Steinman, 1991). DC maturation is a process whereby TLR recognition of cognate ligand initiates the upregulation of surface costimulatory molecules, secretion of cytokines and chemokines, enhancement of antigen presentation and the subsequent migration of DCs to secondary lymphoid tissues (Munz et al., 2005). This TLR-induced maturation of DCs promotes the differentiation of naïve T cells to effector T cells (Hou et al., 2008). Signaling through TLRs initiates the differentiation of immature DC into mature DC that have potent effector functions and are able to trigger specific immune responses (Banchereau et al., 2000; Banchereau and Steinman, 1998; Mellman and Steinman, 2001).

1.2.1.1 DC and T cell polarization

DCs play a role in the development of effector Th cells from naive Th cells. Immature DC reside in locations where they are exposed to pathogen. This pathogen exposure initiates DC maturation, a process that includes the recruitment of innate effector cells such as neutrophils and macrophages to the site of invasion by DC secretion of chemokines and cytokines (de Jong et al., 2005). Upon antigen capture and
recruitment of innate effector cells, DCs migrate to T cell zones within lymphoid tissues and present the processed peptides on major histocompatibility complex (MHC) II molecules to naïve T cells. Antigen uptake, DC migration (as a result of chemokine receptor expression modulation and production of chemokines and cytokines) and antigen presentation to naïve T cells is regulated by the downstream effects of TLR signaling. The recognition of pathogen by TLRs directs all downstream events mediated by DC.

There are three signals provided during DC maturation that determine the fate of naïve Th cells. The first signal is the up-regulation of the expression of MHC class II bearing pathogen-derived peptides on DC that determines the specificity of the T cell response. The second signal involves the expression of costimulatory molecules such as CD80 and CD86 on DC and CD28 on T cells that determine the ability of naive T cells to expand (Kapsenberg, 2003). The quality of signal 3 is dependent on the particular TLR that is activated during DC uptake of pathogen. Depending on the type of pathogen encountered, DCs will express a select group of T-cell polarizing soluble or membrane-bound molecules. DC exposed to intracellular pathogens promote Th1 responses, whereas certain helminthes promote DC to drive the development of Th2 cells. The TLRs and soluble or membrane-bound factors that promote a T_{H1} subset are TLR2, TLR3, TLR4, TLR7, TLR9, IFN-γ, IFN-α, IL-18 and CCR5. Development of a T_{H2} subset is promoted by histamine, PGE$_2$, and TSLP; which TLRs can mediate a T_{H2} subset has yet to be established. A regulatory T cell subset, is promoted by TLR2, IL-10 and TGF-β (Kapsenberg, 2003). A T_{H17} subset is promoted by TLR3, TLR4, TLR9, IL-6, IL-17 and TGFβ (Veldhoen et al., 2006) (Table 2).
Table 2. Polarizing factors of T cell subsets

<table>
<thead>
<tr>
<th>Polarizing Factors</th>
<th>$T_h1$</th>
<th>$T_h2$</th>
<th>$T_R$</th>
<th>$T_h17$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLRs</td>
<td>TLR-2,3,4,7,9</td>
<td>N/A</td>
<td>TLR2</td>
<td>TLR-3,4,9</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IFN-(\gamma), IFN-(\alpha), IL-18, IL-12</td>
<td>IL-4, IL-5, IL-9, IL-13, IL-25</td>
<td>IL10, TGF(\beta)</td>
<td>TGF(\beta), IL-6, IL-17,</td>
</tr>
<tr>
<td>Chemokines</td>
<td>CCR5</td>
<td>CCL-7,8,13, 17</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Others</td>
<td>N/A</td>
<td>Histamine, PGE2, TSLP</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Information within table compiled from (Kapsenberg, 2003; Veldhoen et al., 2006; Zou and Restifo, 2010)
1.2.2 TLR-mediated control of B-cell responses

While several studies have addressed the involvement of DC and T-cell activation in regulating T-dependent (TD) antibody responses (Akira et al., 2001; Edwards et al., 2002; Kalinski et al., 1999; Maldonado-Lopez et al., 2001; Schnare et al., 2001), the contribution of B-cell-TLR interaction in these processes is less well-defined. A recent study concluded that TLR signals in B cells are required for T-dependent immune responses (Pasare and Medzhitov, 2005a). In this work, MyD88 deficient or sufficient B cells were transferred into lymphopenic μMT and these mice were immunized with human serum albumin (HSA) and lipopolysaccharide (LPS), the TLR4 ligand. HSA antibody titers were reduced in mice containing MyD88 deficient B cells. Additionally, using a CD11c-MyD88 transgenic mouse that results in TLR signaling only in DC and not in B cells, immunizing with HSA and LPS had no effects on T-cell activation, but did impair HSA antibody titers (Pasare and Medzhitov, 2005a). These findings suggest that B-cell-intrinsic TLR signaling is required for antibody responses, and that this constraint is not dependent on TLR signaling through DC or subsequent T-cell activation (Pasare and Medzhitov, 2005a).

Later work however, demonstrated normal TD antibody responses in mice lacking both MyD88 and TRIF, thereby questioning these previous findings (Gavin et al., 2006; Meyer-Bahlburg et al., 2007). Gavin et al. demonstrated that B-cell responses to antigen are completely intact in mice deficient in TLR signaling. Using mice deficient in both MyD88 and Trif, the two adaptor proteins through which all known TLRs signal (Hoebe et al., 2003; Janssen et al., 2006; Yamamoto et al., 2003), Gavin et al. showed
that antibody responses to trinitrophenol-hemocyanin (TNP-Hy) or TNP-keyhole limpet hemocyanin (KLH) in a number of adjuvants are comparable to those generated in BL/6 control mice (Gavin et al., 2006).

The work of Meyer-Bahlburg et al. combined experimental elements of these two contrasting studies and found that B-cell intrinsic TLR signaling enhanced, but was not required for antibody responses (Meyer-Bahlburg et al., 2007). Specifically, this group adoptively transferred MyD88 deficient or sufficient B cells into µMT mice immunized with (4-hydroxy-3-nitrophenyl) acetyl-chicken gamma globulin (NP-CGG) in alum and measured NP-specific antibody titers. They found that BL/6 and MyD88 deficient B cells produce comparable amounts of antibody. Additionally, using an NP-CGG/alum with the addition of LPS immunization system, they showed that B-cell intrinsic MyD88-dependent signaling can amplify early immune responses, most notably IgM production (Meyer-Bahlburg et al., 2007). Collectively, these findings support the work of Gavin et al., showing that TLR signaling is not required for B-cell responses.

Recent findings by Palm and Medzhitov (Palm and Medzhitov, 2009a) suggest that differences in model systems in studies that did not find a requisite for TLR signaling in antibody responses account for the divergent findings. Specifically, the use of haptenated proteins elicits a TLR-independent antibody immune response, whereas the use of native proteins results in TLR-dependent humoral immunity. Palm and Medzhitov found that haptenating HSA, which in their hands fails to induce a robust HSA antibody response in BL/6 mice, with DNP results in a strong DNP-specific (and an almost undetectable HSA-specific) response. From this, they concluded that haptenating a non-immunogenic protein resulted in a hapten-focused immunogenicity (Palm and Medzhitov, 2009a). Additionally they showed that when immunized with HSA and LPS,
MyD88/Trif double deficient mice generated very poor HSA-specific antibody responses as compared to wild type controls, whereas DNP-HSA immunization elicited DNP-responses that are closer to those generated in the control mice (Palm and Medzhitov, 2009a). These opposing findings prevent the clear characterization of the contribution of TLR signaling to B-cell antibody responses.

1.2.2.1 Haptens as immunogen

Landsteiner and Jacobs used the Greek word “hapten” which means “to fasten”, to describe the synthetic molecules that they covalently linked to carrier protein and used in their work establishing the specificity of antibody. Haptens are low molecular weight chemicals that while inherently antigenic, must bind to a carrier molecule to be immunogenic. The carrier is usually a protein to which the hapten is covalently bound. Immunization with the hapten-carrier conjugate elicits antibodies specific for: (1) the hapten, (2) epitopes on the carrier, and (3) epitopes created by the coupling of the hapten to the carrier (Landsteiner and van der Scheer, 1931).

The development of haptenated proteins allowed for the study of antibody specificity and affinity. Landsteiner immunized rabbits with a hapten-carrier conjugate, collected serum from the rabbits and tested the reactivity of the immune serum to the hapten, as well as to the hapten conjugated to different carriers. This approach allowed for the measurement of hapten-specific antibodies, without having to account for antibodies to the original carrier protein (Landsteiner and van der Scheer, 1931).

Following Landsteiner’s pioneering work, Rajewsky and Mitchison showed using the hapten-carrier conjugate system that what we now know as B and T cells collaborate to induce immune responses: following hapten-carrier immunization, B cells recognize hapten and T cells recognize the carrier and promote the activation of hapten-specific B
cells. It was also found that the conjugation of the hapten and carrier was necessary to elicit response (Britton et al., 1971). Unanue and colleagues introduced the concept of antigen processing and provided key evidence showing that T cells were activated as a result of recognition of Ag fragments rather than intact antigen (Unanue and Katz, 1973; Ziegler and Unanue, 1981).

Rock and Lanzavecchia then demonstrated independently that the presentation of these Ag fragments took place within the context of the major histocompatibility complex (MHC). Rock, using mouse models, and Lanzavecchia, human cells, demonstrated that B cells present processed Ag to T cells via their class II MHC and that B cells recognize hapten epitopes whereas T cells recognize carrier epitopes (Abbas et al., 1985; Lanzavecchia, 1985; Rock et al., 1984; Rock et al., 1986).

Because of the ease with which hapten antibodies can be quantified, use of the hapten-carrier conjugate system to examine humoral immune responses to immunogen has become orthodox. Recently, however, the use of this system to establish a role for TLR signaling in the humoral response to T-cell dependent antigen has brought into question the fundamental nature of the hapten-carrier conjugate system. In studies that found a role for TLR signaling in humoral responses, unmodified native proteins were used (Palm and Medzhitov, 2009a; Pasare and Medzhitov, 2005a). Studies showing that humoral responses are independent of TLR signaling used haptenated proteins (Gavin et al., 2006; Nemazee et al., 2006). Claims have now been made that the process of haptenation confers a unique TLR-independent immunogenicity to protein and elicits humoral responses.
1.2.3 Mechanisms of B-cell tolerance

The specificity and diversity of the antibody repertoire generated by B cells provide for rapid and efficient protection against the broad array of antigens encountered by the immune system. Because diversity generating mechanisms are random, they generate BCRs that can recognize self-antigens. Due to the randomness of the mechanisms that generate diversity, there is a propensity for the development of autoreactive clones. The elimination of self-reactive clones minimizes damage to self. These tolerance checkpoints are present early during development of the nascent B-cell population in the BM (central tolerance), as well as in antigen-challenged B cells in the periphery (peripheral tolerance).

1.2.3.1 B-cell Central tolerance

Although our understanding of the mechanisms that mediate central tolerance of B cells in the bone marrow is not complete, three tolerance mechanisms have been described: receptor editing, anergy, and deletion. Work done by Tonegawa shows that during B-cell development, the assembly of genes that encode heavy (H) and light (L) chains of the BCR or its secreted form, antibody, is achieved by rearranging individual gene segments through VDJ recombination (Tonegawa, 1983). This process generates immature B cells that cross-react with self-Ags which are removed from the mature pool of B cells during development in the BM (central tolerance) or in secondary lymphoid tissues (peripheral tolerance) (Basten and Silveira, 2010). It was first shown that self-reactive immature B cells could be tolerized (upon exposure to self Ag) by the elimination of these cells (clonal deletion) or by rendering them non-responsive to activating signals (clonal anergy) (Goodnow et al., 1989; Hartley et al., 1991).
The third tolerance mechanism utilized in the BM is receptor editing. Two laboratories independently describe receptor editing as a secondary Ig gene rearrangement that can replace self-reactive light chains with non-self-reactive specificities (Gay et al., 1993; Tiegs et al., 1993).

It is generally thought that if an immature B cell binds to self-Ag or to nucleic acids in the bone marrow, the cell down-regulates surface BCR and induces secondary light chain gene rearrangement. This is a random process that continues until the specificity to self is lost. If production of a successful rearrangement fails, the cell is then susceptible to apoptosis-mediated deletion or anergy (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993).

It has been estimated that 80-90% of B cells are eliminated prior to entering the mature B-cell compartment (Allman et al., 1993; Rolink et al., 1998). More recently, it has been shown that at least 50% of developing B cells are initially self-reactive (Nemazee, 1995; Wardemann et al., 2003). Since autoreactive B cells can be generated at such high frequencies in the BM, the characterization of B-cell tolerance mechanisms is crucial to our understanding of B-cell development.

Our laboratory recently contributed to the understanding of central B-cell tolerance by showing that AID expression in BM immature and transitional B cells suppresses the development of autoreactivity (Kuraoka et al., 2011). Utilizing a 3H9 autoreactive heavy chain transgenic system that confers self-reactivity in association with approximately 60% of κ-light chains (Chen et al., 1995), we showed that crossing these 3H9 mice onto an AID deficient background substantially restores the immature and transitional B-cell populations (Kuraoka et al., 2011), populations usually subject to tolerizing deletion in 3H9 knick-in mice (Ibrahim et al., 1995). Furthermore, we showed
that AID-deficient immature (imm)/T1 B cells are more resistant to BCR-induced apoptosis. Imm/T1 B cells from AID-sufficient mice carry point mutations, suggesting DNA damage. This same cell population in AID-deficient mice do not carry these mutations, suggesting that AID might exert its tolerizing effects by generating low levels of DNA damage that can elicit apoptosis in autoreactive immature/T1 B cells (Kuraoka et al., 2011).

Independent work done by Meffre and colleagues showed that the Ig repertoire of new emigrant/transitional B cells in AID deficient humans is enriched for a VH gene segment known to encode autoreactive antibodies. Additionally, AID deficient humans had a higher frequency of long IgH complementarity determining regions 3 (CDR3s), which are known to favor antibody self-reactivity (Meyers et al., 2011).

Our laboratory's observation that AID mediates central tolerance in B cells is confirmed in humans by Meffre and colleagues. The mechanisms by which AID mediates tolerance remain to be determined.

1.2.3.2 Peripheral tolerance

While central tolerance effectively eliminates the large majority of self-reactive clones from the B-cell repertoire, there is a population of immature B cells released from the BM to the periphery that harbor self-reactive clones (Bolland, 2008). As compared to new emigrant B cells arriving from the BM, there is a reduced frequency of self-reactive mature naïve B cells in the periphery, revealing a peripheral tolerance checkpoint (Meffre and Wardemann, 2008). Immature B cells that pass or escape central tolerance migrate to lymphoid organs where they may encounter peripheral self-antigens not
Peripheral tolerance checkpoints ensure that these autoreactive B cells are counterselected.

Additionally, due to events such as somatic hypermutation (SHM) within the heavy chain and light chain (HC and LC) during the germinal center (GC) reaction and affinity maturation (AM), B cells may acquire self-reactivity (Basten and Silveira, 2010). This self-reactivity that occurs during activation is greatly reduced by tolerizing mechanisms such as anergy and deletion (Goodnow, 1996).

Goodnow, Basten and colleagues were the first to show that self-reactive B cells could be tolerized through clonal deletion or by clonal anergy (Goodnow et al., 1988; Hartley et al., 1991). Using mice that carry either Ig HC and LC transgenes encoding anti-hen egg-white lysozyme (HEL) antibody or crossing those animals with mice expressing the HEL ligand, they found that HEL-binding B cells from double transgenic mice were excluded from follicles and died within three days, whereas anti-HEL single transgenic B cells persisted (Goodnow et al., 1988; Hartley et al., 1991). This finding was the first demonstration of clonal deletion. Using the same transgenic system, double transgenic mice that express high levels of HEL maintained a population of mature, anti-HEL B cells. These cells, however, were functionally nonresponsive or anergic; they expressed both IgM and IgD, confirming that anergy was induced at the mature stage and IgM expression was approximately 20-fold less than anti-HEL single transgenic B cells (Goodnow et al., 1988; Hartley et al., 1991). B-cell anergy in the periphery is characterized by muted BCR signaling and failure to signal through NFkB. This results in poor antigen presentation, antibody production and interaction with B-cell activating factor (BAFF) (Basten and Silveira, 2010).
B cells that survive the initial binding of either foreign or self-antigen relocate to the T-cell zone to receive co-stimulatory signals from helper T cells. If B cells fail to encounter help from T cells, B cells die by apoptosis (Goodnow, 1996). If autoreactive B cells present self-antigen to T cells, engagement of the Fas receptor by the Fas ligand on T cells can result in the induction of apoptosis, yet another mechanism of tolerance for self-reactive B cells (Rathmell et al., 1995; Rothstein et al., 1995).

Tolerizing mechanisms also occur within germinal centers. Those B cells which receive T-cell help either develop into short lived plasma cells (PC) or enter into the germinal center (GC) reaction within follicles where they undergo affinity maturation and somatic hypermutation (SHM) to increase the specificity and affinity of the B-cell repertoire (Jacob et al., 1991b). During SHM, dividing cells undergo a high rate of mutations. These mutations are in the form of single base substitutions. Specifically, an enzyme called activation induced cytidine deaminase (AID) deaminates cytosine to uracil, a residue not normally found in DNA. This causes a DNA mismatch repair enzyme, uracil-DNA glycosylase, to remove the uracil bases. The resultant abasic sites created by the enzyme are filled in by error-prone DNA polymerases (Nussenzweig and Alt, 2004; Odegard and Schatz, 2006). This attempt at diversifying the B-cell repertoire can result in the creation of self-reactive receptors from previously productive receptors. Following this diversification step, B cells can be selected for and become long-lived PC or memory B cells based on their affinity, or they can be negatively selected against self-reactivity due to competition for antigen presented on follicular DCs and access to help provided at the T-cell zone (Basten and Silveira, 2010).
1.2.3.3 TLR signaling in B-cell development and tolerance

While much is known about TLR signaling and B-cell activation and differentiation, not much is known about TLR expression and function during B-cell development. Our laboratory initially found that induction of inflammation by the TLR4 ligand LPS, can mobilize and redistribute myeloid and lymphoid compartments in the BM (Ueda et al., 2005; Ueda et al., 2004). Subsequent studies by Nagai et al., showed that TLR-mediated recognition of microbial and viral products by hematopoietic progenitors can modulate hematopoietic differentiation (Nagai et al., 2006). Key findings in this work were that stem cells and some early hematopoietic progenitors express TLRs 2 and 4 and the subsequent responses from the ligation of these receptors require signaling through MyD88. Additionally, signaling through TLR removed some of the normal differentiative signals required during hematopoiesis and promoted the development of inflammatory monocytes and macrophages. TLR signals were also shown to promote the commitment of lymphoid-biased progenitors to a DC phenotype (Nagai et al., 2006). These findings suggest that engagement of TLRs during early cellular development could provide a mechanism that modulates downstream innate immune responses at the hematopoietic level.

Interestingly, Isnardi et al. have identified TLR signaling pathways as key to modifying the human antibody repertoire during early B-cell development (Isnardi et al., 2008). This group examined three components of the TLR pathway: IRAK-4 and MyD88, signaling components of all TLRs except for TLR3, and UNC93B, which is essential for signaling of endogenous TLRs and functions to transport TLRs from the endoplasmic reticulum (ER) to endolysosomes (Kim et al., 2008). Gene expression analysis revealed expression of TLR7, TLR10, IRAK-4, MyD88, and to a lesser extent
TLR9 in all B-cell fractions. UNC93B1 could be detected weakly in new emigrant, mature naïve and memory B cells. Additionally, this work shows that both IRAK-4 and MyD88 deficient patients exhibit auto-reactive phenotypes: emigrant immature B cells were self-reactive and the self-reactivity persisted at the mature stage. The autoantibodies produced as a result of the deficiencies were specific for double-stranded DNA or nuclear antigens. This group also finds increased frequencies of autoreactive mature naïve B cells in the periphery of IRAK-4, MyD88 and UNC93B1-deficient patients. Collectively these results establish a role for TLR signaling in both central and peripheral B-cell tolerance checkpoints.
2. *MyD88* Mediates Central Tolerance in B Cells

In this chapter, I demonstrate that the TLR adaptor MyD88 suppresses the development of autoreactive B cells in the BM. This work was conducted in equal collaboration with Masayuki Kuraoka, a senior post-doctoral fellow in the laboratory.

2.1 Introduction

Recognition of self-proteins by cells of the immune system results in immune responses that can be detrimental to self. The removal and repression of these autoreactive cells during development ensures efficient functioning of the immune system. The adaptive immune system has therefore evolved tolerance mechanisms that facilitate the elimination of self-reactive cells (Billingham et al., 1953). B cells are subject to tolerizing mechanisms in both the BM (central tolerance) and in lymphoid tissues and organs (peripheral tolerance).

Upon BCR binding of Ag, many factors determine if the antigen engaged by the BCR is self. Some factors suggested to contribute to the identification of Ag as “self” by the BCR are concentration of Ag and duration of binding; high Ag concentration and prolonged binding are indications that the BCR is engaged by a self-peptide (Basten and Silveira, 2010). All of these elements contribute to the categorization of Ag as self by the BCR and induces mechanisms to replace the autoreactive BCR, to eliminate the autoreactive cell altogether, or to render the cell unresponsive or anergic. One aspect of tolerance that has resulted in drastically different findings is Ag affinity. Whereas some groups suggest high affinity Ag induces tolerance, others have shown that B cells induce
tolerance to very low affinity Ag. (Enders et al., 2003; Goodnow et al., 2005; Lang et al., 1996).

A relatively unexplored characteristic of B cells that may influence the recognition of Ag as “self” is the expression of TLRs by B cells. It is known that some TLRs, specifically the endogenous TLRs, TLR -3,-7, and -9 are able to recognize self-Ag (Marshak-Rothstein, 2006). Additionally, it has been shown that BCR triggering together with simultaneous endosomal TLR engagement can lead to enhanced B cell differentiation and antibody responses (Pone et al., 2010). Leadbetter et al. have shown a link between BCR-endogenous TLR synergy and development of autoimmunity. Specifically, this group demonstrated that activation of B cells expressing a self-reactive antigen receptor (specific for rheumatoid factor, RF) required the engagement of both the BCR and the endogenous TLR9 (Leadbetter et al., 2002). A later study showed that while there is an increased frequency of peripheral autoreactive B cells in human IRAK-4, MyD88 and UNC93B deficient patients, these patients did not have anti-nuclear antibodies (ANAs) nor did they show an increase in polyreactive antibodies in the serum (Isnardi et al., 2008). While endogenous TLR signaling mediates B-cell tolerance in the BM, BCR-TLR synergy facilitates activation and subsequent antibody production in autoreactive B cells. Collectively, these studies highlight a dynamic role for endogenous TLR signaling in both B-cell tolerance and activation.

Our laboratory has also contributed to the understanding of central B-cell tolerance. We showed that AID expression in BM immature and transitional B cells suppresses the development of autoreactivity (Kuraoka et al., 2011). It has been previously shown that the relative AID expression of pre-B and imm B cells in MyD88 deficient mice is markedly reduced (70-75% in pre-B; 60-70% in imm) (Han et al.,
Additionally, we saw that the synergistic signaling of BCR and TLR (initiated by anti-\(\mu\) and CpG, respectively) resulted in increased AID expression in immature and T1 B cells (Fig. 2).

These findings have lead us to hypothesize that B-cell central tolerance is mediated by processes that are independent of Ag affinity; tolerance of B cells is initiated by the internalization, processing and presentation of Ag to endogenous TLRs. It is the engagement of intracellular TLRs by endogenous self-ligands that can lead to the elevated expression of AID which induces DNA damage. This damage must be repaired in autoreactive B cells, otherwise, they die. This apoptotic death may represent immunological tolerance (Fig 3).
Figure 2. BCR and TLR synergism induces increased AID expression.

Immature/T1 B cells from BL/6 mice were cultured in media containing 0.5 µg/mL CpG or LPS with or without 10 µg/mL of anti-IgM (anti-µ) Ab for 24h. The relative expression of AID in immature/T1 B cells is shown.
Figure 3. Proposed Pathway for MyD88 and AID mediated B-cell central tolerance

Self-Ag induced BCR stimulation leads to trafficking of self-ligand to endogenous TLR-containing endosomes. Synergy of TLR and BCR signaling leads to the elevation of AID expression in autoreactive B cells leading to increased DNA damage and consequent clonal deletion. Ung and Msh2 are DNA repair mechanisms. P53 is a cell cycle regulator that if silenced, leads to growth arrest and apoptosis.
2.2 Materials and Methods

2.2.1 Mice

C57BL/6 (CD45.2), 3H9 heavy chain knock-in (Chen et al., 1995), 3H9.Myd88−/−, 3H9.Aicda−/−, Myd88−/− mice (all BL/6 background) were bred and maintained in specific pathogen-free conditions at the Duke University Animal Care Facility and with sterile bedding, water, and food. 3H9.Myd88−/− and Myd88 mice received water containing the antibacterial drug Septra (trimethoprim and sulfamethoxazole) and ALPHA-dri® bedding. All studies were approved by the Duke University Institutional Animal Care and Use Committee.

2.2.2 Flow Cytometry

BM cell suspensions were exposed to ammonium chloride buffer to lyse RBC and live mononuclear cells enumerated in Trypan Blue; typically, 2 x 10⁶ nucleated cells were suspended in 100 µl of labeling buffer (PBS with 2% FCS and labeled mAb) for 20 minutes on ice with propidium iodide (Sigma-Aldrich) included to identify dead cells. Labeled cells were analyzed on a FACS Canto (BD Biosciences). Flow cytometric data were analyzed with FlowJo software (Tree Star). Specific BM compartments were discriminated as previously described (Kuraoka et al., 2011).

2.2.3 Cell Culture

Sorted imm/T1 B cells (2.5 x 10⁴) from BL/6 mice were cultured in media (IMDM/10% FCS) containing 0.5 µg/mL CpG (InvivoGen) or LPS (Sigma) with or without 10 µg/mL of F(ab)’2 anti-IgM (anti-µ) IgG (Jackson Immunoresearch) for 24 hours. Total RNA was extracted from the cells and cDNA was prepared by standard methods (Mao et al., 2004).
2.2.4 Quantitative RT-PCR and Quantification of AID Expression

cDNA from cultured cells and AID cDNA templates were amplified in a primary PCR using Ramp-Taq DNA polymerase (Denville Scientific) with AID188 and AID119 primers (Reynaud et al., 1994). Expression of AID mRNA was determined by a quantitative RT-PCR as described previously (Kuraoka et al., 2011).

2.2.5 Statistical Analyses

Statistical significance (at levels of $P \leq 0.05$; $P \leq 0.01$; $P \leq 0.001$) in paired data was determined by Student's $t$ test. Data are presented as mean ± SE.

2.3 Results

2.3.1 MyD88 deficiency impairs central B-cell tolerance

It has been recently reported that humans deficient for MyD88, IRAK-4 and UNC-93B suffer from defective central and peripheral B-cell tolerance checkpoints as evidenced by increased numbers of autoreactive new emigrant/ mature naïve B cells in the blood (Isnardi et al., 2008). To determine whether central tolerance is impaired in MyD88$^{-/-}$ mice, we generated MyD88 deficient mice homozygous for the 3H9 VDJ knock-in allele (Chen et al., 1995). 3H9 knock-in mice contain a V(D)J rearrangement in the heavy chain of antibody that is reactive against dsDNA when in association with approximately 60% of $\kappa$-light-chains (Ibrahim et al., 1995). Rearrangement to this autoreactive heavy chain results in the tolerizing deletion of 30-50% of immature B cells (Chen et al., 1995). If MyD88 mediates central tolerance, deletion of autoreactive immature B cells will be alleviated in 3H9.MyD88$^{-/-}$ mice.
Flow cytometric analysis of BM cells from BL/6, MyD88−/−, 3H9 and 3H9.MyD88−/− revealed a MyD88-dependent tolerance checkpoint in autoreactive (3H9) mice. The BM B-cell compartments of MyD88−/− mice were comparable to those of BL/6 controls. The numbers of immature and T1 B cells in 3H9 mice, however, were significantly reduced (P ≤ 0.001). In contrast, numbers in the immature and T1 B-cell compartments in 3H9.MyD88−/− mice were double those in 3H9 mice (P ≤ 0.01 and P ≤ 0.001, respectively) (Fig. 4). Interestingly, the rescue observed was gene dose dependent. Mice hemizygous for MyD88 (MyD88+/-) were able to rescue the imm/T1 B cell population that is eliminated in their 3H9 deficient litter mates (Fig. 5).
Figure 4. MyD88 deficiency impairs central B-cell tolerance

B-cell development in BM of BL/6, Myd88<sup>−/−</sup>, 3H9, and 3H9. Myd88<sup>−/−</sup> mice was compared. Representative flow data for IgM/IgD expression of BM immature, T1/T2, and mature B cells are shown. Absolute cell number of the indicated B-cell compartments in WT (Myd88<sup>−/−</sup>, dark gray bars, Myd88<sup>−/−</sup> pink bars) and 3H9 (Myd88<sup>−/−</sup>, dark gray bars, Myd88<sup>−/−</sup> pink bars) mice are shown. Histograms represent mean values ± SEM (n=4-6). (**P ≤ 0.01; ***P ≤ 0.001)
Figure 5. Gene dosage effect of *MyD88*.  

B-cell development in BM of BL/6, *Myd88*<sup>+/+</sup>, *Myd88*<sup>−/−</sup>, 3H9, 3H9. *Myd88*<sup>+/−</sup> and 3H9. *Myd88*<sup>−/−</sup> mice was compared. Absolute cell number of the indicated B-cell compartments in mice are shown (n=4-6).
2.4 Discussion

The recent identification of AID-mediated central B-cell tolerance in both mouse and human furthers our understanding of clonal deletion in the BM (Kuraoka et al., 2011; Meyers et al., 2011). The mechanisms by which AID facilitates the removal of developing autoreactive B cells however are not known. A recent human study showed that patients deficient in the TLR signaling pathway components IRAK4, MyD88 and UNC93B had impaired central and peripheral B-cell tolerance (Isnardi et al., 2008).

Because of the observed reduction of AID expression in MyD88 deficient developing B cells (Han et al., 2007) and our observation of a BCR-TLR synergism in the induction of AID expression in immature and T1 B cells (Fig. 2), we hypothesize that engagement of intracellular TLRs by endogenous self-ligands can lead to the elevated expression of AID which induces DNA damage and ultimately eliminates autoreactive B cell clones by clonal deletion (Fig 3).

In order to test this hypothesis, the demonstration of MyD88-mediated central B-cell tolerance in mouse is essential. Indeed, using a mouse model system that induces clonal deletion of autoreactive B cells by clonal deletion, I show that removing MyD88 significantly impaired the deletion of immature and T1 B cells (Fig. 4). Interestingly, the rescue of these populations appeared to be gene dose dependent. Mice hemizygous for MyD88 had increased numbers of imm/T1 B cells as compared to 3H9 controls, albeit the population was smaller than that observed in their homozygous deficient litter mates, indicating that the deletion of even half of MyD88 gene expression was sufficient to
rescue autoreactive immature B cells (Fig. 5). Together, these observations coupled with the established role for AID in central tolerance suggest a model whereby self-Ag induced BCR stimulation leads to trafficking of self-Ag to endogenous TLR-containing endosomes. Synergism of TLR and BCR signaling elevates AID expression in developing autoreactive B cells leading to increased DNA damage and consequent clonal deletion (Fig. 3).
3. Comparable Humoral Responses to Native and Haptenated Proteins in MyD88 Deficient Mice

3.1 Introduction

Haptens are recognized by antibodies but induce humoral immune responses only when covalently ligated to immunogenic carriers. Antibodies produced against hapten-protein conjugates can be hapten- or carrier-specific, or can recognize neo-determinants specific to the hapten/carrier complex. Responses to hapten-carrier immunogens are often dominated by hapten-specific antibody, defining the hapten as the immunodominant epitope (Landsteiner and van der Scheer, 1931).

The utility of hapten-protein immunogens in fundamental immunologic research is difficult to overstate. Indeed, the roster of paradigmatic discoveries based on studies of immune responses to hapten-protein conjugates includes cognate T- and B-cell interaction (Cumano and Rajewsky, 1986; Mitchison, 1971; Rock et al., 1984), affinity maturation of antibody (Eisen and Siskind, 1964; Siskind et al., 1968), class-switch recombination (Hirano et al., 1983; Muller and Rajewsky, 1983), Ig somatic hypermutation (Gearhart et al., 1981; Kaartinen et al., 1983) and antigen-driven clonal selection and competition (Jacob et al., 1991a; Sablitzky et al., 1985; Wysocki et al., 1986). These foundational studies assumed that the immunologic properties of hapten-protein conjugates were identical to those of naturally occurring protein antigens, and therefore, generalizable to infectious agents, pathologic autoimmunity, responses to transplanted tissues, etc.

This long held assumption was recently cast into doubt by a series of studies designed to determine the significance of TLR signaling in the generation of antibody
responses to thymus-dependent protein antigens (Gavin et al., 2006; Meyer-Bahlburg et al., 2007; Palm and Medzhitov, 2009a; Pasare and Medzhitov, 2005a). Initial reports (Pasare and Medzhitov, 2005a) indicated that MyD88, a critical signalling component of many TLR (Akira and Takeda, 2004) was required for antibody responses to a native protein antigen. Later studies using haptenated protein antigens (Gavin et al., 2006) did not confirm a requirement for TLR signaling in adjuvant-enhanced antibody responses and in an effort to resolve these discordant results, Palm and Medzhitov observed that haptenated protein antigens, unlike native proteins, are fundamentally distinct in that they do not require MyD88 activity (Palm and Medzhitov, 2009a). This claim is both intriguing and concerning as hapten-carrier proteins are routinely used as model antigens to study physiologic immunity.

Immunization with chicken γ-globulin (CGG), hen ovalbumin (Ova), (4-hydroxy-3-nitrophenyl acetyl)-CGG (NP-CGG), or NP-Ova precipitated in alum elicits robust antibody and germinal center (GC) responses that have long been used to detail humoral immunity (Jack et al., 1977; Kontiainen and Mitchison, 1975). Immunization with NP-CGG/native CGG and NP-Ova/native Ova allows us to quantify hapten- and protein specific antibody and GC responses to each immunogen and to determine any role for MyD88 signaling in each response. Here, we demonstrate that both native and haptenated protein elicit comparable antibody and GC responses that are independent of MyD88 signaling. We conclude that native and haptenated proteins are comparable immunogens in both MyD88 deficient or –sufficient mice and that humoral responses to hapten-protein immunogens can faithfully be generalized to natural immunity.
3.2 Materials and Methods

3.2.1 Mice

C57BL/6 (CD45.2) and MyD88−/− from Jackson Laboratories (Bar Harbor, ME) were bred and maintained in specific pathogen-free conditions at the Duke University Animal Care Facility and with sterile bedding, water, and food. MyD88−/− received water containing the antibacterial drug Septra (trimethoprim and sulfamethoxazole) and ALPHA-dri® bedding. Mice used in experiments were 8 to 12 weeks of age. All studies were approved by the Duke University Institutional Animal Care and Use Committee.

3.2.2 Antigens and immunizations

The succinic anhydride ester of nitrophenyl (NP) (Cambridge Research Biochemicals, Cambridge, UK) was reacted with chicken gamma globulin (CGG) (Accurate Scientific, Westbury NY) or hen ovalbumin (Ova) (Sigma, St. Louis, MO) and precipitated in alum (NP-CGG/alum; NP-Ova/alum). NP-CGG contained 9-11 mol NP/mol CGG. NP-Ova contained 6 mol NP/mol Ova. Groups of C57BL/6 and MyD88−/− mice were immunized with a single i.p. injection of 20 µg NP-CGG/alum, CGG/alum, NP-Ova/alum or Ova/alum suspended in 200 µl PBS. For humoral memory experiments, mice were immunized with a secondary i.v. injection of soluble NP-CGG or CGG (10 µg) in 100 µl PBS.

3.2.3 Quantification of total serum immunoglobulin

Serum IgM (non-reduced) IgG, and IgG subtypes was quantified by ELISA on Ig κ/λ-coated (2 µg/ml each) 96-well plates (Falcon 3912; Becton Dickinson, Oxnard, CA). Three-fold serially diluted sera (initial dilution: 1:500) were added in duplicate and
incubated for 1 hour at room temperature. Each plate included serially diluted standard IgM (B1-8µ, IgM/λ.1) and IgG (H33Lγ1, IgG1/λ.1) (Takahashi et al., 1998) (Kelsoe et al., 1981) at an initial concentration of 2µg/ml. After washing, bound Ab was revealed by HRP-conjugated goat anti-mouse IgM or IgG (Southern Biotechnology Associates, Birmingham, AL) using a TMB peroxidase substrate kit (Bio-Rad Laboratory, Hercules, and CA). Serum Ab concentrations were determined by comparison of titrated samples to standards.

3.2.4 Quantification of serum NP-specific or protein-specific Abs

Serum IgM (non-reduced) or IgG specific for NP, CGG or Ova was quantified by ELISA on NIP5-BSA, CGG or Ova-coated (2µg/ml) 96-well plates (Falcon 3912; Becton Dickinson, Oxnard, CA) as described (Kuraoka et al., 2009). Three-fold serially diluted sera (initial dilution: 1:100 or 1:1000) were added in duplicate and incubated for 1 hour at room temperature. Each plate included serially diluted standard monoclonal Ab specific for CGG (Cl-8, IgG1/κ), NIP (H33Lγ1, IgG1/λ.1; B1-8µ, IgM/λ.1) (Takahashi et al., 1998) (Kelsoe et al., 1981), and Ova (OVA-14, IgG1/κ; Sigma, St. Louis, MO) at an initial concentration of 2µg/ml. After washing, bound Ab was revealed by HRP-conjugated goat anti-mouse IgM or IgG (Southern Biotechnology Associates, Birmingham, AL) using a TMB peroxidase substrate kit (Bio-Rad Laboratory, Hercules, and CA) (Kuraoka et al., 2009). Serum Ab concentrations were determined by comparison of titrated samples to standards.
3.2.5 Histology of spleen sections

Mice were killed at 0, 8, 16 or 24 days post immunization, their spleens removed and embedded in TissueTek OCT compound (Fischer Scientific, Silver Spring, MD) by freezing in liquid N\textsubscript{2} chilled 2-methylbutane; tissues were stored at -80°C. Histologic sections (5 µm) sections were prepared and fixed in 1:1 Acetone: Methanol for 10 minutes at -20°C (Kuraoka et al., 2009); histologic sections were stored at -20°C until use. Sections were labeled with B220-biotin, TCR\textbeta-PE, and GL-7-FITC mAb. FITC signal was amplified using anti-FITC-AlexaFluor (AF) 488 mAb (Invitrogen); streptavidin-AF350 (Invitrogen) was used to amplify B220-biotin signal. Scale bar equals 50 µm for all images. Images were acquired using a Zeiss Axiovert 200M confocal immunofluorescent microscope at 200x magnification.

3.2.6 Flow cytometry

FITC-, PE-, APC-AF780-, PE-Cy7-, biotin-, or Pacific blue-conjugated mAb specific for mouse GL7, IgD (11-26), TCR\textbeta (H57-597), IgM (II/41), Fas, B220 (RA3-6B2) were purchased (BD Pharmingen or eBioscience). Streptavidin-APC (eBioscience) identified biotinylated mAb. Spleen cell suspensions were exposed to ammonium chloride buffer to lyse RBC and live mononuclear cells enumerated in Trypan Blue; typically, 10\textsuperscript{6} nucleated cells were suspended in 100 µl of labeling buffer (PBS with 2% FCS and labeled mAb) for 20 minutes. on ice with propidium iodide (Sigma-Aldrich) included to identify dead cells. Labeled cells were analyzed on a FACS Canto or a FACS Vantage with DIVA option (BD Biosciences). Flow cytometric data were analyzed with FlowJo software (Tree Star).
3.2.7 Amplification of V\textsubscript{H}DJ\textsubscript{H}2 rearrangements

Genomic DNA was isolated from sorted B-cell subsets by phenol-chloroform extraction; specific VDJ rearrangements were amplified by a nested PCR by *Pfu* polymerase (Stratagene) (Han et al., 1997) and forward primers specific for the V3 subfamily of V\textsubscript{H}1 gene segments and a reverse primer specific for J\textsubscript{H}2 (Jacob et al., 1991b). VDJ amplicands were ligated into pBS II SK\textsubscript{(+)} plasmid (Stratagene), cloned by bacterial transformation (Jacob et al., 1991b), and sequenced using an Applied Biosystems automated DNA sequencer. VDJ sequences were analyzed using IMGT/V-QUEST (http://imgt.cines.fr) and NCBI blast search software.

3.2.8 Statistical analysis

Statistical significance (at levels of *P* ≤ 0.05 or ≤ 0.01) in paired data was determined by Student's *t* test. Data are presented as mean ± SE.

3.3 Results

3.3.1 Native and haptenated proteins induce antibody responses in MyD88\textsuperscript{−/−} mice

To determine whether MyD88 deficiency might affect Ig production generally, we quantified by ELISA serum levels of IgM, total IgG, and specific IgG subtypes in naïve BL/6 and congenic MyD88\textsuperscript{−/−} mice (Fig. 6). Compared to normal controls, MyD88\textsuperscript{−/−} mice exhibited modest but significant (*P* ≤ 0.05) reductions in serum IgM but similar
Figure 6. Serum immunoglobulin levels in C57BL/6 and MyD88 knockout mice.

Serum IgM, IgG and IgG subclass levels were measured by ELISA on naïve sera isolated from WT C57BL/6 mice (black) and MyD-88-deficient (gray). MyD88<sup>−/−</sup> mice exhibited modest but significant reductions in serum IgM but similar concentrations of total IgG. Serum IgG1, IgG2b, IgG2c, and IgG3 in naïve MyD88 deficient mice differed from BL/6 controls, with significantly lower concentrations of IgG2c and IgG3, but substantial increases of IgG1. *<sub>P</sub> ≤ 0.05; **<sub>P</sub> ≤ 0.01. Mean ± SEM from ≥3 independent experiments shown (N = 12 BL/6; 8 KO).
concentrations of total IgG. As reported (Gavin et al., 2006), serum IgG1, IgG2b, IgG2c, and IgG3 in naïve MyD88 deficient mice differed from BL/6 controls, with significantly lower concentrations of IgG2c and IgG3 (82± (24) µg/ml vs. 248 (±61) µg/ml and (14(±5) µg/ml vs. (36 (±8) µg/ml, respectively; P≤ 0.05) and very substantial increases of IgG1 (483± (116)µg/ml vs. 93 (±30) µg/ml; P≤ 0.002) (Fig.6). Whereas the absence of MyD88 signaling altered the distribution of IgG isotypes, presumably by moderating dendritic cell activation (Hou et al., 2011), it did not preclude their expression.

A major oversight in work that supports a role for TLR-dependent responses to native proteins is the lack of rigorous quantitation of response to these proteins (Gavin et al., 2006; Palm and Medzhitov, 2009a; Pasare and Medzhitov, 2005a). While much work was done to quantify the amount of antibody against native protein following immunization with carrier alone and antibody against hapten following immunization with hapten-carrier, the contribution of antibody against the carrier following hapten-carrier immunization has been wholly disregarded. To determine whether native- and haptenated proteins elicit qualitatively different humoral responses through a pathway that is MyD88 dependent, we compared IgM and IgG Ab responses to CGG, NP\textsubscript{10}-CGG, Ova and NP\textsubscript{6}-Ova in BL/6 and congenic MyD88 deficient mice. Control and knockout mice were immunized with 20 µg NP\textsubscript{10}-CGG, CGG, NP\textsubscript{6}-Ova or Ova precipitated in alum; NIP\textsubscript{5}–specific and CGG-specific IgM and IgG Ab levels were determined against monoclonal Ab standards 8, 16 and 24 days post immunization while NIP\textsubscript{5}– and Ova-specific IgG serum Ab was quantified on days 8 and 16.

Immunization with NP\textsubscript{10}-CGG elicited comparable IgM responses in both
MyD88−/− and BL/6 mice. In unimmunized BL/6 mice NIP5-reactive serum IgM was undetectable, but by day 8 post immunization IgM Ab peaked at 48 (±12) µg/ml then
declined on days 16 (6 ±2 µg/ml) and 24 (3 ±1 µg/ml) (Fig. 7A). Naïve, MyD88 deficient mice also had undetectable levels of NIP<sub>5</sub>-reactive IgM, but like their normal controls, generated peak IgM Ab responses by day 8 (37 ±15 µg/ml) that subsequently declined, albeit not so precipitously (day 16, 21 ±9 µg/ml (P ≤ 0.01); day 24, 17±8 µg/ml) (P ≤ 0.01) (Fig.7A).

CGG-reactive, serum IgM also waxed and waned in BL/6 and MyD88 deficient mice immunized with NP-CGG with similar kinetics (Fig.7B).

NP<sub>10</sub>-CGG/alum elicited qualitatively similar IgG responses in both BL/6 and MyD88<sup>−/−</sup> mice. In the sera of naïve BL/6 mice no NIP<sub>5</sub>-reactive IgG could be detected; however, immunization elicited strong IgG Ab responses that peaked by day 16 post immunization and were maintained through day 24 [day 8, 566 (±160) µg/ml; day 16, 1204 (±195) µg/ml; day 24, 1016 (±121) µg/ml] (Fig. 8A). Immunization of MyD88 deficient mice elicited similar IgG Ab responses—from undetectable levels of NIP<sub>5</sub>-binding IgG at the time of immunization to a peak at day 16 post immunization that was insignificantly higher than that of controls [day 8, 200 (± 40) µg/ml; day 16, 1960 (±450) µg/ml; day 24 1750 (±910) µg/ml] (Fig. 8A).

CGG-reactive IgG in naïve BL/6 mice was below the limit of detection; immunization with NP-CGG/alum induced small (3%-4% of NIP IgG levels) but significant increases of IgG Ab that peaked on day 16 [41 (±7) µg/ml] and held steady through day 24 [35 (±6) µg/ml] (Fig. 8C). Immunization of MyD88 deficient mice with NP-CGG resulted in CGG IgG Ab responses with similar kinetics and quantity-from
undetectable levels to a peak on day 8 [22 (±8) µg/ml] that was maintained on days 16 [16 ±1) µg/ml] and 24 [21(±4) µg/ml] (Fig. 8C).

Figure 7. IgM antibody responses to haptenated or native proteins in MyD88 knockout mice as compared to WT controls.

C57BL/6 and MyD88−/− mice were immunized i.p. with 20 µg NP-CGG (A,B) or CGG (C,D) in alum; serum IgM specific for NIP(A,C) or CGG (B,D) were measured by ELISA on days 0, 8, 16 and 24 post immunization [BL/6 (---) or MyD88−/− (---) mice immunized with NP-CGG (■) or CGG (□)]. Mean values ±SEM from 3 independent experiments shown (n = 5 BL/6; 5 KO each point; *P≤ 0.05; **P≤ 0.01).
IgG Ab responses elicited by NP₆-Ova/alum in BL/6 and MyD88⁻/⁻ mice were also similar. In BL/6 mice, levels of NIP5-reactive IgG were undetectable, increasing on days 8 (773 ±145 µg/ml) and 16 (1076 ±244 µg/ml). MyD88 deficient mice had undetectable levels of NIP₅-reactive IgG at the time of immunization, increasing and stabilizing on days 8 and 16 to 1000 (±455) µg/ml and 1008 (±192) µg/ml, respectively (Fig. 8B). Concentrations of Ova-reactive IgG in BL/6 mice following immunization with NP-Ova in alum were undetectable immediately following immunization, remaining low at day 8 (1.4 ±0.4 µg/ml), and increasing on day 16 to 27.5 (±3) µg/ml (Fig. 8D). MyD88 deficient mice exhibited similar kinetics, with undetectable levels of Ova-reactive IgG immediately following and 8 days after immunization followed by an increase on day 16 to 6 (± 2.5) µg/ml. Collectively these results show that immunization with the haptenated protein antigen induced equivalent high concentrations of NIP₅—specific IgG and lower concentrations of CGG-specific and Ova-specific IgG in both MyD88 sufficient and deficient mice (Fig. 8A-D).

We next examined the antibody response to CGG and Ova in alum. Immunization with CGG elicited similar IgM responses in both MyD88⁻/⁻ and BL/6 mice. Levels of CGG-reactive IgM in BL/6 mice were undetectable at the time of immunization, peaked on day 8, decreased on day 16 and remained constant on day 24 (Fig. 7D). Similarly, levels of CGG-reactive IgM in MyD88 deficient mice were undetectable at the time of immunization, peaked on day 8, and remained constant on days 16 and 24 (Fig. 7D). Due to the known stickiness of non-reduced pentameric IgM, low but non-significant
levels of non-specific IgM (2-5 µg/ml) could be detected following immunization with CGG (Fig. 7C).

Levels of CGG-reactive IgG in BL/6 mice following immunization with CGG in alum were undetectable immediately following immunization, rose to 78 ±14 µg/ml by day 8, and increased steadily on days 16 and 24 to 320 ±24 µg/ml and 440 ± 59 µg/ml, respectively (Fig. 8C). MyD88 deficient mice had undetectable levels of CGG-reactive IgG 8 days after immunization (Fig. 8C). By day 16 however, CGG-reactive IgG levels peaked at 439 ±152 µg/ml and were sustained on day 24, measuring 315 ± 81 µg/ml (Fig. 8C).

Levels of Ova-reactive IgG in BL/6 mice following immunization with Ova in alum were undetectable immediately following immunization, increasing to 5 (±3) µg/ml by day 8, and to 105 (±7) µg/ml by day 16 (Fig. 8D). Levels of Ova-reactive IgG in MyD88 mice following immunization with Ova in alum were undetectable immediately following immunization, remained low 8 days following challenge 1.4 (±0.4), and increased to 67 (±7) µg/ml by day 16 (Fig. 8D).

While there was an initial delay in response to CGG in MyD88 deficient mice, by days 16 and 24 post immunization, both MyD88 sufficient and deficient animals produced comparable levels of IgG reactive to CGG (Fig. 8C). The kinetics of the response to Ova was largely comparable between MyD88 deficient and congenic controls (Fig. 8D).
Figure 8. Haptenated or native protein immunization elicits robust antibody responses in MyD88 knockout mice.

C57BL/6 and MyD-88<sup>−/−</sup> mice were immunized i.p. with 20 µg NP-CGG (A,B,E), CGG (C,D,G) NP-Ova(F), or Ova(H) in alum; serum IgM (A-D) or IgG (E-H) specific for NIP, CGG, or Ova were measured by ELISA on days 0, 8, 16 and 24 post immunization [BL/6 (—) or MyD-88<sup>−/−</sup> (---) mice immunized with NP-CGG (NIP [■] or CGG [□] Ab), NP-Ova (NIP [●] or Ova [◊] Ab), CGG (CGG [□] Ab), or Ova (Ova [◊] Ab)]. Mean values ± SEM from 3 independent experiments shown (n = 5 BL/6; 5 KO each point; **P ≤ 0.01).
3.3.2 The NP hapten is the immunodominant epitope

Ab responses elicited by hapten-carrier conjugates are most often directed against the hapten, *i.e.*, the hapten is the immunodominant epitope (Landsteiner and van der Scheer, 1931). This immunodominance was present in our studies as well as NP-CGG and NP-Ova elicited hapten-specific IgG responses that were ≈20- to 100-fold greater than to the carrier (Fig. 8). Regardless of MyD88 signaling, native CGG or Ova elicited significantly less serum IgG Ab (300 µg/ml and 80 µg/ml, respectively; Fig. 8C-D) than did their haptenated forms (Fig. 8A-B).

3.3.3 Immunization with native and haptenated proteins elicits robust germinal center response in MyD88 deficient mice

To determine whether the GC response against native and haptenated protein is comparable in MyD88 deficient mice, knockout and control animals were immunized with NP-CGG, CGG, NP-Ova, or Ova in alum and on days 8, 16, and 24 (NP-CGG/CGG) or days 8 and 16 (NP-Ova/Ova). The splenic GC response was analyzed by flow cytometry and histology (Kuraoka et al., 2009; Zaheen et al., 2009).

Naïve BL/6 mice contained few [0.5 (±0.5)%] splenic GC B cells (GL-7⁺Fas⁺B220hi), but by day 8 after immunization with NP-CGG/alum, this frequency became significantly elevated [4.1 (±2)%; P ≤ 0.01] and gradually declined on days 16 [2.3 (±1)%; P ≤ 0.01] and 24 [1.5 (±1)%; P ≤ 0.05] (p value relative to naïve) (Fig. 9A-B).

Similar kinetics were seen in control mice following immunization with CGG in alum; the frequency of GL-7⁺Fas⁺B220hi splenocytes increased substantially by day 8
[3.0 (±0.9) %; P ≤ 0.01], was stable through day 16 [2.5 (±1) %; P ≤ 0.01], and returned to background by day 24 [1 (±0.3) %; P ≤ 0.05] (p value relative to naïve) (Fig. 9C).

Frequencies of splenic GC B cells were similar in naïve MyD88 mice [0.4 (±0.4) %]; 8 days after immunization with NP-CGG/alum their frequency increased significantly [3.6 (±1)8%; P ≤ 0.01] and subsequently declined to naïve levels by day 24 [day 16, 2 ± (0.3) %; P ≤ 0.01; day 24, 0.5 (±0.2) %;] (Fig. 9A-B). Immunization with CGG/alum elicited a GC reaction that was comparable in magnitude to that driven by NP-CGG but with a slower kinetic (Fig. 9C). The frequency of splenic GL-7+ Fas+B220hi cells was not significantly increased at day 8 after immunization [0.7 (±0.4) %]. However, the frequency peaked on day 16 (1.5 ± 1.0%; P ≤ 0.05) and began to decline by day 24 (0.9 ± 0.4%) (Fig. 9C). While there was a reduced frequency of splenic GC B cells in MyD88 deficient mice as compared to controls 8 days following immunization with CGG (Fig. 9C) that correlated with a significant reduction in antibody concentration (Fig. 9C), histologically, splenic GC, while slightly smaller following CGG immunization at day 8 in MyD88 deficient mice, were comparable in frequencies and size in both MyD88 deficient and sufficient mice when immunized with either native or haptenated protein. (Fig.10).

Immunization with NP-Ova in alum elicited robust GC responses in both MyD88 deficient and congenic control mice. In BL/6 mice, the frequency of GL-7+, Fas+ cells peaked 8 days after immunization (9 ± 0.1%; P ≤ 0.01) and began to decline by day 16 (2 ±0.2%; P ≤ 0.01) (Fig. 9D). Similar kinetics were seen in control mice following immunization with Ova in alum; the frequency of splenic GC B cells peaked at day 8 (8 ±0.7%; P ≤ 0.01), and began to decline on day 16 (4 ±0.7%; P ≤ 0.01) (Fig. 9E).
In MyD88 mice following NP-Ova immunization, the frequency of splenic GC B cells peaked 8 days after immunization (6 ±0.5%; P ≤ 0.01) and began to decline by day 16 (2 ±0.2%; P ≤ 0.01) (Fig. 9D). Similar kinetics were seen in control mice following immunization with Ova in alum; the frequency of splenic GC B cells peaked at day 8 (6 ±0.7%; P ≤ 0.05), and began to decline on day 16 (2 ±0.3%; P ≤ 0.01) (Fig. 9E).

We conclude that immunization with native or haptenated proteins induces robust GC formation and that signaling through MyD88 is not required for the antibody response. Additionally, our findings that the response to native protein, while initially delayed, was intact in MyD88 deficient mice and, at later time points, comparable to the response mounted against haptenated protein further demonstrates that antibody responses to both native and haptenated protein is MyD88 independent.
Figure 9. Haptenated or native protein immunization elicits robust germinal center responses in MyD88 knockout mice.

Spleen cells were harvested from naive WT C57BL/6 (3A), and WT C57BL/6 or MyD-88⁻⁻ mice after immunization with 20µg NP-CGG (3B), CGG (3C), NP-Ova (3D) and Ova (3E) in alum. Germinal center cells (B220⁺ Fas⁺ GL7⁺) were analyzed by flow cytometry. NP-CGG imm. (▲); NP-Ova imm. (●); CGG imm. (△); Ova imm. (○); BL/6 (—) and MyD-88⁻⁻ (---) mice. Mean values ±SEM from 3 independent experiments shown (n = 5 BL/6; 5 KO each point; *P<0.05, **P<0.01). Representative flow cytometric data shown.
Figure 10. Haptenated or native protein immunization elicits robust germinal center formation in MyD88 knockout mice.

Splenic sections from naïve (A) and NP-CGG or CGG immunized (B) mice were labeled with B220-biotin, TCRβ-PE (red) and GL-7-FITC (green) mAb. FITC signal was amplified using anti-FITC-AlexaFluor (AF) 488 mAb. Streptavidin-AF350 was used to amplify B220-biotin signal (blue). Scale bar equals 50µm for all images.
3.3.4 Somatic hypermutation is MyD88 independent

A hallmark of the GC reaction is V(D)J hypermutation (SHM) in B cells (Jacob et al., 1991b). To determine the role of MyD88 signaling in SHM within GC during T-dependent responses, splenic GC B cells were sorted from MyD88 deficient and congenic BL/6 controls 16 days after immunization with NP-CGG. PCR was used to amplify H-chain V regions encoded by the V3 subfamily of V\textsubscript{H}1 gene segments, and determine the diversity and frequency of mutations within this cell population. There was no significant difference in mutation frequency between BL/6 [20.5 x 10\textsuperscript{-3} mutations per base pair sequenced (mutations/bp)] and MyD88 (15.2 x 10\textsuperscript{-3} mutations/bp), indicating that MyD88 is not required for IgH SHM. There was more diverse V\textsubscript{H} gene usage in MyD88 deficient mice (Table 2; Fig. 11), which may be explained by the initial delay in antibody response and lower frequency of GC seen in MyD88 deficient mice (Figs. 8 and 9). The delayed response in deficient animals coupled with more varied V gene usage within GC relative to control suggest that V gene usage has not become as restricted in MyD88 deficient animals when compared to BL/6 controls. Together, our findings support previous work demonstrating that TLR signaling is not required for T-dependent Ab responses, but can increase Ig production (Meyer-Bahlburg et al., 2007).
Figure 11. MyD88 signaling not required for somatic hypermutation of antibody during T-dependent responses within germinal centers.

$V_{\text{H}}1DJ_2$ rearrangements were amplified by nested PCR from genomic DNA of day 16 splenic GC cells isolated from BL/6 or MyD88$^{-/-}$ mice immunized i.p. with 20µg NP-CGG/alum. MyD88$^{-/-}$ mice exhibited more diverse $V_{\text{H}}$ gene usage than BL/6 controls, but no difference in mutation frequency.

Table 3. Somatic hypermutation in GC B cells following immunization

$V_{\text{H}}1DJ_2$ rearrangements were amplified by nested PCR from genomic DNA of day 16 splenic GC cells isolated from BL/6 or MyD88$^{-/-}$ mice immunized i.p. with 20µg NP-CGG/alum. The mutation frequency is calculated by numbers of unique mutations/base pairs sequenced.

<table>
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<th></th>
<th>Nos. VDJ Rearrangements</th>
<th>Basepairs Seq.</th>
<th>Nos. Mutations</th>
<th>Nos. Mutated Rearrangements</th>
<th>Mutation Freq. ($x10^3$)</th>
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<td>BL/6</td>
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<tr>
<td>MyD88$^{-/-}$</td>
<td>12</td>
<td>$3.22 \times 10^3$</td>
<td>49</td>
<td>11 (91.7%)</td>
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3.3.5 Haptenated and native proteins elicit secondary antibody responses independent of MyD88 signaling

Whereas B-cell-intrinsic TLR signals are not required for humoral memory responses to haptenated proteins (Meyer-Bahlburg et al., 2007), the requirement for TLR signaling in memory responses to native proteins has not been tested. To determine the capacity of haptenated and native proteins to generate humoral memory and the effects of MyD88 signaling on both types of antigen, MyD88 deficient and sufficient mice were immunized i.p. with either 10 µg haptenated or native protein in alum; 42 days after this primary immunization, mice were given an i.v. challenge with 10 µg of homologous antigen in PBS and one week later IgG antibody against haptenated and native proteins were quantified by ELISA.

In BL/6 mice immunized with NP-CGG, NIP$_5$-reactive serum IgG levels at day 35 post-immunization averaged 1328 (±128) µg/ml; the i.v. challenge boosted these levels to 1932 (±44) µg/ml by day 49 (Fig. 12A). In MyD88 deficient mice, serum IgG reactive with NIP$_5$ were significantly lower [316 (±94.7) µg/ml; $P \leq 0.01$] at day 35 as compared to BL/6 mice, but the i.v. challenge increased the serum IgG to 2044 (±421) µg/ml ($P \leq 0.01$) (p value= day 35 vs. day 49), comparable to controls (Fig. 12A).

The concentration of CGG-reactive IgG in BL/6 mice increased from 11 (±2) µg/ml on day 35 to 248 (±81) µg/ml ($P \leq 0.01$) following NP-CGG immunization (Fig. 12A). Similarly, levels of CGG-reactive IgG in MyD88 deficient mice increased from 8 (±2) µg/ml on day 35 to 513 (±95) µg/ml ($P \leq 0.01$) 49 days following NP-CGG immunization (Fig. 12A).
In BL/6 mice, 35 days after primary immunization with CGG, levels of CGG-reactive IgG averaged 80 (±8) µg/ml, increasing to 1012 (±345) µg/ml (P ≤ 0.05) on day 49 (Fig. 12B). In MyD88 deficient mice 35 days after primary immunization with CGG, levels of CGG-reactive IgG averaged 76 (±21) µg/ml, increasing to 1425 (±477) µg/ml (P ≤ 0.05) on day 49 (Fig. 12B). NIP$_S$-reactive IgG was undetectable at all time-points following CGG immunization in both BL/6 and MyD88 deficient mice (Fig. 12B). Secondary Ab responses against haptenated and native proteins are equivalent and neither require MyD88 signaling.
Figure 12. Haptenated and native proteins establish humoral memory in MyD88 knockout mice.

WT C57BL/6 and MyD88−/− mice were immunized i.p. with 20µg NP-CGG (A) or CGG (B) in alum, bled on day 35, and then received a secondary alum free i.v. immunization at day 42. On day 49 after immunization, NIP- and CGG-specific IgG was measured by ELISA. Dark gray bars, wild-type C57BL/6; light gray bars, MyD88−/− mice. *, *< .05; **, *< .01. Mean values ±SEM from ≥3 independent experiments shown (n = 5 BL/6; 5 KO each point).
3.4 Discussion

In our hands, immunization of BL/6 and congenic MyD88 deficient mice with haptenated- or native protein antigens elicits serum Ab, GC responses and establishes humoral memory; all processes which require adjuvant (unpublished observations). Comparison of Ab elicited by immunization with native or haptenated protein, revealed average serum Ab concentrations (Fig. 8) were more than ten-fold higher than those observed in studies that state that “haptens are highly immunogenic whereas native proteins are non-immunogenic” (Palm and Medzhitov, 2009a). Additionally, we found that haptenated proteins, without the assistance of adjuvant, are incapable of inducing a humoral antibody response (data not shown). This finding further substantiates that globally, haptenated proteins do not qualitatively change the humoral immune response, but rather act as the immunodominant epitope. Another finding in our studies which refutes the idea of hapten as immunogen is the effect of haptenation on the magnitude of the carrier response. If hapten is immunogenic, then the magnitude of the response to carrier in the context of hapten-carrier conjugation should be greater than that elicited against the unhaptenated native protein. This was not the case in our studies; native protein-specific IgG responses were ≈10-fold greater than to the carrier in the hapten-carrier conjugate.

In work claiming that haptens are immunogenic, peak concentrations of antibody generated against the hapten-carrier conjugate are quite low and atypical of robust humoral immune responses (Palm and Medzhitov, 2009a). The suboptimal responses generated against both native and haptenated proteins in this work may quite possibly
be due to the use of Imject alum as an adjuvant instead of using the classical immunoprecipitation of aluminum hydroxide. It has been recently shown that the formulation of Imject alum is not equivalent to either of the aluminum-containing adjuvants that are used in licensed vaccines (Hem et al., 2007). Indeed, we have immunized mice with aluminum hydroxide precipitate, Alhydrogel (Brenntag Biosector), and Imject (Pierce) alum adjuvants and found Imject to be significantly inferior to classical precipitated and Alhydrogel alum adjuvants in eliciting primary IgM and IgG Ab responses (D.W. Cain et al., manuscript in preparation).

While several groups have demonstrated that B-cell intrinsic MyD88 signaling is not required to elicit Ab antibody responses (Gavin et al., 2006; Meyer-Bahlburg et al., 2007), a recent study confirms and extends this finding, showing that the physical context in which TLR ligands are presented determines whether or not B cells require signaling through MyD88 to elicit a humoral response (Hou et al., 2011). By using a TLR agonist-as-adjuvant system, Hou et al. prevent their ability to test the recent proposition that haptenated proteins qualitatively change the antibody response from a TLR-dependent event (when responding to native protein) to a TLR-independent event due to a “unique immunogenicity” conferred by the hapten (Palm and Medzhitov, 2009a). We contend that a qualitative difference in antibody response that has been ascribed to haptens is really a quantitative event that is masked by a poor immunization system. The “unique immunogenicity” assigned to haptenated protein is simply a misguided description of immunodominance, a phenomenon extensively described almost a century ago (Landsteiner and van der Scheer, 1931). Our data demonstrates the well-established concept of haptenic immunodominance, whereby haptens are not uniquely immunogenic, but serve as the immunodominant epitopes during the humoral response.
to the hapten-carrier protein. Additionally, we substantiate findings that antibody responses against T-dependent antigen do not depend on MyD88 signaling. We believe that these series of tests support the hypothesis that humoral immunity is elicited against both native and haptenated proteins in a MyD88 independent manner.
4. Overall Conclusions

TLRs, specifically endogenous TLRs that are able to recognize self-Ag, are attractive candidates for facilitating B-cell central tolerance. Work done by Wardemann et al. shows that autoantibody production in humans requires two distinct B cell developmental checkpoints. The first occurs in the BM and counterselects mostly antibodies that react against nuclear components such as ss- and ds-DNA. The second checkpoint occurs in the periphery and deletes mostly those antibodies antibodies that bind to cytoplasmic antigens (Wardemann et al., 2003). It is already well established that B cells express endogenous TLRs that are able to recognize self-Ag (Marshak-Rothstein, 2006; Pone et al., 2010). Additionally, there is evidence that BCR-endogenous TLR synergy in the periphery directs B-cell differentiation and activation in response to self-Ag (Pone et al., 2010). Recently, early AID expression in immature and transitional B cells during their maturation in the bone marrow was shown to be required for the counterselection of self-reactive cells (Kuraoka et al., 2011; Meyers et al., 2011). The mechanisms used to mediate this tolerance checkpoint have remained largely undefined. Because tolerance is induced mostly by B-cell intrinsic factors that distinguish between self and nonself Ag (Goodnow, 1996; Nemazee et al., 2000), it is likely that AID mediated B-cell central tolerance relies on some of these same self-discriminating factors. It is therefore reasonable to propose that BCR and TLR work together to discriminate self-Ag from nonself-Ag and to induce central tolerance mechanisms that modify self-reactive B cells.
Our work that elucidated a role for AID, an enzyme thought to be restricted to mediating CSR and SHM in peripheral mature B cells in the periphery (Muramatsu et al., 2000), in central B-cell tolerance (Kuraoka et al., 2011) re-emphasizes the dynamic roles that well-defined factors can play in numerous aspects of both cellular development and differentiation.

The same can be said for TLRs. TLRs, well-established as mediators of both innate and adaptive immune responses, have recently been highlighted for their additional role in hematopoiesis (Nagai et al., 2006). Specifically, TLR-mediated recognition of microbial and viral products by hematopoietic progenitors was shown to modulate hematopoietic differentiation (Nagai et al., 2006). These findings suggest that engagement of TLRs during early cellular development could provide a mechanism that modulates downstream innate immune responses at the hematopoietic level.

Our current work has demonstrated by way of studying the universal TLR adaptor protein MyD88, that in addition to their contribution to regulating hematopoiesis, TLR signaling is required for B-cell central tolerance. Following our discovery of the requirement of AID for mediating central tolerance, we began to explore possible mechanisms by which AID mediates tolerance in the BM. It has been previously shown that the relative AID expression of pre-B and imm B cells in MyD88 deficient mice is markedly reduced (70-75% in pre-B; 60-70% in imm) (Han et al., 2007). Additionally, we saw that the synergistic signaling of BCR and TCR (initiated through receptor engagement by anti-\(\mu\) and CpG, respectively) resulted in increased AID expression in immature and T1 B cells. I therefore hypothesized that engagement of intracellular TLRs by endogenous self-ligands can lead to the elevated expression of AID that in turn induces DNA damage. The resultant damage must be repaired in autoreactive B cells,
otherwise, they die. This apoptotic death may represent immunological tolerance.

Utilizing mice deficient in MyD88, I showed that TLR signaling through MyD88 does in fact mediate the central tolerance of B cells. Specifically, crossing the MyD88 deficiency onto a 3H9 autoreactive heavy-chain knock-in rescued the immature and transitional 1B-cell compartments in the BM (Fig. 4). This finding demonstrates the requirement for MyD88 signaling in the removal of autoreactive B-cell clones at the first tolerance checkpoint. It also identifies a mechanism that is independent of the antibody-affinity selection model that is thought to initiate the removal of autoreactive B cells from the B-cell repertoire in the first tolerance checkpoint (Bolland, 2008).

While this work greatly enhances our understanding of TLR signaling in B-cell development, the role of TLR signaling in humoral immune responses remains a point of contention in the field.

Direct B-cell activation by TLR ligands such as LPS and CpG has been observed for some time (Kearney and Lawton, 1975a; Kearney and Lawton, 1975b; Krieg, 2006; Moller, 1965). In both models, B-cell activation is achieved solely as the result of TLR ligand engagement. These findings aren’t surprising as LPS is an established thymus independent antigen (TI Ag) and polyactivator of B cells (Andersson et al., 1972; Coutinho and Moller, 1975) and CpG recognition by the BCR results in internalization and interaction with its endogenous cognate TLR receptor, TLR9 (Poeck et al., 2004).

Recently, establishing a role for TLR in TD antibody responses has been hotly debated. Initial experiments that found TLR signaling to be required for TD antibody responses against native proteins employed a model system where B-cell deficient mice received B cell transfers from wild-type, TLR4- or MyD88-deficient mice and were subsequently immunized with native proteins and the TLR ligands LPS or flagellin
Studies that found TLR signaling to be completely dispensable in TD antibody responses immunized mice doubly deficient in the TLR adaptor proteins Trif and MyD88 with haptenated native proteins and a number of different adjuvants, some containing TLR ligands (Gavin et al., 2006). Medzhitov and colleagues proposed that haptenated proteins elicit a TLR independent antibody response whereas native and unhaptenated proteins require TLR signaling to initiate TD humoral responses. Indeed they suggest that haptenated proteins “possess a unique, hapten-focused immunogenicity that affects the requirements for adaptive immune activation” (Palm and Medzhitov, 2009a).

Historically, haptens have been used solely as a quantitative tool to measure affinity and specificity. Haptens, which are inherently non-immunogenic, must be coupled to an immunogenic carrier to elicit antibody response (Landsteiner and van der Scheer, 1931). It has also been observed that while antigenic haptens must be coupled to an immunogenic carrier to elicit an antibody response, the antibody response against the hapten is often of a greater magnitude than that against the carrier, i.e. the concept of immunodominance (Landsteiner and van der Scheer, 1931).

Close examination of the model systems used to make the claim that TLR signaling is required for TD antibody responses and that haptenation confers immunogenicity on nonimmunogenic protein carriers and elicits TLR independent antibody responses reveals two flaws: immunizations within this study elicited sub-par antibody responses and the quantity of antibody against the carrier following hapten-carrier immunization was not measured (Palm and Medzhitov, 2009a).

We corrected these oversights by inducing robust immune responses in MyD88 deficient and sufficient mice that were immunized with both native and haptenated
proteins. We quantified serum antibody against both the hapten and carrier in all immunization models. With these corrections, we were able to definitively test our hypothesis that MyD88 signaling is not required for TD antibody responses and that the “unique immunogenicity” attributed to haptenated proteins was really an observation of immunodominance, a concept described almost a century prior (Landsteiner and van der Scheer, 1931).

I found that MyD88 signaling is not required for antibody, GC or memory responses to native or haptenated protein. While a delay in serum antibody production and GC formation was observed in MyD88 deficient mice as compared to congenic BL/6 mice 8 days following immunization with native protein, antibody production and GC formation was intact in MyD88 deficient and sufficient mice at later time points when immunized with either native or haptenated proteins. Affinity maturation, determined by the frequency of VH point mutations in GC B cells was comparable between MyD88 deficient and sufficient mice. Both MyD88 deficient and sufficient mice were able to elicit secondary immune responses to native and haptenated proteins. Furthermore, I demonstrated that the MyD88 independent immunogenicity attributed to haptenated protein is a misinterpretation of the established concept of immunodominance—haptenated proteins elicited hapten-specific responses that were ≈20- to 100-fold greater than to the carrier. Regardless of MyD88 signaling, native proteins elicited significantly less serum Ab than did their haptenated forms.

The results of my studies establish a role for MyD88 signaling in central B-cell tolerance and definitively settles the controversy surrounding the contribution of TLR signaling in TD antibody responses. I have shown that MyD88 plays a role in the counterselection of autoreactive immature and transitional B cells during their maturation.
in the bone marrow. While the mechanism for this mediation remains to be elucidated, preliminary findings suggest interplay between MyD88 and AID. Additionally I have shown that the antibody response to haptenated protein is not qualitatively different than that to native protein and that MyD88 is not required for TD antibody responses.
References


**Biography**

Pilar Brooke Snowden was born on June 23, 1982 in Annapolis, Maryland to Rodney B. Harvey and Patti O. Harvey. She attended Howard University and graduated with Bachelor of Science degrees in Biology and Chemistry in May, 2005. She married Brandon J. Snowden on January 3, 2009. Since joining the laboratory of Dr. Garnett H. Kelsoe in 2006, Pilar has received an NIH predoctoral traineeship (2010).

**Honors and Awards**

- NIH Predoctoral Traineeship, Duke University May 2010- present
- Fellowship, H. Lee Moffitt Cancer Center & Research Institute March 2009

**Publications**

- Cain DW, Snowden PB, Sempowski GD, Kelsoe G. *Inflammation triggers emergency granulopoiesis through a density-dependent feedback mechanism*. PLoS One. 2011;6(5)e19957