ABSTRACT

Title of Document: THE ACTIVATION OF MEMORY B CELLS TO GENERATE HIGH AFFINITY ANTIBODY RESPONSES *IN VITRO* AND *IN VIVO*.

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Immunological memory is the hallmark of the adaptive immune system. The humoral branch of the immunological memory is mediated by memory B-cells (mB). Memory B cells are marked by longevity, expression of antibodies with high affinity, and ability to generate robust antibody responses upon reencountering pathogens. However, requirements for the activation of mB cells and the induction of humoral memory responses are not well understood. This thesis examines the role of Toll-like receptors (TLRs) in mB activation using an immunized mouse model. TLRs are a family of receptors that recognize common molecular patterns of microbial pathogens and stimulate innate immune responses. Our study found that mouse mB expressed TLR9 and 4, and responded to their agonists *in vitro* by differentiating into high affinity IgG secreting plasma cells. However, TLR agonists alone were not sufficient to activate memory B cells *in vivo*. Antigen was required for the clonal expansion of

antigen-specific memory B cells, the differentiation of mB cells to high affinity IgG secreting plasma cells, and the recall of high affinity antibody responses. The Ag-specific B cells that had not yet undergone isotype switching showed a relatively higher expression of TLR4 than memory B cells, which was reflected in a heightened response to its agonist, but in both cases of TLR4 and 9 yielded mostly low affinity IgM secreting plasma cells. When immunized together with the antigen, TLR agonists not only boosted the antigen-specific titers, but also increased affinity and isotype switching of the immunoglobulin. Thus, while TLR agonists alone are unable to activate mB *in vivo*, they can enhance humoral memory responses induced by the antigen.

THE ACTIVATION OF MEMORY B CELLS TO GENERATE HIGH AFFINITY ANTIBODY RESPONSES *IN VITRO* AND *IN VIVO*

By

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Dedication

I dedicate this thesis to my mother, Dr. Gabriele Richard, who has been my strongest supporter throughout the years in every aspect, and to my sister, Annika Richard, who has been my personal cheer leader when the going got tough and rejoiced in every bit of progress made on it, no matter how small.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
AID	Activation-induced cytidine deaminase
Alum	Aluminium hydroxide gel
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BA	Binding affinity
BCAP	B cell cytolplasmic adaptor protein
BCR	B cell antigen receptor
BLNK	B cell linker protein
BM	Bone marrow
Btk	Bruton's tyrosine kinase
C or C_μ	Constant domain of immunoglobulin / corresponding gene segment
Ca^{2+}	Calcium ion
CD	Cluster of differentiation
CpG	Cytosine-guanine motif, unmethylated
CRAC	Ca^{2+} release-activated Ca^{2+} channel
CREB	cAmp response-element-binding protein
CSR	Immunoglobulin class-switch recombination

D	Diversity segment of Ig gene
DAG	Diacylglycerol
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
EU	ELISA unit
Fc	Crystallizable fragment of immunoglobulin
FDA	U.S. Federal food and drug administration
fDC	Follicular dendritic cell
FO	Follicular B cell
Gard.	Gardiquimod
GC	Germinal center
HSC	Hematopoietic stem cell
HRP	Horseradish peroxidase
IFNγ	Inteferon γ
Ig	Immunoglobulin

IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
ITAM	Immunoregulatory tyrosine-based activation motif
ITIM	Immunoregulatory tyrosine-based inhibition motif
J	Joining segment of the immunoglobulin gene
JNK	c-Jun NH2-terminal kinase (a MAPK)
KLH	Keyhole-Limpet hemoycanin, used as vaccine carrier protein
L	Ligand of a receptor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
mB	Memory B cell
MHC-II	Major histocompatibility complex class II
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response gene 88 / encoded protein
NF-κB	Nuclear factor κB
NP	4-hydroxy-3-nitrophenylacetyl

OD	Optical density
ODN	Oligodeoxynucleotide, usu. CpG ODN
PAMP	Pathogen-associated molecular pattern
PC	Plasma cell
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositide-3-kinase
$PI(4,5)P_2$	Phosphatidyl inositide 4,5-bis-phosphate
PLCγ2	Phospholipase Cγ2
Pro-B	Progenitor B cell
PRR	Pattern recognition receptor
R	Receptor
RAG1/2	Recombination activating genes 1 and 2 / encoded proteins
Ribi	Ribi's adjuvant
SH2	Src homology 2 domain
SHIP	SH2 domain-containing inositol polyphosphate 5'-phosphatase
SHM	Somatic hypermutation
Syk	Spleen tyrosine kinase
TD	T cell-dependent (thymus-dependent)

TDM	Trehalose dicorymycolate
TdT	Terminal deoxynucleotidyl transferase
TGFβ	Transforming growth factor β
T_H	Helper T cells, (CD4+)
TI	T cell-independent (thymus-independent)
TLR	Toll-like receptor
V	Variable domain of immunoglobulin / corresponding gene segment

Chapter 1: General Introduction

Immune systems are necessary for multicellular organisms to protect themselves from harmful substances and invading organisms. Plants and fungi rely primarily on the production of toxic chemicals to prevent reproduction of the microorganisms that infect them. Animal defenses rely predominantly on digesting pathogenic microorganisms, which is carried out by specialized phagocytic cells (reviewed by Menezes and Jared, 2002) [1]. Phagocytosis is especially efficient with opsonized microorganisms. Several different mechanisms of opsonization have evolved in the animal kingdom. One of them, which arose in gnathostomes (jawed vertebrates; reviewed by Pancer and Cooper, 2006) [2], is opsonization by specific antibodies (Ab; reviewed by Aderem and Underhill, 1999) [3]. In addition, Abs are useful tools for activating the complement cascade to opsonize and lyse foreign cells (reviewed by Ochsenbein and Zinkernagel, 2000) [4], and Abs can also directly neutralize and inactivate viruses and bacteria (reviewed by Law and Hangartner, 2008; Bebbington and Yarranton, 2008) [5, 6]. The first advantage of specific Ab is the ability to afford a large repertoire of different receptor specificities. This is useful for competition against microorganisms that can evolve quicker due to their relatively short generation time. Second, the large Ab repertoire can recognize completely new microorganisms and thus allows the jawed vertebrate to travel in search of food rather than being tied to one ecological niche. Third, the repertoire can be specifically enriched for those receptor specificities that have been useful in the individual's prior

life experiences (immunological memory) (reviewed by Danilova, 2006) [7]. Of course the repertoire must be selected to be minimally self-reactive. A mis-trained repertoire on the one hand creates specific disease susceptibility and on the other hand allows for the development of allergies and autoimmune diseases.

In this thesis, the organisms of interest are humans and mice. The mouse, *Mus musculus*, was used as the model organism. Mammals, including human and mouse, have evolved a complicated immune system with many different specialized immune cell types and cross-talk pathways. Traditionally, the immune system can be divided into the two branches of innate and adaptive immune responses (reviewed by Pone *et al.*, 2010) [8, 9]. The innate branch includes a broad range of white blood cells whose receptor repertoires are limited to recognize general patterns of microorganisms. Any changes in innate immune receptors are due to evolution of the host organism. Innate immune cells include macrophages, neutrophils, basophils, eosinophils, dendritic cells, and natural killer cells. In addition, the barrier functions of the epithelium and stomach acidity, entrapment of microbes by platelet clotting, zymogens secreted from liver cells, and control of body temperature by the hypothalamus contribute to innate immune protection. However, the cells responsible for creating the trainable Ab repertoire in the previous example are different: They are the effector cells of the B lymphocyte branch, which forms one arm of the adaptive branch of the immune system. The adaptive branch is evolutionarily younger than the innate branch and is mediated by B and T lymphocytes. B cells are responsible for the humoral immune responses and T cells are responsible for the cellular immune responses. The

adaptive immune cells (B and T cells) can generate a large repertoire of receptors by DNA recombination, a random gene reassembly process. B cells express a large repertoire of immunoglobulin (Ig), which has both secretory and membrane form. Membrane Ig is a part of the B cell receptor (BCR). T cells express a large repertoire of T cell receptor (TCR). Both BCR and TCR serve for recognition of foreign substances, and their ligands (L) are broadly called antigen (Ag). Receptor binding with Ag activates B and T cells to proliferate and to become effector cells. Other activation signals are also required in primary immune responses, without which the lymphocyte will undergo an abortive activation and become tolerant. The humoral effect of fully active primary immune responses initially is secretion of IgM antibodies by plasma cells. The cellular effect is secretion of cytokines by helper T cells and secretion of cytolytic proteins by cytotoxic T cells. Some of the activated B and T cells become memory cells to provide immunity in subsequent exposures to pathogens with that Ag. Activation of memory immune cells induces a secondary immune response, which is typically faster and more robust than primary immune responses. Immunological memory is the hallmark of the adaptive immunity and has been exploited by immunization (reviewed by Rappouli *et al*, 2002) [10]. Immunization is the most effective prevention method against infectious diseases.

Interactions between different immune cells are essential for providing the system of checks and balances. The spleen and lymph nodes are organs that facilitate the adaptive immune cells' encounter with Ag as well as with other activated immune cells. These lymphoid organs have a complex micro-architecture to segregate

immunologically naïve T and B cells while they interact with Ag presented by dendritic cells (DCs) and follicular dendritic cells (fDCs), their respective Ag presenting cells (APCs) (reviewed by Mueller and Germain, 2009) [11]. Sometimes interaction with Ag and the APC is sufficient for complete lymphocyte activation. For B cells this is called T cell-independent (TI) activation. TI activation of B cells is often associated with Ag that are high in valency and organization (i.e. viral coat "quasicrystals") and the presence of polyclonal activators (adjuvants) (reviewed by Hinton et al, 2008) [12]. The activation of B cells is enhanced through direct interaction with helper T (T_H) cells, which leads to T cell-dependent (TD) responses. The T-B cell interaction is primarily mediated through CD40 and CD154 (CD40L) and through cytokines that activated T_H cells secrete (paracrine stimulation) (reviewed by McHeyzer-Williams *et al*, 2005) [13]. Ag-activated B cells request and reciprocate the T cell help by processing and presenting Ag in the context of major histocompatibility complex class II (MHC-II) to T_H cells, providing costimulatory signals such as through CD28 and CD80/CD86, and secreting cytokines (reviewed by Harris *et al*, 2000) [14]. For these events to take place, B cells and T cells that recognized the same Ag (B cell in its native form, T cell in its processed form) have to find each other [13]. Segregating un-activated B and T cells in the secondary lymphoid structure keeps them out of the way of activated lymphocytes trying to find each other on the borders between B and T cell zones to enhance each other's activation [11]. T-B cell interaction induces rapid proliferation of B cells, leading to germinal center (GC) formation for fine-tuning of antibody responses and developing immunity. GCs are again aided by the micro-architecture of the secondary lymphoid

structures to spatially separate competing B cells that must interact with an fDC from mutating ones that can interact with other fibroblasts (reviewed by Mueller and Germain, 2009) [11].

Section 1 B cells

B cells were first discovered in chickens. They are named for the bursa of Fabricus, a specialized organ in birds for B cell development (reviewed by Warner and Szenberg, 1964) [15]. In mammals, B cells develop in the liver of fetuses and in the bone marrow of adults (reviewed by Duchosal, 1997) [16]. B cells can be divided into many phenotypically and functionally distinct subsets (Table 1.1). This thesis focuses on memory B cells (mB) that are responsible for generating robust secondary humoral immune responses. So far, the majority of studies have focused on follicular naïve B cells (FO).

Naïve FO B cells are mature B cells found in blood and secondary lymphoid organs. Each B cell expresses Ig of a unique specificity as the Ag recognition subunit of its BCR. The Ig in the BCR contains two Ig light chains (IgL) and two Ig heavy chains (IgH) with an extended transmembrane domain in their C-terminals. The BCR is composed of a membrane Ig and two signaling chains (Igα and Igβ) (Figure 1.1). The specificity of the BCRs is determined by variable domains of the IgH and IgL. The Ig genes are assembled by a recombination activating genes 1 and 2 (RAG1/2) enzyme-dependent mechanism during B cell development. The IgH gene is assembled during Pro-B cell stage with over 6000 possible combinations of variable (V), diversity (D), and joint region (J) gene segment arrangements. The IgL is assembled during Pre-B

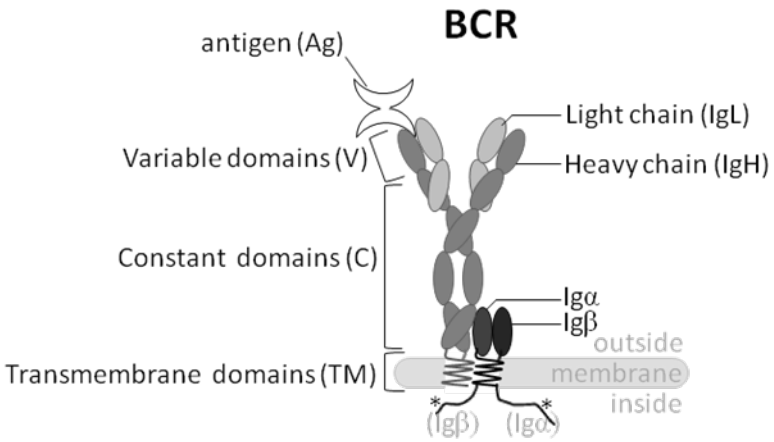
cell stage with almost 400 possible combinations of its V and J gene segments. The IgH and IgL gene repertoires are further expanded by insertion of up to 20 random nucleotides between the Ig segments by a terminal deoxynucleotidyl transferase (TdT) (reviewed by Hardy and Hayakawa, 2001) [17]. Even after accounting for silent mutations (same protein sequence after translation), out-of-frame Ig genes (check-points at end of Pro- and Pre- B cell stages), and elimination of self-reactive cells (negative selection in immature B cell stage), an individual's naïve B cell

Table 1.1a: Major B cell subsets during B cell development

B cell subset name	Phenotypic identifiers (mouse)	Function/Developmental Event	Location	Transcription master regulator	References
Common lymphoid progenitor	IL-7Rα^{+}	Precursor to B, T, and NK cells	embryo: Liver adult: Bone marrow	c-KIT	[17, 18]
Progenitor B (Pro B)	B220^{+} Early: CD19^{-} AA4.1^{+} Late: CD19^{+} surrogate light chain CD43^{+} BP-1^{-} HSA^{+} IL-7R^{+}	Commit to B cell lineage IgH gene rearrangement	embryo: Liver adult: Bone marrow	PAX-5	[17, 19]
Pre-B	B220^{+} CD19^{+} Igμ^{+} CD43$^{+/-}$ BP-1^{+} CD25^{+} HSA^{high} AA4.1^{+} CD138^{+}	IgL gene rearrangement	embryo: Liver adult: Bone marrow	PAX-5	[17, 19, 20]
Immature	B220^{+} CD19^{+} IgM^{high} (μ+κ) IgD^{low} AA4.1^{+}	Negative selection of self-reactive B cells (deleted, become anergic, or BCR editing)	embryo: Liver adult: Bone marrow Peripheral blood	PAX-5	[17]
Transitional	B220^{+} CD19^{+} CD138^{-} IgM^{high} IgD$^{low->high}$ AA4.1^{low} HSA^{higl} CD23$^{+/-}$ CD21$^{+/-}$		Peripheral blood Spleen (white pulp), Tonsil Lymph nodes	PAX-5	[17, 20]

Table 1.1b: Mature B cell subset

B cell subset name	Phenotypic identifiers (mouse)	Function/Developmental Event	Location	Transcription master regulator	References
B-1	$B220^{+}$ $CD19^{+}$ $CD5^{+/-}$ $CD11b^{+}$ IgM^{high} $IgD^{low/neg}$ $CD23^{+/-}$ $CD21^{int/-}$ $CD43^{+}$ $AA4.1^{-}$ HSA^{int}	Production of natural antibody (CD5 is an activation marker used to distinguish the antigen-experienced B-1a cells)	Peritoneal cavity, Pleural cavity	PAX-5	[17, 21, 22]
Marginal zone (MZ)	$B220^{+}$ $CD19^{+}$ IgM^{high} IgD^{low} $CD1^{high}$ $CD23^{low}$ $CD21/35^{high}$ $AA4.1^{-}$ HSA^{int}	Uptake soluble antigens→IgM Ab	Spleen (border between red and white pulp)	PAX-5	[17]
Follicular (FO)	$B220^{high}$ $CD19^{+}$ IgM^{int} IgD^{high} $BAFF\text{-}R^{+}$ $CD38^{+}$ HSA^{low} $CD23^{high}$ $CD21/35^{int}$ $CD138^{-}$ $AA4.1^{-}$	Primary immune response	Spleen (white pulp), Tonsil Lymph nodes Peripheral blood	PAX-5 +BCL-2	[17, 20, 21, 23-25]
Effector B cells (B_E1, B_E2)	$B220^{+}$ $CD19^{+}$?	Cytokine secretion B_E1: IFNγ, IL-12, -10 B_E2: IL-2, -4, -6, -10	Spleen, Tonsil Lymph nodes Tissue	PAX-5 (?)	[14]
Germinal center (GC)	$B220^{+}$ $CD19^{+}$ PNA^{+} $GL\text{-}7^{+}$ $CD95^{+}$ (FAS) $BCL2^{-}$ $CD38^{low}$ $MHC\text{-}II^{high}$	Antibody affinity maturation	Spleen (white pulp), Tonsil Lymph nodes	PAX-5 + BCL-6	[21, 23, 24, 26-28]
Memory (mB)	$B220^{+}$ $CD19^{+}$ $IgM^{+(?)/-}$ IgD^{-} hyper-mutated BCR, $CD38^{high}$	Secondary immune response	Spleen (white pulp), Tonsil Lymph nodes Peripheral blood	PAX-5	[13, 21, 26, 29, 30]
B10	$CD19^{+}$ $CD23^{+}$ $CD5^{+}$ $CD1d^{high}$	IL-10 secretion, limit autoimmune activity	Spleen Lymph nodes	PAX-5	[31-33]
Regulatory (B_{reg})	$CD19^{+}$ $FasL^{+/-}$ $CD80^{+/-}$	TGF-β1 secretion Recruit T_{reg} cells	Spleen Lymph nodes	PAX-5	[33]
Anergic/ Tolerized	$B220^{+}$ $CD19^{+}$ IgM^{low} IgD^{high} $CD23^{+}$ $Fas^{+/-}$ Ca^{2+} high baseline	Central/peripheral Tolerance	Spleen Lymph nodes	PAX-5	[34-36]
Short-lived Plasma Cell (sl-PC)	$CD138^{+}$ $B220^{-}$ $CD19^{-}$ $CD38^{low}$	Antibody secretion (IgM, IgD)	Spleen (red pulp) Lymph nodes (medulla)	BLIMP-1	[20, 24, 37]
Long-lived Plasma Cell (ll-PC)	$CD138^{+}$ $B220^{-}$ $CD19^{-}$	Antibody secretion (IgG, IgA, IgE)	Bone marrow	BLIMP-1	[38-41]

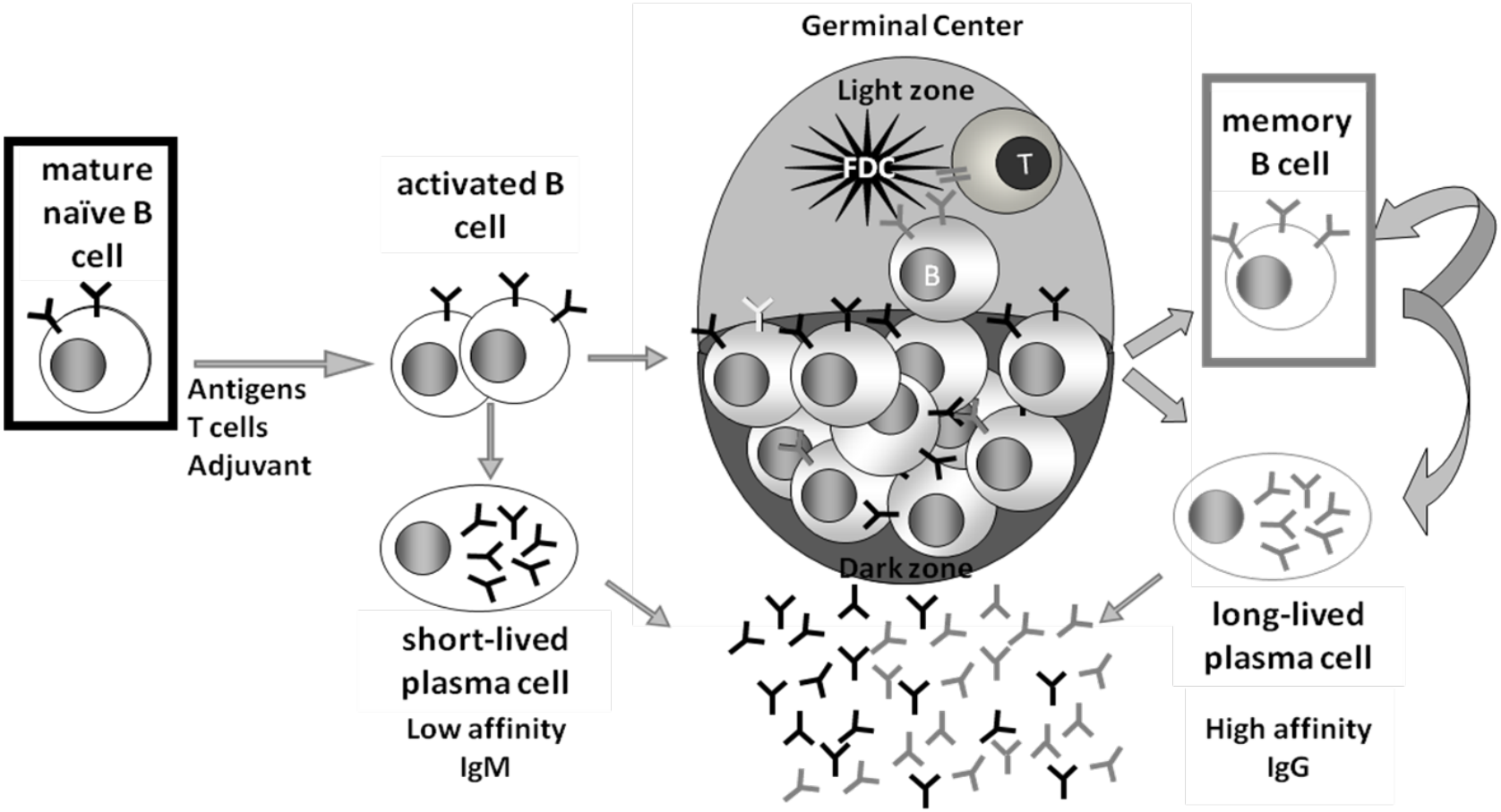


Adapted from Tolar *et al.* 2009 Immunolgical Reviews 232:34-41.

Figure 1.1: The B cell receptor (BCR). This is a diagram of an IgM isotype BCR as it would appear on a mature naïve B cell when it has first bound an antigen. Antigen binding site is near the tip of the variable domains, composed of three hypervariable loops on each domain. The $C_{\mu}3$ constant domains are held apart from each other by glycosylation. Immunoregulatory tryrosine-based activation motifs (ITAMs) are indicated by an asterisk.

repertoire is estimated to contain 10^7-10^9 different BCRs (Abbas and Lichtman, Eds., 2005) [9]. Importantly, the rearrangement of the immunoglobulin heavy chain (IgH) in one chromosome suppresses the rearrangement and expression of IgH on the other chromosome, so that each B cell expresses BCRs of only one unique specificity [42-46]. Constant regions (C) of the Ig gene determine the isotypes of Ig and do not undergo RAG-dependent rearrangement. The default isotype of the BCR is IgM. FO B cells also express IgD isotype BCRs, an alternate splice form of the IgM segment (reviewed by Preud'homme *et al*, 2000) [47, 48].

Memory B cells (mB) are descendants of antigen-experienced FO B cells (Figure 1.2) (reviewed by McHeyzer-Williams, 2005) [13]. In the primary immune response, antigen encounter by the mature yet immunologically naïve B cell (nB) with stimuli from T_H cells (TD) or adjuvants (TD or TI) lead to full B cell activation. Activated B cells undergo a burst of proliferation and have two possible cell fates: terminal differentiation into short-lived plasma cells (sl-PC) (reviewed by Calame, 2003) [49] or formation of a germinal center (GC) (reviewed by Tarlinton, 2006; MacLennan, 1994) [50, 51]. Sl-PCs have direct effector function, secreting antibody. These antibodies are encoded in the same rearranged DNA as the BCR, only spliced to an alternate 3' ending to produce the secretory form, and hence the antibody specificity and affinity for antigen are exactly the same as those of the BCR of the responding naïve B cell. In TD responses, germinal centers for the formation of memory B cells (mB) and long-lived plasma cells (ll-PC) is possible with B cell interaction with the Ag through the BCR and with T_H cells through antigenic peptide-MHC class II



Adapted from Tarlinton, 2006 Nature Reviews Immunology 6(10): 785-790.

Figure 1.2 Generation of memory B cells. See text for description .

complex and through CD40-CD40L interactions [52, 53]. In the GC, activated B cells will undergo isotype switching and affinity maturation. These processes require activation-induced cytidine deaminase (AID) [54-62]. AID mediates somatic hypermutation (SHM) of the immunoglobulin (Ig) gene (reviewed by Stavnezer, 1996; McHeyzer-Williams, 2005) [13, 63] as B cells divide in the "dark zone" of GCs [64, 65]. SHM is also frequently accompanied by Ig isotype class switching. Which isotype a B cell switches to is determined by the cytokines secreted by T_H cells and the properties of antigens [66-70]. Whether T_H cells are required for the class switch recombination (CSR) [54, 69, 71-79] and whether all B cells in GC reaction undergo CSR are controversial questions (reviewed by Klein, 1998) [80-84]. Between rounds of division, B cells migrate to the GC "light zone" containing resident follicular dendritic cells (fDC) (reviewed by Mueller and Germain, 2009) [11, 64]. Interaction with antigen displayed on the fDCs provides survival signals to B cells. B cells expressing high affinity receptors, resulting from somatic hypermutation, are better able to compete with other B cells to interact with the fDC, and thus are selected to survive. In a typical immune response, the B cells undergo 25-60 rounds proliferation and competition [51, 65, 85-87] until the GC eventually dissolves [85, 86]. Therefore, post-GC B cells have a higher affinity for the specific antigen than the original cell that formed the GC [50]. Many of the post-GC cells also undergo terminal differentiation into PCs [38-40]. The major cell type to provide survival signals to long-lived (ll-)PCs are bone marrow stromal cells, and hence most ll-PC are found in the bone marrow where they occupy a limited number of specific niches [38, 40, 41]. Ll-PCs secrete the higher affinity antibody. The other GC products are

memory B cells that are precursors to ll-PCs. In contrast to ll-PCs, mB do not secrete Ab until re-exposure to the antigen. Then the memory B cells can lend immunity in subsequent waves of recurrent epidemics, such as measles (reviewed by McHeyzer-Williams and McHeyzer-Williams, 2005; Andersen *et al.*, 2006; Höfer *et al.*, 2006; and Lanzavecchia *et al.*, 2006) [13, 88-90]. Both the mB and ll-PC contribute to humoral immune memory.

Memory B cells (mB) are high affinity antigen-specific B cells that persist long after an infection is resolved. Most reports can only present anecdotal evidence for the duration of immunological memory, however, a few controlled studies exist: According to Dr. Peter Ludvig Panum, among the elderly inhabitants of the Faroe Islands (life expectancy 80-90 years, excluding infant mortality) 100% of those who had contracted the measles in the 1781 outbreak had immunity in 1846—a maintenance of memory over a span of 65 years after single exposure to the virus [91]. Isolation of the faroese villages from each other provided an ideal age-matched control population who had been successfully quarantined in the first outbreak, and all of these were as susceptible as their younger kinsfolk, ruling out age as a confounding variable to disease resistance [91]. Due to the unique circumstances of this population, this epidemiological report is the first to be able to include an immunologically naive control population. However, since the 19th century Faroese were also insulated from other infectious diseases, it was uncertain if memory maintainance to this extent could be achieved in larger populations with exposures to many pathogens. Dr. Shane Crotty and colleagues were able to address this point by

studying a group of non-isolated smallpox vaccine recipients more than 25 years after extinction of the virus for variola-specific antibodies and lymphocytes. The antigen-specific mB were maintained at ~0.1% of IgG^+ B lymphocytes in blood of volunteers who had received the vaccine 8-60 years before. These mB were able to respond to re-vaccination [92]. Dr. Erika Hammarlund and collegues performed similar studies and found that although T cell memory gradually declined, in their cohort variola-specific antiserum titers maintained at similar levels from 1-75 years after immunization (mB cells were not quantified) [93]. Dr. Mark Slifka's laboratory performed a longitudinal study of antibody maintainance to common vaccines and pathogens including measles, mumps, rubella, Epstein-Barr virus, varicella-zoster virus, vaccinia, tetanus, and diphtheria. They determined 95% confidence intervals for IgG antiserum half-lives, which ranged from 10-14 years (tetanus) up to 104-∞ years (measles) [94]. This means that ll-PCs keep secreting these antibodies, because human IgG molecules in serum only have a half-life of 21 days [95]. Memory B cells were also quantified in the blood of the volunteers and for most vaccines correlated with the titers (except tetanus) [94]. This longevity of immunological memory is remarkable and still raises many questions: Which cells are responsible for future immunity and how can they be distinguished from other B cells prior to re-infection? What is the mechanism to maintain the pool of antigen-specific memory B cells? And how are memory B cells reactivated to form the high affinity secondary immune responses?

Identifying memory B cells is a controversial subject. At the basic definition, memory B cells simply are those B cells that provide the specific protection in re-exposure to a pathogen. Which properties uniquely define a memory B cell or are essential to the function of producing the secondary immune responses, opposed to features that are just common in memory B cells but are not definitive, or whether the memory pool might actually consist of several distinct subpopulations of cells is unclear. In human, the majority of memory B cells express a TNF-receptor family member that serves as a cell surface marker for memory B cells: CD27 [81]. Cross-linking of the CD27 prevents differentiation into plasma cells and enhances production of memory B cells [96]. For the mouse model, to date no single marker for memory B cells has been found.

Most mouse memory B cells are generated in response to T cell-dependent antigens in germinal centers [97]. The most reliable feature of these memory B cells is that they carry mutations in the gene segments for the immunoglobulin heavy and light chain variable domains that are absent in other cell types (Lalor *et al*., 1992) [98]. However, determining somatic hypermutation in memory B cells requires sequencing of PCR-amplified sections of the V gene segments of individual cells, which is difficult, expensive, and impractical for experiments.

Isotype switched B cells appear to be enriched in memory B cells by 20-30-fold [99, 100]. This was also shown by their adoptive transfer into immunologically naïve animals lending greater protection in a late challenge than transfer of unswitched B

cells, while both B cell subsets are protective in early challenges [101, 102]. The isotype-switched cell population also contains germinal center cells from on-going immune responses [86, 98] and cells that switched outside of germinal centers [103, 104]. One interesting debate concerns a small population of hyper-mutated cells that express IgM. In Klein *et al.* and Kruetzman *et al.* the human memory B cell marker CD27 also stains some IgM-expressing B cells, and these cells had undergone somatic hypermutation [81, 84]. These cells may be memory B cells that underwent germinal center reaction without isotype switching. They may also have received signals to switch back to IgM using the second immunoglobulin allele, which has been shown to occur in some cancer lines [105]. (Switching between different alleles, although not necessarily to IgM, has been shown to occur in about 5% of CSR in rabbits [106]. The signals that drive trans-switching are unknown.) Weller and colleagues provide an alternative explanation for this phenomenon: fetal marginal zone B cells may undergo somatic hypermutation as part of their diversification [82, 83]. Studies of human tonsil B cells revealed that a subset of adult marginal zone B cells can form germinal centers and undergo hypermutation as well [107]. Whether these IgM^+ isotype switched cells are memory B cells is unclear. McHeyzer-Williams and collegues also described a population of mB that have downregulated their B220 B cell marker expression [108]. Typically, however, a loss of B220 indicates differentiation into plasma cells [97].

We chose to study the least controversial memory B cell subpopulation in the mouse: B cells specific for an antigen that was introduced in a T cell-dependent

immunization at least six weeks earlier and are isotype switched. We identify these cells by flow cytometry using fluorescently labeled antibodies against B220 (pos), CD138 (neg), IgM (neg), IgD (neg), and the antigen (pos). The majority of mouse serum antibodies is of IgM and IgG isotypes; so some investigators choose to show isotype switching by determining if it is IgG^+ [109, 110]. For studies in mice, this is not recommended as all commercially available anti-mouse IgG antibodies developed against all subtypes of IgG have a strong tendency to cross-react with IgM antibodies. Furthermore, B cells display captured IgG via Fcγ receptors on their surface [111]. Therefore, it was necessary that we used the indirect measurement of lack of IgM and IgD to represent isotype switching. Probably most of these cells are IgG^+.

Several studies have addressed the requirements for mB maintainance. Whether antigen (Ag) is required for memory B cell re-activation is controversial [112]. Ag appears to be required for the survival of adoptively transferred mB cells [113, 114]. Ag can be maintained at pg-levels in immune complexes on follicular dendritic cells (fDCs) (reviewed in Tew *et al.,* 1990; Mandel *et al.,* 1980) [115-118]. Since it would be impossible to remove Ag completely after immunization, whether the maintained Ag actually is relevant for the maintainance of mB could not be tested until the advent of transgenic mice. In mice that cannot form immune complexes due to being restricted to the membrane-bound form of IgM antibody [119] and in mice where the BCR is engineered to switch to the second IgH allele to recognize the phycoerythrin dye rather than the Ag specifically in antigen-experienced (memory + GC) B cells [120], memory B cells are maintained.

ll-PCs survive by resisting apoptosis rather than homeostatic proliferation. ll-PCs are known to be maintained without antigenic stimulation in the bone marrow, where stromal cells provide the necessary environment for their longevity [90, 121-130]. ll-PCs also intrinsically express high levels of anti-apoptotic genes [131-133]. In specific pathogen free-housed laboratory mice, about 10% of the Ag-specific ll-PCs generated in GCs are maintained for more than one year [123, 125]. In humans this frequency over an equivalent time frame adjusted for lifespan is likely to be lower due to increased diversity of microbial exposures putting selective pressure on availability of bone marrow niches [124, 134]. However, in *ex vivo* cell culture all PC, including ll-PC, die within a few days [122]. In contrast, although circulating memory B cells may visit the bone marrow, they are predominantly found in the spleen [84, 103, 135, 136]. The BCR is required for mB maintainance [137], but tonic BCR signaling is required for the survival of all B cells [138, 139] (except PCs [126]). In addition, both the Tangye and Noelle labs showed that antigen-stimulated memory B cells become more responsive to anti-apoptotic effects of the B cell activating factor (BAFF), a TNF family member, due to increased expression of one of its receptors B cell maturation antigen (BCMA) [128, 140]. Resting memory B cells in mice, however, only express the TACI receptor for BAFF, but blocking this receptor with antibodies did not impact mB survival significantly [141]. Recently activated mB also down-regulate surface expression of the major interacting partner with T_H cells CD40, which serves to stimulate B cell proliferation [140, 142], but T_H cells are not required for the survival of resting mB [143]. TNFR1-/- mice do not

allow follicular dendritic cell (fDC) maturation, and consequently cannot form GCs or develop mB. However, when mB were adoptively transferred into these mice, they were still present one year later, indicating that fDCs are not required for mB maintainance either [144]. Several labs showed that during the GC phase and during *in vitro* stimulation, activated B cells express telomerase [145-150]. Telomerase function is to lengthen chromosome telomeres, which is associated with greater proliferation potential. In general, the gene expression profiles of resting mB, as analyzed by micro-array, are very similar to those of resting FO B cells despite large-scale cell-program changes during GC reaction [151]. Whether mB can sporadically leave the G0 cell cycle phase for homeostatic proliferation [120, 152-154] and which cellular signals are required for the maintenance of resting memory B cells are presently unknown.

Ag activation of memory B cells leads to the high affinity secondary immune responses. At re-encounter with the antigen, mB are thought to differentiate into ll-PC directly [13, 50]. A few gene expression differences have been identified between mB and naïve B cells sofar that may impact mB activation. Tangye and colleagues and Klein and colleagues found an approximately 100-fold increased expression of the interleukin-2 receptor (IL-2R) on human memory B cells [142, 151]. This suggests that mB may be more sensitive to this cytokine, at least in the human. *In vitro* stimulation with IL-2 in addition to CD40 and IL-10 increased the number of *in vitro* formed antibody secreting cells (ASCs) from mB origin compared with only CD40L + IL-10 stimulation [142].

Whether mB cell activation requires help from T_H cells and/or adjuvant is debated. While T_H cells are not required for mB maintainance [143], for some antigens mB re-activation required MHC-II expression [155], suggesting necessity for T_H cell interaction. *In vitro*, human mB were significantly less responsive to CD40L than naïve FO B cells for IL-10 secretion [156]. BCR cross-linking plus stimulation with CD40L caused FO B cells to switch from secreting IL-10 to secreting lymphotoxin (LT) and TNFα. In this regard, mB produced significantly more LT and TNFα than naïve B cells [156]. CD40 stimulation of human mB leads to rapid differentiation into ASCs, where they produce 5-8 fold more ASCs than naïve FO B cells [157, 158]. Therefore, Memory B cells are generally considered to be more sensitive to stimulation, however, inhibitors of activation, such as CD200R, are up-regulated in mB as well [159].

Polyclonal activation of mB has been proposed as a mechanism for re-activation to form ll-PCs by Dr. Bernasconi and colleagues [112]. This model is still controversial. While human mB up-regulated expression of the Toll-like receptors implicated for this type of activation [160] and B cells activate well in response to their ligand *in vitro* [112, 160-162], this has so far not been observed *in vivo*. Some periodic turn-over of mB into PCs may indeed be necessary to maintain the ll-PC pool as in the absence of mB, ll-PCs were shown to decline [125, 158]. However, to date the only evidence of turn-over *in vivo* was seen in latent infection or chronic inflammation [163, 164].

Thus, naïve FO B cells and mB differ in several ways, including the isotype of their receptors, their gene expression profiles, their ability to respond to polyclonal stimulation *in vitro*, and their cytokine secretion. Our interest lies in studying the signals that allow mB to activate to form ll-PC and to maintain their own population. The next section looks at the mechanism of activation to identify where there may be differences between naïve FO B cells and mB.

Section 2 Antigen-specific B cell activation through the BCR

Signaling mechanisms in memory B cells are not well understood. Most current knowledge of BCR-mediated signaling is based on the data generated in FO B cells. BCR signaling leading to B cell activation underlies the important B cell functions of antigen presentation to T cells and differentiation into plasma cells. The cell signaling for both of these processes occur simultaneously. B cell signaling induced by encounter with antigen leads the B cells to spread and contract, accumulating antigen on its BCR in the process (reviewed by Harwood and Batista, 2008) [165]. Then BCR and bound Ag are internalized by clathrin-mediated endocytosis [166-169]. The signaling events described herein start upon first contact with the antigen and continue through internalization [166, 170, 171].

BCR signaling is initiated by engagement with antigens or receptor cross-linking with anti-IgM antibodies, which causes oligomerization of the BCRs into microclusters on the B cell plasma membrane [172-174]. BCRs and antigen accumulate at the site of

antigen contact, forming either a B cell cap (soluble antigens) [170, 175, 176] or an immune synapse (membrane-bound antigens) [177-180]. The conformational change in the BCR associated with oligomerization allows recruitment of the adapter B cell linker protein (BLNK, also named SLP-65) [181, 182] and the BCR Igα-Igβ signaling chains to spread out [174, 183]. Within the first minute of contact with multivalent or membrane-associated antigen, Förster/fluorescence resonance energy transfer (FRET) efficiency between the membrane-bound Ig and the Igα chain decreases, indicating a conformational change in the majority of the BCRs from closed to open conformation [183, 184]. These events likely precede even the earliest tyrosine phosphorylation-based signaling events since they are not affected by treatment with the src-family kinase inhibitor PP2 [183, 185]. BCR microclusters transiently interact with lipid rafts [183, 186], where Src family tyrosine kinases, such as Lyn, phosphorylate the Igα and Igβ chains on their ITAM (D/E- x_7-D/E-x_2-Y-x_2-L/I-x_7–Y-x_2-L/I; where x is any amino acid) (reviewed by Dykstra *et al*, 2001) [187]. Phosphorylated ITAMs provide a binding site for spleen tyrosine kinase (Syk) via its two SH2 domains (reviewed by Sada *et al*, 2001) [188]. Binding to the Igα-Igβ ITAMs activates Syk by interfering with Syk auto-inhibition (reviewed by Kulathu, 2009) [173, 189, 190]. Syk phosphorylates the adaptor protein BLNK, which can then recruit Syk downstream targets [191, 192].

Among the most important Syk targets for B cell activation are phospholipase Cγ2 (PLCγ2), Bruton's typrosine kinase (Btk), and Vav. PLCγ2 is responsible for initiating calcium signaling by generating the second messenger inositol-1,4,5-

trisphosphate (IP_3) (reviewed by Cambier and Campbell, 1992) [193, 194]. Calcium signaling is required for the activation of transcription factors NF-κB and NFAT and the MAPK c-Jun NH2-terminal kinase (JNK) [195]. Btk provides positive feedback for the signaling downstream of Syk, including activation of PIP5K that generates $PI(4,5)P_2$, the substrate for PLCγ2 and PI3K (reviewed by Kurosaki and Hikida, 2009) [194, 196]. Btk is involved in mediating B cell spreading through crosstalk with the actin cytoskeleton [197]. Btk and Syk together recruit and activate the adaptor B cell cytoplasmic adaptor protein (BCAP) [196] that recruits the constitutively active class I phosphatidylinositide-3-kinase (PI3K) [198]. Mutations in Btk cause B cell immunodeficiency diseases, XLA (X-linked agammaglobulin-emia) in humans [199], Xid in mice [200]. Recruitment of PI3K increases the local concentration of $PI(3,4,5)P_3$ (PIP_3) , the membrane docking sites of both Btk and PLCγ2 to PIP_3 via their pleckstrin homology (PH) domain [194, 196, 201] as well as activating the serine/threonine-specific protein kinase Akt with anti-apoptotic effect (reviewed by Deane and Fruman, 2004) [201]. Vav is the guanine nucleotide exchange factor (GEF) of Rho family small G proteins Rac1 and Cdc42 [195]. These GTPases, regulate MAPK p38 activation. In concert with calcium signaling, this cascade also leads to activation of JNK [195, 202]. Ras protein is involved in activating the MAPKs extracellular signal-related kinases 1 and 2 (ERK1/2). In summary, the BCR orchestrates signaling in micro-clusters that are transiently associated with lipid rafts [186]. BCR oligomerization triggers the activation of Syk, which propagates the BCR signaling cascade at the local membrane through PLCγ2, Btk, and Vav. These mediate the downstream signaling events of calcium influx,

activation of small G proteins, and activation of MAP kinase cascades to induce transcription factor activation.

BCR internalization also depends on actin rearrangement (reviewed by Schafer, 2002) [203, 204]. Internalized antigens are transported to antigen processing compartments in a BCR signaling and phospholipase D (PLD)-dependent pathway [205]. Here, antigens are digested to short peptides and deposited into major histocompatibility class II complexes (MHC-II) (reviewed by Clark *et al*, 2004; McHeyzer-Williams *et al*, 2006) [206-208]. Peptide-loaded MHC-II complexes are targeted to the B cell surface. BCR signaling also leads to the display of activation markers on the B cell surface, including B7-1 and B7-2 that interact with CD28 on T cells, providing co-stimulatory signals for $CD4^+$ T_H cells [208]. Thus, these antigen-experienced B cells can interact with antigen-binding $CD4^+$ T_H cells. PI3K is involved with regulating microtubule assembly [209, 210], important for transport to and from the immune synapses, including antigen presentation to $CD4^+$ T_H cells. The binding of TCR to peptide-loaded MHC-II triggers T cell activation, which leads to CD40L expression and cytokine secretion for B cell activation [207, 208].

BCR activation can also result in plasma cell differentiation (reviewed by Calame, 2003) [49]. BCR signaling suppresses the transcription factor Pax-5, which functions to maintain B cell phenotype and the expression of the master regulator of plasma cell differentiation, Blimp-1 [211]. Under the control of Blimp-1, transcription factor XBP-1 upregulates proteins involved in the secretory pathway [212, 213]. And

interferon regulatory factor 4 (IRF-4) is associated with the B-cell intrinsic cell fate decision of either developing into a plasma cell or transition to GC B cell state. IRF-4 is a critical transcription factor of both the Prdm1 (Blimp-1) and Aicda (AID) loci, at low and high IRF-4 expression level, respectively [214, 215].

The rapid and robust secondary antibody response suggests a lower signaling threshold and faster kinetics of mB activation. IgG-expressing B cells were recently shown to be more efficient than IgM-expressing B cells in their ability to form microclusters and gather antigen [216]. This must be especially important since mB cells express fewer BCR complexes than their naïve counterparts [217]. As previously noted, the majority of memory B cells generated in response to T cell-dependent antigen immunization have undergone isotype switching to IgG. The cytoplasmic tail of IgG molecules is 25 amino acids longer than that of IgM molecules, which is necessary for secondary immune responses [218]. Several signaling pathways for the IgG tail have been proposed. The Tsubata laboratory showed that the IgG tail can inhibit the phosphorylation of CD22, a negative regulator of B cell signaling [219]. However, expression of transgenic IgG1 tail expression did not increase global levels of BCR signaling [220]. Using mice in which the transmembrane and intracellular tails on their IgM have been substituted with those from IgG1 (IgMG), the Goodnow laboratory showed enhancement of antibody secretion in naïve IgMG B cells similar to those by transgenic IgG1-expression in B cells [221]. The IgMG-expressing B cells do have robust calcium signals in response to Ag stimulation, but also show 50-90% lower expression than IgM-expressing B

cells for more than half of the molecules that are associated with B cell activation in microarray, including IRF-4, IL-1R, and Myc [220]. The ERK, NFAT, and NF-κB pathways were unchanged by the IgG1 tail [220]. The IgG tail also contains a conserved tyrosine which can be phosphoylated by Syk [222]. Mutation of this tyrosine results in lower phosphorylation of Igα/Igβ, BLNK, and PLCγ2, while phosphorylation of this tyrosine allows recruitment of the adaptor Grb2 and enhanced calcium signaling and MAPK activity [222]. However, the ability to gather more antigen was associated with the membrane-proximal amino acids of the IgG tail rather than the tyrosine [216]. The other feature of mB is a higher affinity for the specific Ag after GC reaction. Not surprisingly, high affinity of the BCR for its antigen also allows more potent activation of B cells in IgM and IgG expressing B cells [216]. By definition, a BCR has higher affinity for its Ag if it spends more time in contact with the Ag within a given time frame compared to lower affinity receptors. Thus, high affinity receptors have more opportunities to signal through the BCR.

B cell activation is modified by other cell surface receptors. BCR signaling can be markedly enhanced by co-engagement of the co-receptor CD19 through complement factor C3d opsonized antigen. CD19 complexes with CD21, a complement receptor, and CD81, a tetraspan membrane protein [223]. CD19 recruits PI3K and Vav, effectively lowering the antigen threshold for B cell activation by 1,000-10,000-fold (reviewed by Tedder *et al.* 1997) [223-225]. CD19 may be necessary for B cell activation in physiological settings [180], but this hypothesis could not be tested *in*

vivo as CD19 is required for B cell development [223]. When B cells encounter antigen-antibody complexes, the BCR co-engaged with Fcγ receptor IIB, which activates Src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP-1) and leads to a conversion of PIP_3 to PIP_2 and inhibition of Btk, PLCy2, and PI3K (reviewed by Takai, 2005) [226]. Additional regulatory co-receptors of the BCR include Toll-like receptors.

Section 3 Polyclonal B cell activation through Toll-like receptors (TLR)

Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) that bind to pathogen associated molecular patterns (PAMPs) and play essential roles in innate immunity. This family of receptors was first discovered in *Drosophila* where one of its function is in preventing fungal infections [227]. Thus far 14 mammalian homologs have been described, TLRs 1-13 and a 105 kDa radioprotective protein (RP105) [228-241]. Their natural and synthetic ligands, where known, are listed in table 1.2. TLRs fall into two broad categories: (1) those that are localized to the plasma membrane and detect bacterial/fungal/viral surface components (TLRs 1, 2, 4-6, 10, 11 and RP105) and (2) those that are localized to endosomes and lysosomes, and recognize nucleic acids (TLRs 3, 7, 8, and 9) [242, 243]. The intracellular localization of these TLRs may be necessary to prevent receptors from interacting with DNA and RNA leaked from dying host cells, which could provoke an autoimmune reaction. However, their endosomal location requires pathogen's genetic material to be delivered to the receptors through the endocytic pathway [244].

Table 1.2 Mammalian Toll-like receptors and their ligands.

Receptor	Ligands	Reference
TLR1	(Dimerizes with TLR2) Lipoproteins, Pam_3CSK_4	[245]
TLR2	Peptidoglycan (PGN), Lipoteichoic acid, Lipopolysaccharides, Lipoproteins, lipopeptides Zymosan, Pam_3CSK_4	[236, 245-250]
TLR3	Double-stranded RNA, poly(I:C)	[251]
TLR4	Lipopolysaccharides (LPS), Monophosphoryl lipid A, Endotoxin, taxol, some Glycoproteins	[229, 230, 234, 237]
TLR5	Flagellin	[252]
TLR6	(Dimerizes with TLR2), Mycoplasma lipopeptides, Zamosan	[245]
TLR7	Single-stranded RNA, viral RNA, imidazoquinolines, siRNA, guanosine-containing compounds	[245, 253-255]
TLR8	Single-stranded RNA, viral RNA, imidazoquinolines	[245, 254]
TLR9	Hypomethylated CpG-motif containing oligodeoxynucleotides, oligoDNA phosphodiester sugar backbone, Hemozoin	[161, 231, 256-262]
TLR10	(Dimerizes with TLR1, 2)	[263]
TLR11	Profilin-like protein, uropathogenic bacteria	[238, 264]
TLR12	(unknown, high degree of homology with TLR11)	[239]
TLR13	(unknown, high homol. w/ TLR3, ↑ in yeast and parasite infect., ↓ with LPS, PGN treatment)	[239, 265-267]
RP105	(Modifies TLR2 and TLR4 function), LPS	[268, 269]

All known TLRs are type 1 transmembrane proteins with an extracellular domain composed of tandem leucine-rich repeats (LRR), a transmembrane domain, and an intracellular Toll/IL-1R (TIR) domain [270]. The LRR domain mediates the interaction with their ligands as well as TLR oligomerization. The transmembrane domain aids in the oligomerization, which appears to be necessary for the initiation of TLR signaling events. The TIR domain is responsible for interaction with TLR signaling adaptor proteins [270]. The two most important adaptor proteins are the

myeloid differentiation primary response gene 88 product (MyD88) and TIR domain-containing adapter inducing interferon beta (TRIF). MyD88 can be recruited to all TLRs except TLR3. MyD88 recruitment eventually leads to nuclear translocation of NF-κB and MAPK phosphorylation (particularly ERK1, ERK2, p38, and JNK) [270]. TRIF is recruited to TLR3 and late TLR4 and leads to the activation of interferon regulatory factor 3 (IRF3) in addition to the NF-κB and the MAPKs, although use of different signaling pathways offsets the molecular kinetics [270].

B cells express a subset of TLRs [160, 263, 271-275], and the expression profile varies among B cell subsets and among species. Existing data for mouse and human FO and mB B cell subsets are summarized in table 1.3. Human naïve FO B cells do not express or only express very low levels of TLRs [160, 263, 271, 272]. Lanzavecchia and collegues showed that BCR cross-linking dramatically up-regulated the mRNA expression of TLR9 and TLR10 within 4 hours of BCR cross-linking [160]. The same study showed that human mB show increased production of TLRs 6, 7, 9, and 10 on the transcriptional level [160]. In contrast, naïve FO B cells from mice express relatively high levels of TLR9 and TLR7, moderate levels of TLR1, TLR2, and TLR4, and low levels of TLR3 and TLR6, but do not express TLR5 and TLR8. There are no data available on the expression of the more recently discovered TLRs (TLR11, TLR12, and TLR13) in B cell subsets [273-275]. The TLR expression profile in different subsets of B cells has not been fully investigated. Gururajan and collegues tested expression level of TLRs in a mixed population of mB and GC B cells from mice and found that these cells express mRNA for the same

Table 1.3 Toll-like receptor expression in follicular and memory B cell subsets

Receptor	Mouse	Human
TLR1	mRNA(++or+++/?)	mRNA(+/+)*
		protein(+/?)
TLR2	mRNA(+or++/?)	mRNA(+/+)
	protein(+/?)	
TLR3	mRNA(±/?)	mRNA(-/-)
TLR4	mRNA(+/?)	mRNA(±/-)
	protein(+/?)	
TLR5	mRNA(±/-)	mRNA(±/±)
TLR6	mRNA(±/?)	mRNA(-/+++)
TLR7	mRNA(+or++/?)	mRNA(+/++)
TLR8	mRNA(±/-)	mRNA(±/±)
TLR9	mRNA(+++/?)	mRNA(+/+++)
		protein(+/++)
TLR10	N/A	mRNA(±/+)
		protein(+/?)
TLR11	?	N/A
TLR12	?	N/A
TLR13	?	N/A
RP105	mRNA(?/?)	mRNA(-/+)
	protein(++/?)	protein (++/+++)
References	[269, 273-277]	[160, 263, 271, 272, 278, 279]

*(Naïve FO / Memory); - none, + low, ++ int, +++ high, ± none or low expression in different sources, ? unknown, N/A not applicable (no functional gene in this species)

TLRs as FO B cells, but at approximately half of the level [275]. However, TLR expression is partially controlled post-translationally [276, 280, 281]. Spleen B cells express low levels of TLR2 protein [276]. TLR4 and RP105 protein expression is intermediate on FO B cells and decreases in GC B cells [269, 277]. TLR expression on mouse mB has not been examined on the protein level. The TLR expression profile of the small memory B cell subset in mice remains elusive, and therefore is one of the subjects of this thesis. One of the major goals of this project is to shed light on whether memory B cells from mice can be polyclonally activated through TLR stimulation.

TLRs play an important role in B cell activation and B cell-mediated immune responses. MyD88$^{-/-}$ mice that have normal IgG1 responses to TD antigen vaccination with alum (no TLR agonists), are defective in generating antibody responses to immunogen containing complete Freund's adjuvant (contains dead mycobacteria that presumably signal through TLRs) [282]. Furthermore, humans with mutations in IRAK-4, a kinase in the MyD88-dependent TLR signaling pathway, fail to attain humoral immunity to both T cell-dependent (TD) and –independent (TI) antigens [283]. These studies indicate a role for TLR signaling in B cell activation. However, to what extend the direct effects from TLR signaling in B cells versus secondary effects from stimulation of innate cells and T cells contribute to B cell activation is debated. The recognition of PAMPs by TLRs could serve as a danger signal to control a checkpoint preventing autoimmune B cell activation by helping B cells to distinguish foreign from self (reviewed by Ehlers and Ravetch, 2007; Deane and Bolland, 2006) [284-287]. In addition, *in vitro* TLR stimulation of human plasma cells has recently been shown to increase antibody production [279].

In support of direct activation of B cells by TLRs, Pasare and Medzhitov show by adoptive transfer of wt or MyD88$^{-/-}$ B cells into the B-cell deficient μMT animals that there was a B cell-intrinsic defect. They first showed that this defect can account for at least 80% of the lack of antibody production seen in MyD88$^{-/-}$ mice. In a second line of evidence, the MyD88$^{-/-}$ phenotype was only partially rescued in a conditional MyD88 knock-in with MyD88 expression limited to CD11c-expressing dendritic cells and help from *in vitro* activated T cells [288]. In contrast, work from the

Nemazee lab shows that both TD and TI antibody responses are relatively normal in MyD88$^{-/-}$Trif$^{Lps2/Lps2}$ mice where TLR signaling pathways are completely disabled [289]. This finding argues for a role of TRIF-dependent TLR signaling in inhibition of the humoral immune response. A recent study using adoptive transfer of MyD88$^{-/-}$ B cells, similar to techniques used by Pasare and Medzhitov, showed that B-cell intrinsic TLR signaling (although able to transiently boost antibody responses *in vivo)* was not required for B cell activation [277].

TLRs can also activate B cells indirectly. Macrophages, dendritic cells (DCs), and T cells are stimulated by TLRs resulting in upregulation of costimulatory molecules and secretion of cytokines. MyD88 recruitment and subsequent nuclear factor κB (NF-κB) activation and MAPK phosphorylation (particularly ERK1, ERK2, p38, and c-Jun N-terminal kinase (JNK)) trigger pro-inflammatory cytokine production in macrophages and DCs (reviewed by Akira, 2006) [270]. *Salmonella* and *Escherichia* LPS can stimulate macrophages to secrete TNF-α through a TLR4- and MyD88-dependent mechanism [237]. TRIF recruitment (TLR3 and late TLR4 signaling) leads to interferon regulatory factor 3 (IRF3) activation in addition to the NF-κB and the MAPKs (although use of different pathways from MyD88 offsets the timing of these molecules activation), which allows the additional up-regulation of IFN-α and IFN-β [270]. Imiquimod and resiquimod, two synthetic TLR7/8 agonists in the imidazoquinoline family, stimulate mouse IFNγ-treated macrophages to secrete TNF-α and IL-12 in a dose-dependent and MyD88-dependent manner *ex vivo* [253]. Resiquimod was shown to stimulate DCs to up-regulate surface expression of the

activation markers and costimulatory molecules CD40, CD80, and CD86 *ex vivo*, as well as stimulating secretion of IFN-α, TNF-α, and IL-12 *in vivo* [253]. And cytosine-guanine motif-containing oligodeoxynucleotide (CpG ODN) stimulation also triggers IFN-α, TNF-α, IL-6 release from DCs (different subset than the LPS-activated DCs), as well as up-regulation of MHC-II, ICAM, CD40, CD54, CD80, and CD86 (reviewed by Krieg, 2002) [262]. TNF-α is required for formation of new B cell follicles and maturation of fDCs [290]. B cells are directly responsive to IL-6, which promotes their differentiation into plasma cells [291]. IFN-α, and IL-6 are also potent activators of inflammation, while MHC-II, ICAM, CD40, CD54, CD80, and CD86 serve for interaction with T cells. IL-12 is the major stimulator in T cell maturation for Th1 response [292]. T cells can also directly respond to TLR agonists as co-stimulatory signals leading to T cell priming and clonal expansion (reviewed by Rahman et al, 2009) [293]. T cell cytokines such as IL-21 are potent stimuli for B cell differentiation into plasma cells [294-297]. Thus, it has been difficult to discern in what ways TLR signaling in B cells affects the immune response.

Recent work using *in vitro* TLR stimulation of primary mouse and human B cells is beginning to shed light on the mechanism for TLR activation in B cells. *In vitro* cultured B cells respond to TLR based activation by proliferation and differentiation into antibody-secreting cells. Even the highly purified FACS-sorted naïve FO B cells became activated after incubation with TLR agonists in the absence of BCR cross-linking, as measured by *in vitro* proliferation rates, up-regulation of activation markers, frequency of developing into antibody-secreting cells (ASCs), transcription

factor activation profile, and the amount of antibody or cytokines secreted into the culture medium [273-275]. Similar activation effects of TLRs were observed in human mB, but not FO B cells, after *in vitro* stimulation with TLR agonists [160].

Recent studies have demonstrated a synergistic relationship between the activation of the BCR and TLR of mouse B cells (reviewed by Pone *et al.*, 2010) [8, 160]. MyD88-dependent TLR signaling stimulates activation of several transcription factors that are also downstream of the BCR, including ERK1/2, AP-1 (JNK and p38) and NF-κB (reviewed by Akira et al, 2006) [270]. Surface TLRs, such as TLR4, can engage with their ligands rather easily, requiring only activation of the co-receptors MD2, CD14, and LPS-binding protein, which is readily expressed on the cell surface as well (reviewed by Akashi-Takemura and Miyaka, 2008) [298]. In contrast, the endosomal TLRs must first be recruited to compartments of antigen processing for exposure to potential agonists. *In vitro* studies of primary mouse FO B cells show that upon BCR stimulation induced fusion of TLR9-containing endosomes with the antigen processing compartment in a PLD-dependent manner, thus exposing the TLRs to Ag-associated PAMPs [206]. The interactions between the TLR and BCR signaling pathways have not been fully understood. *In vitro* B cell co-culture with primed DCs, T cells, and gardiquimod elicited a 2-fold higher antibody secretion than the co-culture without the TLR agonist [299]. Resiquimod was able to stimulate resting mouse B cells in B-cell enriched cultures to secrete Ab [300] and IL-6, IL-12, and IL-10 cytokines [301] although it only provided a weak proliferation stimulus [301].

The finding of up-regulated TLRs in human mB led the Lanzavecchia laboratory to hypothesize that human memory B cells may be prone to polyclonal activation through these receptors, but minimal non-specific antibody responses were detected *in vivo* by their study [112]. This does not preclude the possibility of polyclonal activation to maintain memory B cell pools without stimulating plasma cell differentiation. *In vitro* experiments suggest the possibility of polyclonal B cell activation from exposure to TLR agonists in absence of BCR engagement [142, 160, 302, 303].

Whether TLRs actually play a role in directly activating B cells *in vivo* or are vestigial remnants is somewhat controversial. Knocking out individual TLRs has led to a wealth of information regarding their functions (reviewed by Ehlers and Ravetch, 2007; Deane and Bolland, 2006) [285, 286], but the results can be confusing as deficiency of different receptors have contrasting effects. For example, the role of TLR9 in B cell activation appears to be two-fold. Activation of TLR9 signaling through CpG-ODN triggers B cell activation with enhanced proliferation and antibody production. On the other hand, in absence of agonist, TLR9 appears to suppress TLR7-mediated activation of autoimmune B cells [304-309]. MRL/lpr mice (with mutation in Fas) are autoimmune-prone and exhibit a systemic lupus erythematosous (SLE)-like phenotype [34], which is a B cell-mediated autoimmune disease. In this SLE mouse model, TLR7 is required for induction of autoantibodies against RNA-containing self-antigens [304-306]. SLE phenotype is less severe in MRL/lpr $TLR7^{-/y}$ mice [307]. This is consistent with the hypothesis that TLR7 and

BCR engagement is necessary for autoimmune cell activation [305]. In addition, TLR7 gene duplication [308] and TLR7 protein over-expression by as little as two-fold [309] lead to the autoimmune phenotype of Yaa mice. In contrast, TLR9-/- mice on the MRL/lpr genetic background have elevated IgG titers and succumb to autoimmune disease much earlier than $TLR9^{+/+}$ MRL/lpr mice [304, 307]. TLR9-deficiency also exacerbates autoimmunity in PLCγ2 mutant mice where B cells show hyper-reactive BCR signaling [310]. This contradicts the finding that TLR9 is required for anti-DNA and anti-chromatin autoantibody production in MRL/lpr mice [284, 304, 307, 310, 311]. The Shlomchik group more recently reports that TLR7 and 9 double knock-out mice on the MRL/lpr background (as well as MRL/lpr $MyD88^{-/-}$) do not develop the fatal kidney disease despite the presence of anti-nucleosome antibodies [306]. Thus, although both TLRs 7 and 9 are expressed in B cells, located in endosomes, and signal through MyD88, their contributions to B cell-mediated immune responses appear to be different from each other.

Section 4 Toll-like receptor agonists as vaccine adjuvants

Vaccination can substitute for infection to generate primary immune responses and build immunity. Most successful vaccines (measles, polio, smallpox, yellow fever) stimulate primary humoral responses, generating a life-long supply of protective antibodies (reviewed by Plotkin, 2008) [312]. Current vaccines can be divided into two general categories: (1) attenuated versions of infectious organisms and (2) surface molecules of pathogens aided by adjuvant. While the surface molecules provide specific targets for the immune system, adjuvant plays an essential role in

increasing the antigenic response. Two known mechanisms for increasing the antigenic response are: (1.) to slow the release of antigen and extend the duration of antigen interaction with immune cells (example: aluminum salts) and (2.) to activate innate immune pathways, thereby providing the necessary co-stimulation to adaptive immune pathways. The live attenuated organism not only provides extended exposure of antigens due to its own replication, but also naturally expressed molecules with adjuvant activities. The caveat of this type of vaccine is that antigens and adjuvant molecules are often responsible for the pathology of the microorganism. Thus specific surface molecules that can stimulate the immune response but are no longer able to carry out their other functions are often the safe choices for vaccines. Due to their formidable activation of innate immune cells (reviewed by Akira and colleagues) [270], synthetic TLR agonists have been suggested to be good candidates for vaccine adjuvants. To date, one of the TLR agonists (TLR4 agonist monophosphoryl lipid A), has joined the non-TLR aluminum salts on the short list of adjuvants that have been approved by the FDA for human use [313]. Several more TLR synthetic agonists are undergoing clinical trials. Due to the relatively high mRNA expression of TLRs7 and 9 in mouse FO B cells and activated human B cells, these agonists of these two receptors are particularly promising for enhancing B cell activation directly.

Agonists of TLR4 include lipopolysaccharides (LPS) and monophosphoryl lipid A (MPL). TLR4 binding of MPL and LPS leads to different signaling outcomes (reviewed by Baldridge *et al.*, 2004) [314]. LPS can induce high fever, toxic shock,

and organ failure in human, and thus is not safe for adjuvant usage (reviewed by Karima *et al*, 1999) [315]. In humans, MPL is thought to directly activate only innate immune cells for local cytokine responses and APC activation, since B cells and T cells were not able to respond to MPL stimulation [316]. In mice, LPS is much less harmful than in humans and is commonly used as adjvant. LPS containing adjuvants include Complete Freund's adjuvant (CFA) and Ribi adjuvant [317, 318]. Mouse vaccines using these adjuvants were shown to increase cytokine secretion, antigen specific antibody titers, and recently were also shown to increase antibody affinity maturation (ref) [319-326]. The adjuvant activity of TLR4 agonists was demonstrated by the finding that treatment of anti-TLR4 antibodies that block interaction of TLR4 with agonists decreased the number of germinal centers in the spleens and lymph nodes and reduced antibody affinity maturation in mice immunized with a T cell-dependent antigen + LPS [327].

Agonists for TLR7 are single-stranded RNA (ssRNA) molecules. The more stable synthetic agonists that structurally mimic ssRNA oligomers are in the imiquimod family (reviewed by Ferratini and colleagues) [328]. Imidazoquinolines are used topically in intradermal vaccines such as to human papilloma virus, hepatitis B and hepatitis C, and some cancer antigens [329-333]. The purpose of their usage is to recruit antigen-presenting cells. A clinical trial with the gardiquimod, a new compound of the imidazoquionline family, as adjuvant in anti-tumor therapeutic vaccines has also been shown that it is safe and effective at 1 mg/kg dose [334], and other clinical trials with gardiquimod are ongoing. Lower doses of gardiquimod than

imiquimod are required for TLR7 activation. In the murine model system, gardiquimod has been shown to activate macrophages, DCs, T cells, NK cells, and NKT cells [299, 334, 335]. While one study found that addition of gardiquimod to sub-cutaneous immunization of TD antigen had no effect on antibody response in C57BL/6 mice [336], other studies show that usage of imiquimod increases of IgG1, IgG2a, IgG2b and IgG2c in Balb/c mice [336, 337]. However, Balb/c mice, Ag-specific Ab titers did not respond differently to immunization with TD antigens with resiquimod, an imidazoquinoline that binds to both TLR7 and TLR8, or immunization without adjuvant by both subcutaneous and intramuscular routes [301].

Agonists for TLR9 include phosphorothioated oligomers of DNA containing an unmethylated cytosine-guanosine (CpG) dinucleotide recognition motif (CpG-ODN). There are several classes of CpG-ODN-based TLR agonists. Class A CpG-ODNs (CpG-A) have only one CpG motif but have a poly-G tail. This class of CpG-ODN activates IRF-7 in plasmacytoid DCs to produce type 1 interferon [338]. This TLR9-dependent pathway does not appear to be used in B cells, as B cell response to CpG-A is low and B cell activation is not affected by Irf7 gene knockout [338]. Class B CpG-ODNs (CpG-B) contain multiple CpG motifs and are capable of triggering IL-12 production in DCs (creating a T_H1 cytokine environment) as well the activation of B cells (reviewed by Murad and Clay, 2009) [339]. The cytokines produced by B cells in response to CpG are typically also T_H1 response cytokines [340-342], although with a different route of immunization, T_H2 responses have been found to be induced by CpG ODNs as well [343]. CpG-B has been shown to increase antigen-

specific antibody titers in mice [336, 340, 342-353] and in human clinical trials. Human clinical trials generally show that CpG-B is safe to use, noting mostly only mild side effects such as transient neutropenia [354-361]. Immunization using CpG ODN as adjuvant has been shown to require fewer boosters to reach maximum antigen-specific antibody titers in both malaria and hepatitis B vaccine trials in comparison with the same immunogen formulation lacking CpG ODN [354, 357]. However, whether CpG ODN improves antibody affinity maturation and the generation and activation of memory B cells have not been addressed so far.

Section 5 Hypothesis

This project is focused on mB. Based on our understanding of TLR expression and stimulation on B cells we asked: Do mouse mB cells also alter their expression profile TLRs? And does TLR expression on memory B cells make them more sensitive to re-activation? If memory B cells are more sensitive to TLR activation, is there a chance that TLR stimulation may increase autoimmune disease? If not, and polyclonal mB cell activation is suppressed, can it still directly enhance the activation of those mB cells that also interact with antigen; or does the re-activation depend on T cells and innate immune cells as well? And finally, which of the TLR-based vaccine candidates is most efficient at stimulating high affinity antibody production and lasting immunity?

We hypothesize that in mice, the development of mB is associated with a change in TLR expression compared to FO B cells, similar to that seen in human. We further

hypothesize that TLR stimulation is sufficient for polyclonal activation of mB to differentiate into antibody secreting cells *in vivo*, similar to TLR-induced activation of immunologically naïve B cells. Based on reports of TLR stimulation enhancing B cell activation *in vitro*, we hypothesize that TLR7 and 9 agonists can stimulate an increase in antibody affinity maturation and long term immunity *in vivo*.

Section 6 Significance

This study will enhance our understanding of mB generation and activation and will be useful for vaccine development and prevention of autoimmune diseases. mB are the major players in humoral memory responses. Generation of high quality mB is the optimal goal of vaccines. Understanding how mB are activated upon re-exposure to antigen aids in the development of vaccines, particularly to improve booster vaccines to target and expand pre-existing mB populations. Suppression of autoreactive mB is a major challenge for treating autoimmune diseases. Current treatments that target B cells wipe out large populations of B cells, leaving patients immunodeficient. Understanding how the activation is regulated in mB can lead to the discovery of drug targets and preventive treatments for autoimmune diseases such as systemic lupus erythematosus (SLE) that down-modulate hyperactive cells of certain reactivities rather than depleting the entire repertoire. In this way, personalized medicine will be able to reach autoimmune patients.

The generation of autoreactive B cells is a normal part of B cell development [362, 363], but these cells are usually eliminated by induction of tolerance (reviewed by von Boehmer and Melchers, 2010) [364]. While in autoimmune disease, clearly, there is a breach of tolerance, this in itself cannot fully explain the development of disease symptoms. The majority of healthy individuals also harbor mature autoreactive B cells as well as PCs that contribute to the auto-Ab titer [365]. Subclinical auto-immune responses are speculated to be beneficial by speeding up the clearance of dying cells (personal communication Dr. J. Deane), which increase in frequency as the individual ages (REF). Since autoimmune B cells are frequently exposed to their antigen, once tolerance is broken, most autoreactive B cells will generate mB and ll-PCs. Isotype-switched autoantibodies, particularly the IgG subtypes, are associated with autoimmune diseases in mice and people [366-372]. Targeting the ll-PCs that secrete IgG is difficult. They are resistant to most immunotherapies such as anti-CD20 antibodies and irradiation [373-376]. The anti-thymocyte globulin (used for treating organ rejection) has been able to deplete bone marrow PCs [377], but this antibody causes complete imunoablation and is associated with high risk of infection-related mortality. In mice, blocking TACI (a receptor inducing an anti-apoptotic pathway in PCs and most B cell subsets in response to BAFF and APRIL survival factors) was also able to kill ll-PCs [141]. The problem with this approach is that it leaves the memory B cells, despite TACI being the only BAFF/APRIL receptor mB were shown to express [141], so new activation of autoimmune mB will repopulate the ll-PC compartment. Although probably further steps in autoimmune disease development exist, blocking autoimmune mB activation

to prevent the generation of new ll-PCs may be the most effective method to slow the onset of disease or its progression.

It will be preferential to target only autoimmune B cells rather than all B cells for treatment of autoimmune disease in order to preserve existing non-autoimmune mB repertoire that the patient has acquired during her or his lifetime. One hypothetical approach for this may be to quarantine the patient until all non-autoimmune immune responses have subsided and then target the activated B cells, which, in theory, should be the ones that are still exposed to the self-antigens. However, maintaining quarantine for several weeks is tedious and expensive. Most hospitals are not equipped with the space necessary for this type of endeavor. Furthermore, it is unlikely that all autoimmune memory B cells are activated simultaneously, so that the patient may still retain resting auto-mB that could develop into PC at any future time point. To make an autoimmune disease treatment more effective and available to the general population, resting mB should be blocked from activation, and the blocking agent should be conjugated to the patient's own particular autoantigen(s), bypassing the need to wait for ongoing non-autoimmune immune reactions to settle before treatment is started. Targeting resting autoreactive mB will prevent the development into new ll-PCs [125]. Natural competition for BM niches for ll-PC survival (reviewed by Radbruch, 2006; Tokoyoda *et al*, 2004) [124, 134], will ensure reduction of existing autoimmune ll-PCs as the mB activation blocking treatment would not require the continued use of immunosuppressive drugs. This is expected to enhance the patient's post-treatment quality of life and may encourage patients to

seek treatment earlier in their disease development. Early treatment will lower the time required for the ll-PC competition to reduce autoimmune titers back to below the threshold of disease symptom development.

So what shape might a mB activation-blocking agent have? We already know that TLR stimulation is related to the development of autoimmune diseases in mice and humans [284-286, 304-311, 378-383]. One important question is whether TLR stimulation on B cells themselves is responsible for their ability to overcome tolerance or activation of autoreactive mB. This is the question we try to address in this thesis. If there is indeed a B cell-intrinsic mechanism of TLR-based activation *in vivo*, we can proceed to use molecular tools to dissect this mechanism in order to find differences that are unique to B cells. As the TLR signaling pathway in B cells appears to interact with the BCR signaling pathway at least for the case of TLR9, molecules that enable this cross-talk may be good candidates for blocking targets. The reason we may still need to find the most effective blocker in B cells that will be ineffective in innate immune cells, is that due to being non-specific, innate immune cells are likely to encounter and interact with the therapeutic agent. Conjugation to the patient's particular self-antigens that she or he is forming an immune response against will ensure that only autoreactive B cells are targeted.

For applications in vaccine development, adjuvants that can enhance induction of mB and their ability to activate at reencountering the antigen will be useful tools. Pathogens fall into three categories: those we have learned to control with

vaccination, those we are still struggling to contain, and those that overrun our immune systems. Current vaccination strategies are successful for protection against a wide range of human and livestock pathogens. This has led to the eradication of smallpox [384] as well as dramatic reduction of the global incidence of poliomyelitis [385] and many other diseases. The pathogens we are still struggling with require several cycles of immune boosting until protection is established and to maintain immunological memory, such as hepatitis B and tetanus vaccines [94]. Influenza vaccines are not included in this category as the viral antigen changes in each season and/or pandemic. Immunity to any one strain of influenza does yield life-long protection with a single dose (reviewed by Couch and Kasel, 1983) [386]. For some tropical diseases, such as malaria, however, it is exceedingly difficult (though not completely impossible) to establish immune protection (reviewed by Langhorne *et al.*, 2008; Marsh and Kinyanjui, 2006) [387, 388]. Even in endemic areas it takes about 10 years of recurrent exposure to build protection against the malaria pathogen *Plasmodium falciparum*, putting the population at risk each season, particularly children. Many adults still develop parasitemia and mild disease [387-389]. As "hard-earned" as this protection is, it is not lasting immunity [387, 388, 390]. For infections with human immunodeficiency virus (HIV/AIDS) and *Mycobacterium tuberculosis*, no natural acquisition of memory is known and current vaccine strategies are ineffective (reviewed by Vekemans and Ballou, 2008) [391]. It is for the protection against these last two categories of pathogens that we need to change vaccine design.

In the past, the most successful vaccines (measles, polio, smallpox) stimulated primary humoral responses [312]. New strategies include vaccinations targeting cellular immunity and use of new adjuvants. Synthetic TLR-based adjuvants show particular promise for their relative safety and ability to enhance primary antibody titers (reviewed earlier in this chapter). However, the effects on TLR adjuvants on mB cell formation and affinity maturation have not been sufficiently explored. It is in this regard that we would like to contribute with this thesis.

Chapter 2: Agonists of Toll-like receptors 4 and 9 are sufficient to activate memory B cells *in vitro* but not *in vivo*

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Section 1 Introduction

Immunological memory is a hallmark of adaptive immunity. The humoral branch of immunological memory consists of memory B cells (mB), which are the precursors of high affinity Ab-secreting cells (ASCs), and long-lived plasma cells (ll-PC), which maintain serum Ab levels independent of antigenic stimuli. Recent studies have begun to characterize these two important humoral memory components [13, 88-90]. To date, no distinct surface markers identify mouse mB. In general, mB cells exhibit the characteristics of post-germinal center B cells. Most mB express BCRs that have undergone isotype switching, somatic hypermutation, and affinity maturation, and thus bind to Ags with high affinities. IgM-expressing memory B cells with somatic hypermutation have been reported in human [81, 84]; however, whether these cells are IgM-expressing memory B cells or marginal zone B cells remains controversial [82, 83]. Both long-lived plasma cells and memory B cells persist up to the lifetime of an individual as demonstrated by persistent Ab levels and the ability to mount rapid and robust Ab response to Ag challenge years after immunization. Memory B cells undergo rapid clonal expansion and differentiation to mount high affinity Ab responses upon exposure to Ags. Long-lived plasma cells are terminally differentiated

and continue secreting Abs without antigenic stimulation in the bone marrow that provides the necessary environment for their longevity [90, 123, 124].

The requirements for the generation and maintenance of memory B cells have been studied in some detail [51]. The generation of both memory B cells and long-lived plasma cells requires germinal center reaction, although germinal center-independent generation of memory B cells has also been reported [103, 104]. Both Ag and the interaction of B cells with T_H cells in the context of antigenic peptide-MHC class II complex and through CD40-CD40L are essential for the generation of germinal centers and memory B cells [52, 53]. In contrast, the maintenance of the B cell memory seems to be independent of Ag and T_H cells [92, 120, 143]. Crotty *et al*. [92] reported that small pox vaccine-specific memory B cells in human were detected 60 years post immunization and 25 years after small pox was eradicated worldwide. Maruyama et al. [120] showed that Ag-specific memory B cells persisted in transgenic mice where memory B cells switched their Ab specificity away from the immunizing Ag. Furthermore, mouse memory B cells have been shown to persist in the absence of T_H [143] or follicular dendritic cells [144].

Although the requirements for the generation of B cell memory are becoming better understood, the requirements for the activation of memory B cells are still not clear. The rapid and robust responses of memory B cells to antigenic challenge suggest a low signaling threshold for memory B cell activation. Bernasconi et al. [112] reported that TLR agonists polyclonally activated human memory B cells to proliferate and

differentiate into plasma cells in vitro, and that tetanus toxoid immunization increased serum Abs to unrelated Ags, suggesting that TLRs play a role in the polyclonal activation and long-term maintenance of human memory B cells in the absence of Ag in vivo.

The ability of common microbial products to increase the efficacy of immunization has long been observed [317]. The discovery of TLRs reveals that some of the major components of commonly used adjuvant contribute to immune responses through TLRs. TLRs are responsible for the initiation of innate immune responses and the maturation of dendritic cells that activate T cells, triggering adaptive responses [230, 270]. The importance of TLRs in humoral immune responses was demonstrated using MyD88-knockout mice, where T_H cell activation and T-dependent Ab responses were reduced or completely abolished [282]. In addition to their role in activating T_H cells, TLR agonists can directly act on B cells. Mouse naive B cells express TLRs, including TLR2, 4, 7, and 9, and proliferate and differentiate into plasma cells in response to TLR agonists [161, 272, 319, 344]. In contrast, human naive B cells do not express TLRs, but are induced to express TLRs in response to Ag stimulation through the BCR [112, 160]. Human memory B cells constitutively express TLRs, including TLR2, 6, 7, 9, and 10, and proliferate and differentiate into plasma cells in response to TLR agonists alone in vitro. Using MyD88-knockout mice and LPS as adjuvant, Pasare and Medzhitov [288] demonstrated that TLR signaling in B cells was required for optimal Ab responses to T-dependent Ags. In contradiction, using $MyD88^{-/-}/Trif^{Lps2/Lps2}$ mice, Gavin et al. [289] showed that both T cell-dependent and -

independent Ags induced comparable humoral immune responses in the absence of TLR signaling. Thus, the exact role of TLR in the activation of humoral memory responses requires further examination.

In this study, we examined the role of TLR9 and 4 in the activation of memory B cells in vitro and in vivo using 4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin (NP-KLH)-immunized mice as a model. We show that TLR4 and 9 agonists alone promote the differentiation of memory B cells into high affinity IgG ASCs in vitro; however, TLR agonists alone are not sufficient to effectively activate humoral memory responses in vivo.

Section 2 Materials and Methods

Immunization

All work of this project was performed using wild type C57BL/6 mice, a strain commonly used for immunological studies. This strain of mice expresses the IgG_{2c} allele of IgG_{2a} [392]. T cells from C57BL/6 mice have a stronger tendency to produce T_H1 responses (proinflammatory cytokines) in the lungs and spleen compared to BALB/c and DBA/2 mice (reviewed by Fan *et al*, 2005) [393-395].

To generate humoral memory responses, 6 – 8 week old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were immunized twice, 28 days apart, with 400 μg/mouse NP_{19}-KLH (Biosearch Technologies, Novarto, CA) and Ribi adjuvant (MPL + TDM Adjuvant System; Sigma-Aldrich, St. Louis, MO) by intraperitoneal

route. To activate humoral memory responses in vivo, the immunized mice were rested for more than 6 weeks and boosted for the third time using 400 μg/mouse NP_{19}-KLH and/or one of the following adjuvants: Ribi adjuvant, LPS (50 μg/mouse) from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) in oil in water (PBS) emulsion, or class B CpG containing phosphorothioated oligodeoxynucleotide (ODN) for mouse (50 μg/mouse) (5'-TCCATGACGTTCCTGACGTT-3'; Operon Biotechnologies, Inc., Huntsville, AL) in oil in water (PBS) emulsion.

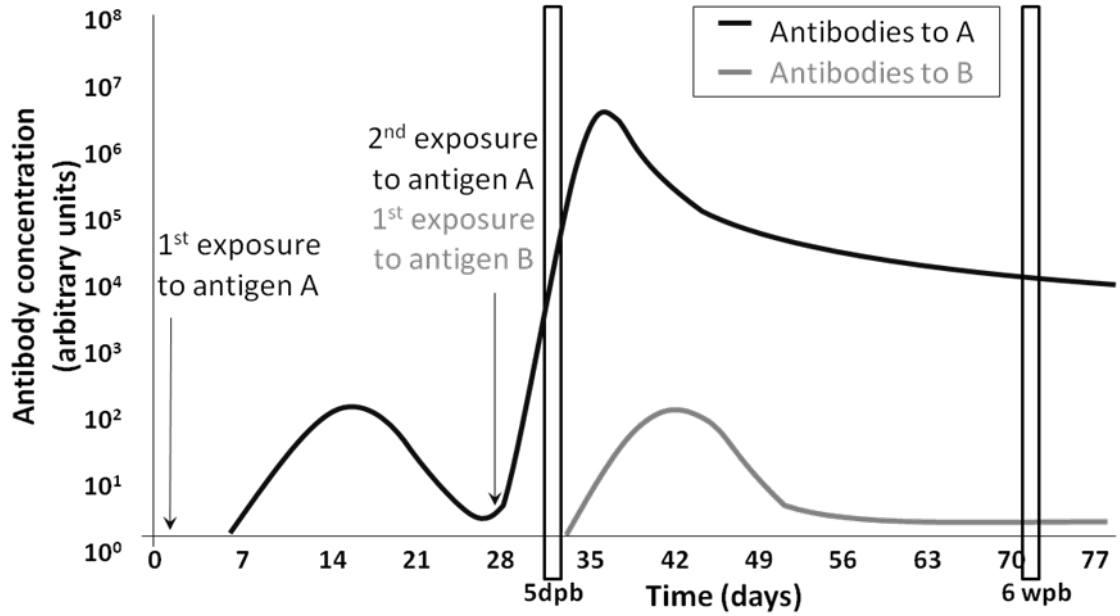
We immunized with (4-hydroxy-3-nitrophenyl)acetyl (NP), a well-characterized hapten, conjugated to keyhole limpet hemocyanin (KLH), a protein carrier for inducing TD responses. Although generation of mBs has been reported in some types of TI immunization, TD responses are the largest source of mB [396]. In C57BL/6 mice, prior to germinal center reaction only B cells with λ1 light chain [397-399] and heavy chain composed of gene segments V186.2, D FL16.1, and J_H2 [400-402] dominate the response to NP. The use of λ1 light chain is relatively rare in C57BL/6 mice [86]. The λ1 light chain-V186.2 pairing can also interact with closely related nitrophenols. The unmutated anti-NP antibody actually has a higher affinity for (4-hydroxy-5-iodo-3nitropheny)acetyl (NIP) than for NP, only requiring about $1/10^{th}$ of the free hapten competitor concentration in a competitive ELISA [403]. (This is a mouse-strain specific phenomenon: Only one of the mouse Ig alleles, the b-allele, is capable of producing these heteroclitic antibodies, and many other commonly used inbred mouse lines such as Balb/c and CBA have different alleles [397].) As mentioned above, the immunogen we used was a protein conjugate to allow T cell

activation, and T cell help promotes GC reactions to promote generation of memory B cells. In vaccination with other protein conjugates, such as NP-chicken γ-globulin, only a small fraction (less than 10%) of NP-specific antibodies cross-reacted with unrelated epitopes (certain myeloma proteins) [397]. During CSR, NP triggers Ig class switching predominantly to the IgG_1 isotype in both C57BL/6 and CBA mice [397].

The timing of booster vaccination and sample collection was also carefully chosen. Primary immune responses have a lag period of approximately 5-7 days, and peak at two weeks past immunization (Figure 2.1). Primary immune responses are typically very short-lived and serum titers return to levels close to baseline within one month. Secondary immune responses have a shorter or no lag period, and titers can be maintained high for a relatively long time. Therefore we chose to administer booster vaccines after at least 28 days to wait for the contraction of the immune response, and we collected samples 5 days after the booster for re-activated memory B cells and their serum antibodies, and at least six weeks after the booster for resting memory B cells (Figure 2.1) [41, 86, 98, 404-406].

B cell isolation

Single-cell suspension of splenocytes was treated with ACK lysis buffer to remove erythrocytes. After washing, cells were incubated with magnetic beads coated with anti-mouse CD4, CD8, CD11b, and CD11c Abs (Miltenyi Biotech), and cells binding to the beads were removed by AutoMACS (Miltenyi Biotech).



Adapted from Janeway, Ed. Immunology, 5th Ed 2006.

Figure 2.1. Humoral immune responses. This chart depicts the typical kinetics of antigen-specific serum antibody titers. Time windows for collecting activated memory B cells (5dpb) and resting memory B cells (6wpb) are indicated. In our model, we do not administer a second antigen (antigen B), but if a second primary response is initiated from the booster immunization we expect that it would follow similar kinetics.

Flow cytometry analysis

Purified B cells were first incubated with anti-mouse CD16/CD32 mAb (BD Biosciences, San Diego, CA) to block FcγRs, followed by FITC-anti-mouse IgD, FITC-anti-mouse IgM (SouthernBiotech, Birmingham, AL), PE-anti-mouse CD138, PerCP-Cy5.5-anti-mouse B220 Abs (BD Biosciences, San Diego, CA) and NP_{19}-allophycocyanin at 4°C in FACS buffer (1% FBS, 20 mM EDTA, and 0.02% NaN_3 in PBS). At time points after dissolution of germinal centers (3 weeks), the small population of antigen-binding B cells that have also undergone isotype switching consist primarily of mB. This was confirmed by sequencing the V186.2 region of individual mB for evidence of somatic hypermutation [98, 100].

To label the surface TLR4, cells were incubated with PE-anti-mouse TLR4 Ab (BD Biosciences, San Diego, CA) or anti-mouse TLR4 Ab (IMGENEX) followed by a pacific blue-conjugated secondary Ab. To label TLR9, cells that were labeled with FITC-anti-IgD, FITC-anti-IgM, PerCP-Cy5.5-anti-B220 Abs and NP_{19}-allophycocyanin were washed, fixed with 4% paraformaldehyde, and permeabilized with a permeabilization buffer (0.1% saponin, 10 mM HEPES, and 10 mM glycine in DMEM). Cells were incubated with anti-mouse TLR9 Ab (IMGENEX) in the permeabilization buffer, followed by a pacific blue- or Alexa Fluor 750-conjugated secondary Ab. After washing with the permeabilization buffer and PBS, cells were fixed with 1% paraformaldehyde and analyzed using CyAN flow cytometer (Dako Cytomation, Seattle, WA).

Cell sorting

Purified B cells were first incubated with anti-mouse CD16/CD32 mAb to block FcγRs, followed by FITC-anti-mouse IgD, FITC-anti-mouse IgM, PE-anti-mouse CD138, PerCP-Cy5.5-anti-mouse B220 Abs and NP_{19}-allophycocyanin in 2% FBS/PBS. After washing, cells were sorted using FACSAria (BD Biosciences, San Jose, CA) into four different populations: $B220^-$ $IgD^-IgM^-CD138^+NP^-$ as plasma cells, $B220^+IgD^-IgM^-CD138^-NP^+$ as memory B cells, $B220^+$ IgD/IgM^+ $CD138^-$ NP^+ as NP-binding unswitched B cells, and $B220^+$ IgD/IgM^+ $CD138^-$ NP^- as non-NP-binding unswitched B cells.

B cell in vitro stimulation

Splenic B cells isolated from mice 5 days or 6 weeks after the second immunization and sorted plasma and B cell subpopulations were incubated at 5×10^5 cells/ml for 5 days with graded concentrations of CpG ODN (Operon Biotechnologies, Inc., Huntsville, AL), LPS (Sigma-Aldrich, St. Louis, MO), or Gardiquimod (InvivoGen, San Diego, CA) at 37°C, 5% CO_2 before ELISPOT analyses.

ELISPOT analysis

Assay plates (Millipore, Billerica, MA) were coated with 10 μg/ml NP3-BSA, NP30-BSA (Biosearch Technologies, Novarto, CA), or anti-mouse IgG+M Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at 4°C and blocked with 10% FBS in PBS for 2 h at 37°C. Cells were serially diluted into wells and incubated at 37°C 5% CO2 for 5 h. Plates were washed with PBS and PBS containing 0.05%

Tween 20. Ab secreted by cells were detected with biotin-labeled anti-mouse IgM Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) or a mixture of biotin-labeled anti-mouse IgG1, IgG2a, IgG2b, and IgG3 Abs (Southern Biotech, Birmingham, AL), followed by HRP-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and visualized by HRP substrate True Blue (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Plates were sent to Cellular Technologies for scanning and spots were counted with software provided by Cellular Technologies Ltd. (Shaker Heights, OH).

ELISA analysis

ELISA plates (Nalge Nunc International) were coated with 40 ng/ml NP5-BSA, NP30-BSA (Biosearch Technologies, Novarto, CA), or 5 μg/ml anti-mouse IgG+M Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) in 50 mM NaHCO3 (pH 9.6) overnight at 4°C and blocked with PBS containing 0.3% milk, 1% FBS, and 0.1% Tween 20 for 1 h at 37°C. Mouse serum was serially diluted into wells and incubated at room temperature for 2 h. After washing, the plates were incubated with a mixture of an equal amount of HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 Abs (SouthernBiotech, Birmingham, AL), and visualized by 2,2'-azino-bis(3-ethylbenz- thiazoline-6-sulfonic acid) (Sigma-Aldrich, St. Louis, MO) and 0.03% hydrogen peroxide in phosphate-citrate buffer (pH 5.0). The reaction was stopped after 5 min with 0.5% SDS. Absorbance at 405 nm was measured using a plate reader (Wallac Victor, PerkinElmer). Titers were determined as highest serum dilution that was ≥3 SD above the average reading for secondary controls.

Section 3 Results

B cell responses to TLR4, 7, and 9 agonists in vitro

The ability of splenic B cells from NP-KLH-immunized mice to differentiate into NP-specific IgG and IgM high affinity ASCs when cultured with TLR4, 7, and 9 agonists was determined. Mice were immunized with 400 μg/mouse NP_{19}-KLH plus Ribi adjuvant, a LPS-based adjuvant, twice, 4 weeks apart. B cells were purified from spleens of NP-KLH-immunized mice 5 days after the second immunization and cultured with graded concentrations of CpG ODN or LPS for 5 days (Figure 2.2, *A–D*). The number of NP-specific Ab ASCs was determined by ELISPOT using NP3-BSA as the coating Ag to capture only high affinity Abs and NP30-BSA to capture both high and low affinity NP-specific Abs. In the absence of CpG ODN or LPS in the culture medium, the splenic B cells did not differentiate into a significant number of NP-specific ASCs (Figure 2.2, *A–D*). When cultured with CpG ODN or LPS, splenic B cells differentiated into NP-specific, IgG ASCs, a significant portion of which exhibited high affinity for NP (Figure 2.2, *A* and *B*). CpG ODN induced relatively fewer NP-specific IgM ASCs (Figure 2.2*C*) than LPS (Figure 2.2*D*). However, the majority of these ASCs showed low affinity for NP (Figure 2.2, *C* and *D*). The numbers of NP-specific ASCs generated in the B cell cultures peaked at 0.25 μg/ml CpG ODN and 0.6 μg/ml LPS (Figure 2.2, *A–D*), suggesting that the stimulatory effect of TLR agonists CpG ODN and LPS was saturable. To test the presence of the precursor B cells for high affinity IgG ASCs in mice long after the immunization, splenic B cells from mice 6 weeks after the second immunization were cultured with graded concentrations of CpG ODN, LPS, or Gardiquimod for 5 days.

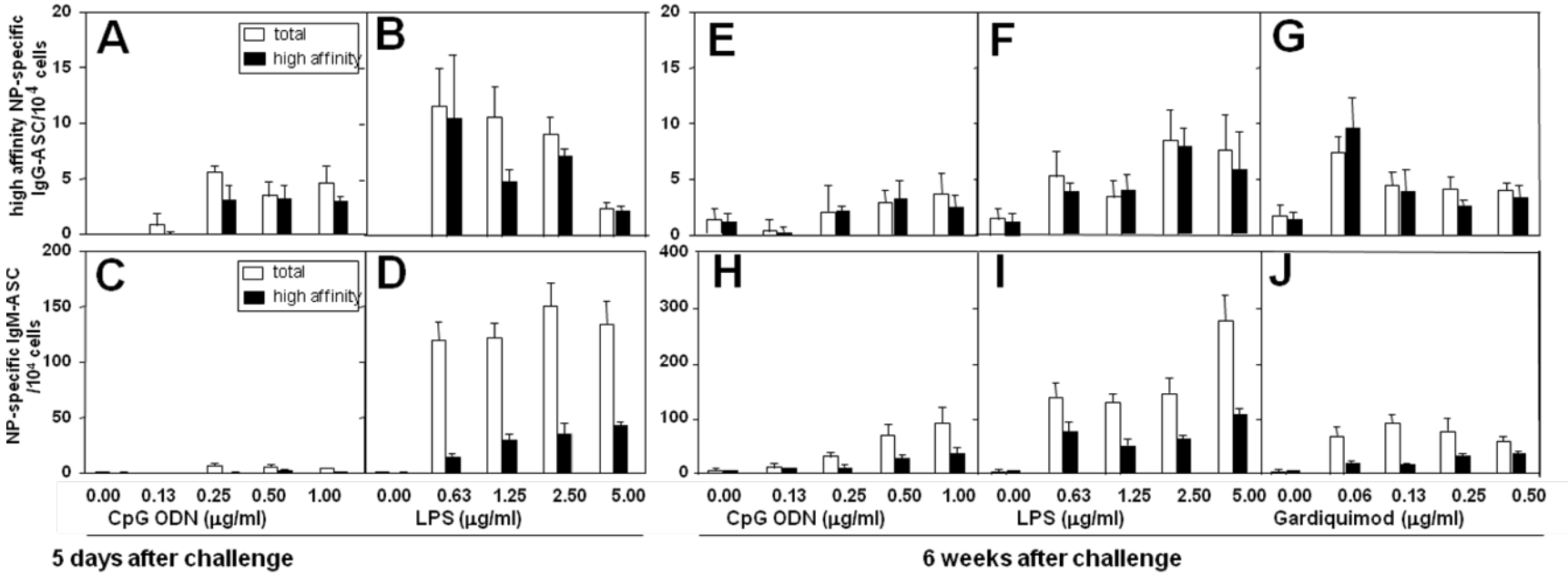

high affinity NP-specific IgG-ASC/10^4 cells
NP-specific IgM-ASC /10^4 cells
A
B
C
D
E
F
G
H
I
J
total
high affinity
0.00 0.13 0.25 0.50 1.00
CpG ODN (μg/ml)
0.00 0.63 1.25 2.50 5.00
LPS (μg/ml)
0.00 0.06 0.13 0.25 0.50
Gardiquimod (μg/ml)
5 days after challenge
6 weeks after challenge

FIGURE 2.2. Differentiation of B cells to Ag-specific ASCs in response to TLR4, 7, and 9 agonists *in vitro*. Splenic B cells were isolated from mice 5 days (*A*–*D*) or 6 weeks (*E*–*J*) after the second immunization and cultured with different concentrations of CpG ODN (*A*, *C*, *E*, and *H*), LPS (*B*, *D*, *F*, and *I*), or Gardiquimod (*G* and *J*) for 5 days. The numbers of NP-specific IgG (*A*, *B*, and *E*–*G*) and IgM (*C*, *D*, and *H*–*J*) ASCs were determined by ELISPOT using plates coated either with NP30-BSA for all NP-specific ASCs (total) or NP5-BSA for high affinity NP-specific ASCs (high affinity). Shown are the representative results of three independent experiments.

Similar to the splenic B cells isolated at 5 days (Figure 2.2, *A–D*), when cultured with TLR4, 7, or 9 agonist, splenic B cells isolated at 6 weeks post the second immunization differentiated into NP-specific IgG ASCs, most of which exhibited high affinity (Figure 2.2, *E–G*). TLR4 agonist induced a greater number of NP-specific IgM ASCs than TLR7 and 9 agonists; however, a significant portion of those exhibited low affinity (Figure 2.2, *H–J*). These results are consistent with previous reports that showed that TLR agonists can stimulate the differentiation of B cells into plasma cells [161, 319]. Our results further showed that TLR4, 7, and 9 agonists induced similar numbers of high affinity IgG ASCs from splenic B cells from 5 days and 6 weeks post immunization, but TLR4 agonist predominantly activated splenic B cells to differentiate into low affinity IgM ASCs.

B cells with memory phenotype differentiate into high affinity IgG secreting cells in response to TLR4 and 9 agonists in vitro

Based on the general properties of memory B cells that have been described so far [13, 89], we defined Ag-specific memory B cells as isotype-switched, Ag-binding B cells ($B220^+$ IgD^- IgM^- $CD138^-$ NP^+). Using flow cytometry, NP-specific memory B cells in the spleen were undetectable in nonimmune mice (frequency of 0.03%) (Figure 2.3). Five days following the second immunization, the frequency of NP-specific memory B cells in the spleen reached ~0.5% of splenic B cells (Figure 2.3). Six weeks after the second immunization, the frequency of NP-specific memory B cells in the spleen declined to ~0.1%, which was still higher than that of nonimmune mice. The frequency of NP-specific memory B cells remained at this level for several

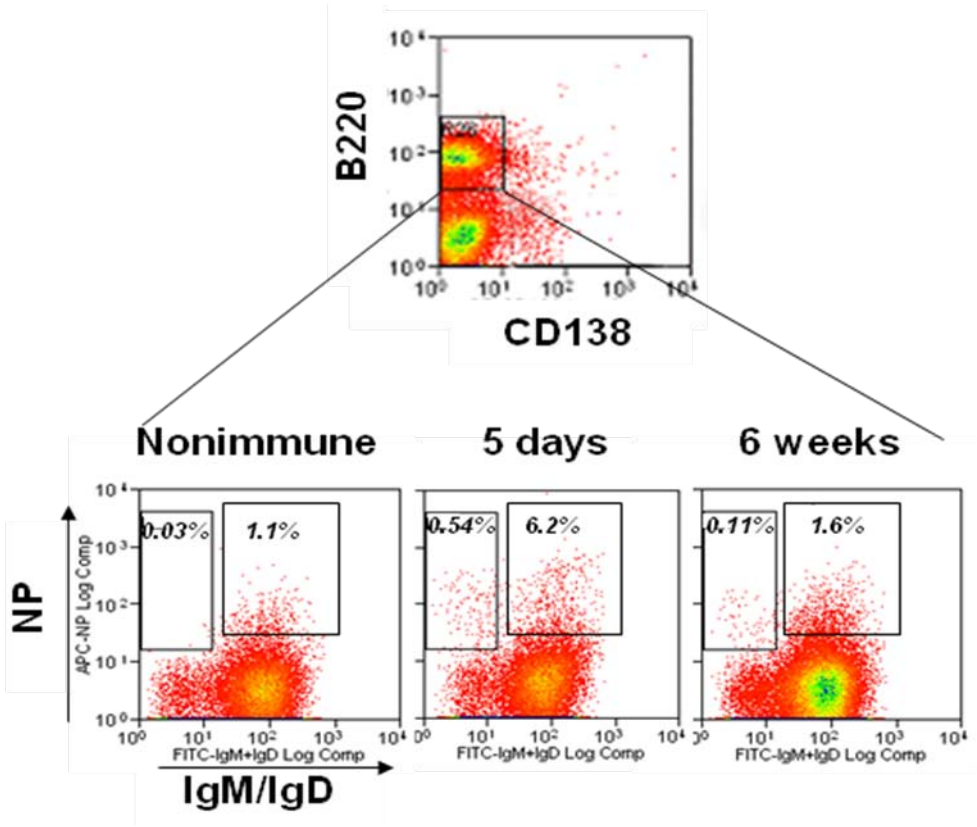
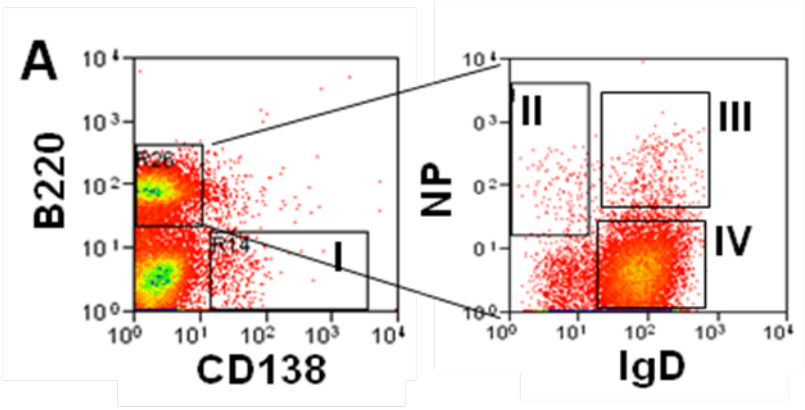


FIGURE 2.3. The frequencies of NP-specific memory B cells in the spleen of immunized mice. Splenic B cells were isolated from unimmunized mice (nonimmune) and mice 5 days and 6 weeks post the second immunization with NP-KLH and Ribi adjuvant. Cells were stained with FITC-anti-IgD, FITC-anti-IgM, PE-anti-CD138, PerCP-Cy5.5-anti-B220 Abs and allophycocyanin-NP_{19} and analyzed using a flow cytometer. Shown are representative data from eight independent experiments.

months (data not shown). The frequency of NP-binding, unswitched B cells ($B220^+$ IgD/IgM^+ $CD138^-$ NP^+) was 1.1% in nonimmune mice, increased to 6.2% following immunization, and decreased to 1.6%, near nonimmune levels, by 6 weeks following the second immunization (Figure 2.3) in contrast to memory B cells. The relatively high frequency of these B cells in nonimmune mice as compared with the predicted frequency suggests that they may be very low affinity cells. These data show that immunization induced rapid increases in the frequencies of NP-specific B cells with the memory phenotype in the spleen. While the frequencies of memory B cells decreased with the time after the second immunization, a significant number of memory B cells persisted in the spleen for several months.

B cells from NP-KLH-immunized mice 5 days after the second immunization were sorted into four different populations (Figure 2.4*A*): (I) $B220^-$ IgD/IgM^- NP^- $CD138^+$ as plasma cells, (II) $B220^+$ IgD/IgM^- NP^+ $CD138^-$ as NP-specific memory B cells, (III) $B220^+IgD/IgM^+$ NP^+ $CD138^-$ as NP-binding unswitched B cells, and (IV) $B220^+$ IgD/IgM^+ NP^- $CD138^-$ unswitched B cells that were not specific for NP. Population III, NP-binding unswitched B cells, might consist of Ag-experienced, unswitched B cells and NP-binding marginal zone B cells. Population IV, unswitched B cells that were not specific for NP, may contain naive B cells and marginal zone B cells specific for unrelated Ags. Before the in vitro culture, only population I, plasma cells, contained NP-specific ASC (Figure 2.4, *B* and *E*); however, these did not survive in the culture (Figure 2.4*C*, *D*, *F*, and *G*). After culture in vitro with CpG ODN and LPS, B cells in population II, memory B cells, differentiated exclusively into NP-specific



	I	II	III	IV	V
CD138	+	-	-	-	nonimmune
B220	-	+	+	+	
IgD/M	-	-	+	+	
NP	-	+	+	-	

	I	II	III	IV	V
CD138	+	-	-	-	nonimmune
B220	-	+	+	+	
IgD/M	-	-	+	+	
NP	-	+	+	-	

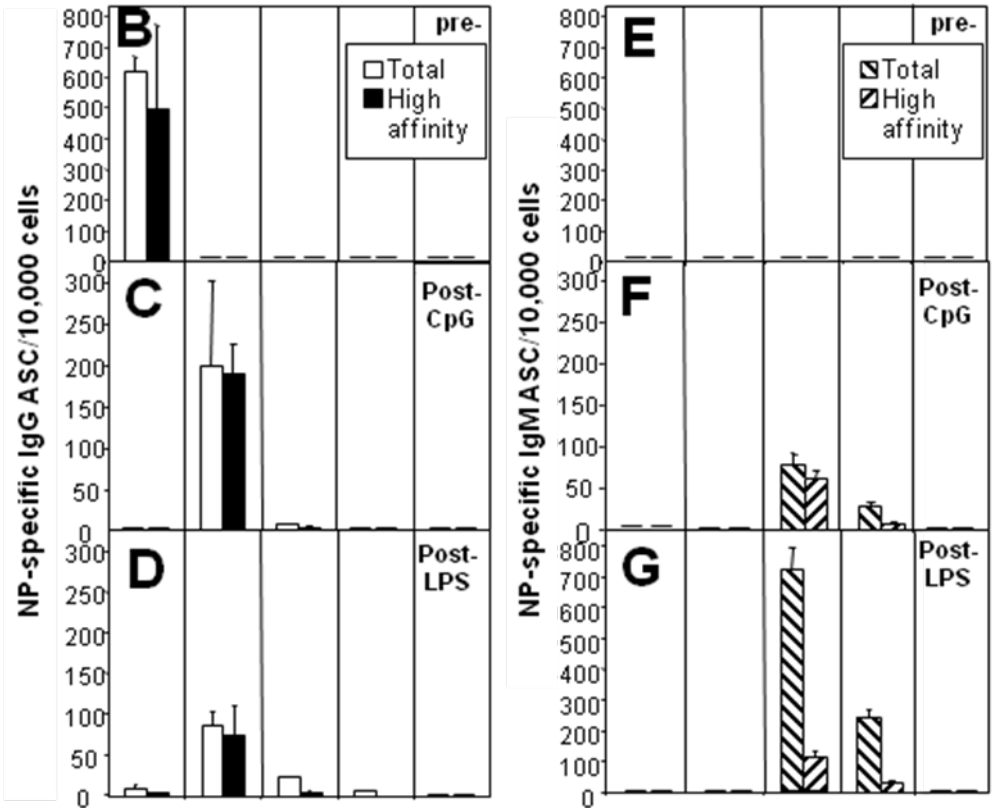


FIGURE 2.4. B cells with the memory phenotype differentiated into Ag-specific, high affinity IgG ASCs in culture with CpG ODN and LPS. *A*, Splenic B cells from NP-KLH-immunized mice were sorted into four populations: $B220^-$ IgM^- IgD^- NP^- $CD138^+$ (I, plasma), $B220^+$ IgM^- IgD^- NP^+ $CD138^-$ (II, memory), $B220^+$ $IgM/IgD^+NP^+CD138^-$ (III, NP-binding, unswitched), and $B220^+IgM/IgD^+NP^-CD138^-$ (IV, unspecific, unswitched). Naive B cells (V) were isolated from the spleen of unimmunized mice. The frequencies of high affinity and total NP-specific IgG (*B–D*) and IgM (*E* and *F*) ASCs were determined by ELISPOT before (*B* and *E*) and after culture in CpG ODN (2 μg/ml) (*C* and *F*) or LPS (5 μg/ml) (*D* and *G*) for 5 days. Shown are representative results from three independent experiments.

IgG ASCs, a large portion of which were high affinity (Figure 2.4*C*, *D*, *F*, and *G*). In contrast, populations III and IV containing unswitched B cells generated mostly low affinity IgM ASCs in response to CpG ODN and LPS (Figure 2.4*C*, *D*, *F*, and *G*). As expected, the NP-binding population of unswitched B cells generated a higher number of NP-specific IgM ASCs as compared with the non-NP-binding population (Figure 2.4, *F* and *G*). Response of the unswitched B populations to LPS was nearly 10-fold greater than to CpG ODN (Figure 2.4, *F* and *G*). These results demonstrate that TLR agonists can directly activate memory B cells in vitro to differentiate into high affinity, isotype switched plasma cells and unswitched B cells to differentiate into low affinity, IgM ASCs.

Differential expression of TLR4 and 9 in populations of B cells

The differential responses of the B cell populations to TLR4 and 9 agonists shown in Figure 2.4 suggest different expression levels of TLR9 and 4. To test this hypothesis, the levels of expression of TLR4 and 9 in the different populations of splenic B cells isolated from NP-KLH-immunized mice 5 days after the second immunization were determined by flow cytometry. The analyses showed higher TLR9 expression in NP-binding B cell populations (II and III) compared with naive B cells (population V) from nonimmune mice (Figure 2.5, *left panels*). In contrast, the TLR4 surface levels of naive (population V) and unswitched B cells (populations III and IV) were significantly higher than that of NP-specific memory B cells (population II) (Figure 2.5, *right panels*). These differences in expression levels provided an explanation for

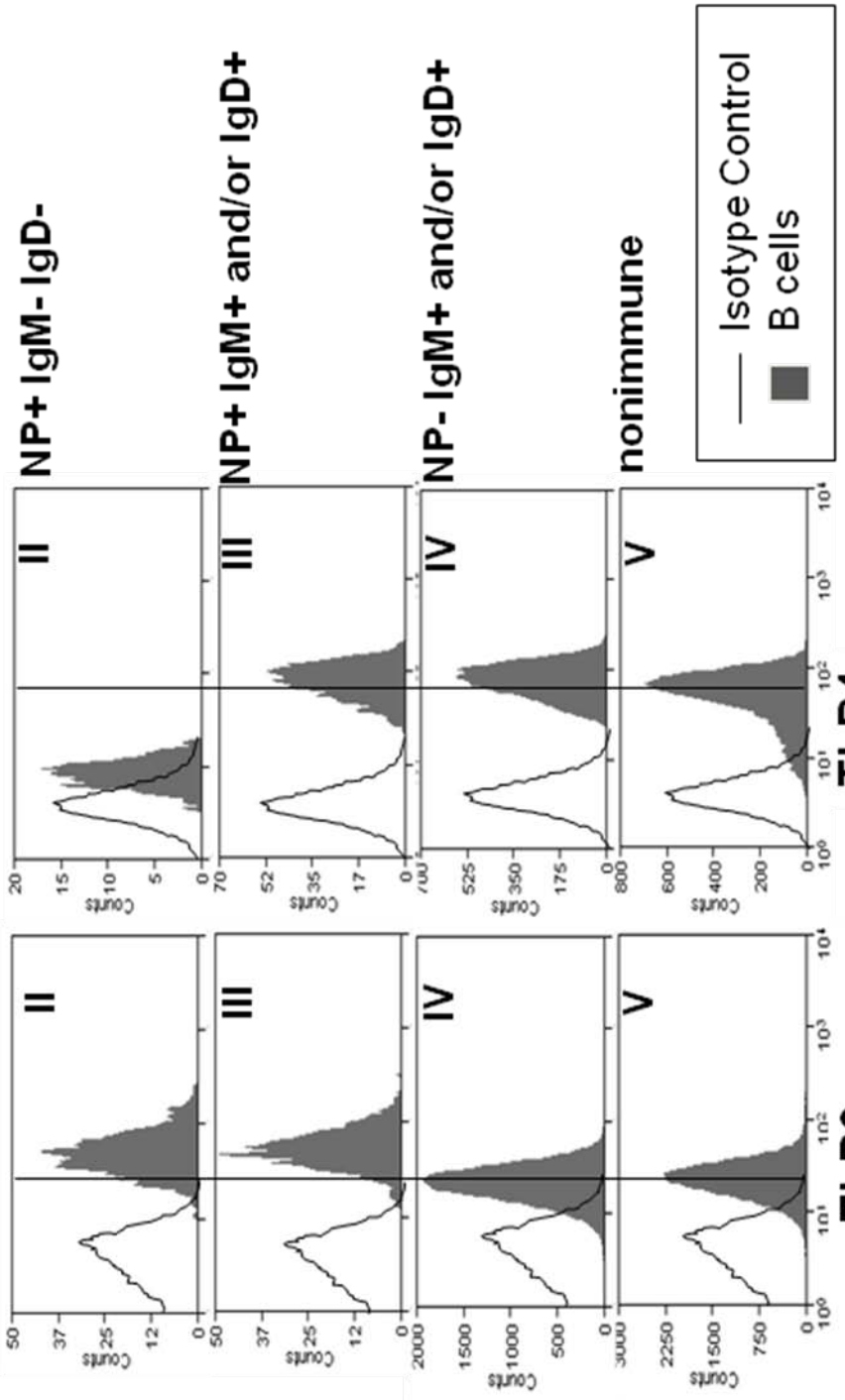

NP+ IgM- IgD-
NP+ IgM+ and/or IgD+
NP- IgM+ and/or IgD+
nonimmune
II
III
IV
V
Isotype Control
B cells
TLR4
TLR9
Counts

FIGURE 2.5. Expression levels of TLR9 and TLR4 in different subpopulations of splenic B cells. Splenic B cells were isolated from unimmunized mice (nonimmune) and mice that were immunized with NP-KLH plus Ribi adjuvant. The splenic B cells were preincubated with the FcγR blocking Ab, followed by PerCP-Cy5-anti-B220, FITC-anti-IgD, FITC-anti-IgM Abs, allophycocyanin-NP_{19}, and PE-anti-TLR4-MD2 Abs for staining TLR4. For staining TLR9, the cells were washed, fixed, permeabilized, and stained with anti-TLR9 Ab and a pacific blue-conjugated secondary Ab. The cells were analyzed by flow cytometry. $B220^{high}$ B cell populations of nonimmune mice (V), NP-specific memory B cells (II), NP-binding, unswitched B cells (III), and nonspecific, unswitched B cells (IV) of immunized mice were gated, and their TLR4 (*right panels*)- and 9 (*left panels*)- staining levels are shown. Shown are representative results from five independent experiments.

the equivalent response of memory B cells to CpG ODN and LPS and the hyper-response of NP-binding unswitched B cells to LPS.

The activation of B cell memory responses by TLR4 and 9 agonists in vivo

To determine whether TLR4 and 9 agonists can stimulate memory B cells in vivo, mice were immunized with NP_{19}-KLH plus Ribi adjuvant twice, 4 weeks apart, to establish a pool of NP-specific memory B cells. Six weeks later, the mice were challenged with NP_{19}-KLH, Ribi adjuvant, CpG ODN, LPS alone, or NP-KLH plus either Ribi adjuvant, CpG ODN, or LPS. Five days after the challenge, high and low affinity serum titers of NP-specific IgG and the number of high affinity, NP-specific IgG ASCs in spleens were determined. A certain level of high affinity serum titer (Figure 2.6*A*) and a small number of high affinity, NP-specific IgG ASCs (Figure 2.6*B*) existed in the spleens before the boost, indicating the persistence of NP-specific IgG ASCs in the spleen even 6 weeks after an immunization. The challenge with Ag alone significantly increased (~10-fold) the serum titer of high affinity NP-specific IgG and the number of high affinity, NP-specific IgG ASCs (Figure 2.6, *A* and *B*). However, the challenge with adjuvant alone, including Ribi adjuvant, CpG ODN, and LPS, did not significantly change either the serum titer of high affinity NP-specific IgG or the number of high affinity NP-specific IgG ASCs in comparison with those before the challenge (Figure 2.6, *A* and *B*). In contrast, challenging with Ag plus adjuvant dramatically increased the number of the high affinity IgG ASCs, with ~56-, ~46-, and ~16-fold increases for Ag plus Ribi, LPS, and CpG ODN adjuvant, respectively (Figure 2.6*B*). LPS or Ribi appeared more effective than CpG ODN as

the adjuvant under this specific immunization condition. At 5 days post the third immunization, the serum titers of mice challenged with CpG or LPS plus Ag were significantly higher than those that did not receive the third immunization and those that were challenged with CpG or LPS alone, but lower than those that were challenged with Ag alone (Figure 2.6*A*). This did not completely mirror what was observed for the numbers of NP-specific ASCs (Figure 2.6*B*) and may reflect different kinetics and timing for B cell differentiation and for NP-specific plasma cells to exit from spleens in mice challenged with Ag alone and Ag plus adjuvant.

Taking advantage of the ability of memory B cells to specifically differentiate into high affinity IgG ASCs in culture with CpG ODN, we estimated the relative frequencies of NP-specific memory B cells in spleen after the different challenges. A significant number of NP-specific, high affinity IgG ASCs was detected in the 5-day culture of B cells from mice rested for 6 weeks without the challenge (Figure 2.6*C*), indicating the persistence of memory B cells in the spleen long after an immunization. The number of high affinity IgG ASCs differentiating from B cells from mice challenged with Ag or adjuvant alone was similar to or lower than that of B cells from mice that were not challenged (Figure 2.6*C*). The number of high affinity IgG ASCs differentiating from B cells from mice challenged with Ag plus adjuvant was 5- to 20-fold higher than those of mice without the challenge (Figure 2.6*C*). Among different adjuvants, Ag plus either Ribi or LPS generated the largest increase. To further confirm this result, we determined the frequency of NP-specific memory B cells by flow cytometry. The challenge with Ag plus adjuvant, but not Ag or adjuvant alone,

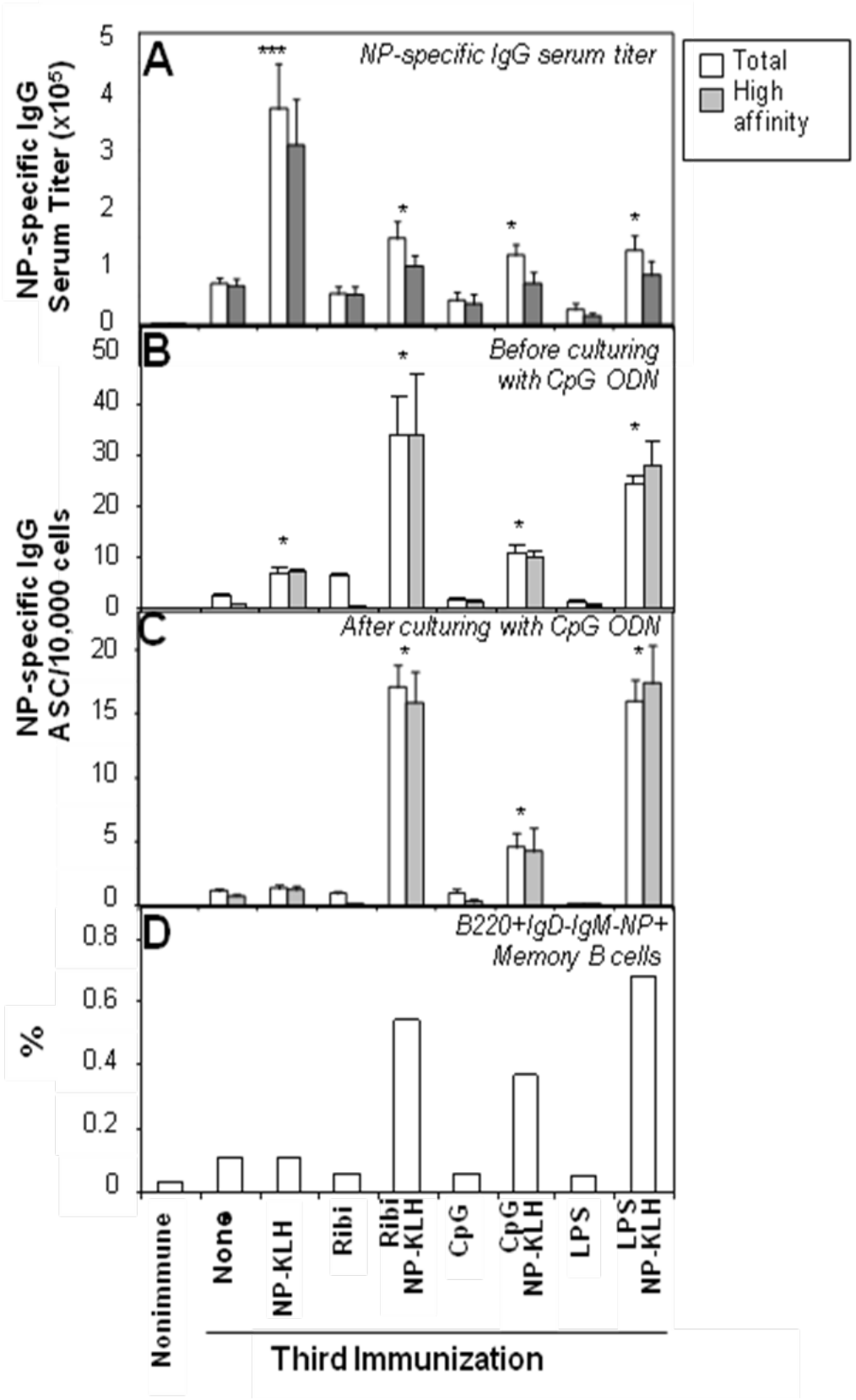

A
NP-specific IgG serum titer
Total
High affinity
NP-specific IgG Serum Titer (x10^5)
B
Before culturing with CpG ODN
C
After culturing with CpG ODN
NP-specific IgG ASC/10,000 cells
D
B220+IgD-IgM-NP+ Memory B cells
%
Nonimmune
None
NP-KLH
Ribi
Ribi NP-KLH
CpG
CpG NP-KLH
LPS
LPS NP-KLH
Third Immunization

FIGURE 2.6. Clonal expansion and differentiation of memory B cells in response to immunization of Ag or TLR agonists alone or in combination. C57BL/6 mice were twice immunized with NP-KLH plus Ribi adjuvant 4 weeks apart and rested for 6 weeks. The mice were immunized for the third time with NP-KLH (400 μg/ml), Ribi adjuvant, CpG ODN (50 μg/mouse), or LPS (50 μg/mouse) alone or in combination. Controls included nonimmune mice and mice that did not receive the third immunization (None). *A*, Serum was collected from 1 day before and 5 days after the challenge and analyzed for NP-specific high affinity and total IgG titers. Shown are average titers (±SE, n = 3~25). The serum titers of mice challenged with NP-KLH plus the adjuvant were significantly higher than those of mice that did not receive the third immunization or received the adjuvant alone for the third immunization. ***, $p \leq 0.001$; *, $p \leq 0.05$. *B* and *C*, B cells and plasma cells were enriched and frequencies of NP-specific IgG ASCs were determined by ELISPOT before (*B*) and after (*C*) culture with CpG ODN (2 μg/ml) in vitro for 5 days. *D*, The percentage of NP-specific memory B cells among the splenic B cells was determined by flow cytometry, as described in Figure 2.3. Shown are the representative results of four independent experiments.

induced significant increases (5- to 20-fold) in the numbers of NP-specific memory B cells (Figure 2.6*D*). Ribi or LPS as adjuvants appeared to be more effective than CpG ODN as an adjuvant in expanding the NP-specific memory B cell pool (Figure 2.6*D*).

Section 4 Discussion

Humoral memory responses consist of two major components, long-lived plasma cells that secrete high affinity Ab and memory B cells, the precursors of long-lived plasma cells. In this study, we examined the role of TLR agonists in activation of humoral memory responses in vitro and in vivo by tracking these two major components of the humoral memory responses. We found that TLR4, 7, and 9 agonists could directly stimulate the differentiation of memory B cells into high affinity plasma cells in vitro; however, TLR agonist alone was not sufficient to stimulate the clonal expansion and differentiation of memory B cells and specific Ab recall responses *in vivo*.

Our study showed that when cultured with TLR4, 7, and 9 agonists, the splenic B cells from NP-KLH-immunized mice, either 5 days or 6 weeks post the second immunization, differentiated into NP-specific ASCs with most of IgG ASCs as high affinity and most of IgM ASCs as low affinity. Using sorted memory B cells, we further showed that among different populations of B cells cultured with TLR4 or 9 agonist, NP-specific memory B cells exclusively differentiated into high affinity, isotype switched ASCs, and NP-binding unswitched B cells mainly differentiated into

low affinity IgM ASCs. This demonstrates that TLR agonists can directly act on memory B cells in vitro and polyclonally stimulate the differentiation of memory B cells into high affinity, isotype switched plasma cells in the absence of T cells. In contrast, TLR agonists stimulated unswitched B cells *in vitro* primarily into low affinity, unswitched ASCs. Specific differentiation of sorted memory B cells into high affinity IgG ASCs indicates that they are the precursors of high affinity plasma cells and confirms that B cells sorted based on the phenotypic characteristics consist of mainly Ag-specific memory B cells. Furthermore, the differentiation specificity in the *in vitro* culture with TLR agonists provides us a good tool to determine the frequency of memory B cells in a mixture of multi-subpopulations of B cells. The finding that splenic B cells isolated from mice 5 days and 6 weeks post the immunization responded to TLR4 and 9 agonists in a similar manner suggests the persistence of Ag-specific memory B cells in mice.

The experimental definition of memory B cells is still controversial, especially in the murine system. McHeyzer-Williams *et al*. [109, 110] established a multicolor flow cytometry method to follow NP-specific memory B cells in NP-KLH-immunized mice based on their phenotypic characteristics of post-germinal center B cells ($B220^+$ IgD^- $IgG1^+$ NP^+) and demonstrated that B cells with such phenotypes exhibited a high frequency of point mutations in their variable regions. Anderson *et al*. [407] recently suggested that CD80 and CD35 expression levels could be used to define B cell subtypes that have or have not undergone somatic hypermutation. Slifka and Ahmed [408] established an in vitro culture system to determine the frequency of acute

lymphocytic choriomeningitis virus-specific memory B cells using the combination of limiting dilution and ELISPOT. Taking advantage of the ability of CpG ODN to stimulate the differentiation of memory B cells into high affinity IgG ASCs in vitro, we determined the frequency of memory B cells using this in vitro culture system in addition to flow cytometry. Both flow cytometry analysis and the *in vitro* culture system showed dramatic increases in the number of Ag-specific memory B cells 5 days after the second immunization and maintenance of a small, but significant, number of memory B cells 6 weeks after the second immunization.

In addition to NP-specific, isotype switched B cells, a population of NP-binding, unswitched B cells were found in the immunized mice. This population of B cells was expanded in response to the second immunization, but returned to the basal level of unimmunized mice by 6 weeks. When cultured with TLR4 and 9 agonists, they differentiated into NP-specific IgM ASCs, most of which exhibited low affinity for the Ag. Although the exact nature of the NP-specific unswitched B cells is unclear, it may contain IgM-expressing memory B cells. The existence of IgM-expressing memory B cells is still controversial. The discovery of a subpopulation of $CD27^{+}IgM^{+}$, somatic mutated B cells in human peripheral blood [81] suggests the presence of IgM expressing memory B cells. Weller *et al*. [83] showed that this population of B cells was circulating spleen marginal zone B cells that mutated their Ig receptors during ontogeny, before their differentiation into T-independent Ag-responsive cells. The role of this population of B cells in humoral memory responses remains to be further defined.

Our studies present a first comparison of B cell responses to TLR4 and 9 agonists *in vitro* and *in vivo*. Our results showed that TLR4 and 9 agonists were equally effective in stimulating memory B cells to differentiate, but TLR4 agonist was much more efficient than TLR9 agonist in stimulating unswitched B cells from immunized mice to differentiate *in vitro*. This differential response can be explained by different expression levels of TLR4 and 9 in different subpopulations of B cells. We found that the immunization increased the expression levels of both TLR4 and 9 in mouse B cells, and the expression level of TLR9 was maintained and the expression of TLR4 down-regulated when activated B cells differentiated into memory B cells. Our finding of an increase in TLR9 expression level by immunization is similar to the report of Bernasconi *et al*. [160] that human memory B cells, but not naive B cells, constitutively express TLR9. The biological significance of this differential expression of TLR9 and 4 among different B cell subpopulations remains to be explored. The higher expression level of TLR4 in naive and activated, unswitched B cells than memory B cells predicts that LPS preferentially induces primary humoral responses.

The direct stimulatory effects of TLR9 and 4 agonists on memory B cells *in vitro* showed in this study and previous studies [112, 160] suggest their potential role in polyclonal activation of memory B cells *in vivo*. Our finding that TLR9 or 4 agonist alone failed to increase the frequencies of either Ag-specific high affinity IgG ASCs or Ag-specific memory B cells, as well as the concentration of Ag-specific IgG in

serum, does not support the polyclonal activation hypothesis. Our result that Ag in combination with TLR9 or 4 agonist was more effective than Ag alone for inducing clonal expansion and plasma cell differentiation suggests that the activation of memory B cells in vivo still requires more than one signal, Ag-triggered BCR-mediated signal in combination with TLR-triggered signals or T cell help. The findings that on day 5 post the third immunization, Ag alone induced a higher serum titer but a lower frequency of high affinity NP-specific IgG ACS in spleens than Ag plus CpG or LPS may reflect differences in the kinetics of B cell differentiation and plasma cell migrating from the spleen to the bone marrow in response to different stimuli. Some other factors may limit the direct role of TLRs on B cells, such as the competition between B cells and dendritic cells to interact with TLR agonists. TLR agonists are very effective in the induction of dendritic cell maturation. Dendritic cell-activated T_H cells provide memory B cells with a second stimulation signal. Above all, requirement of both Ag and TLR agonists for the activation of memory B cells in vivo ensures the specificity of humoral memory responses.

The necessity of TLRs in humoral memory responses is controversial. Disruption TLR signaling by knocking out MyD88 and/or Trif genes either did or did not significantly inhibit Ab responses against T-dependent Ags, depending on the specific experimental system [282, 288, 289]. The study presented here reveals a direct stimulatory role of TLR on memory B cell differentiation in vitro and the requirement of both Ag and TLR agonist for effective activation of humoral memory responses *in*

vivo. These results strongly support a critical role for TLRs in recalling humoral memory responses.

Chapter 3: Agonists of Toll-like receptors 7 and 9 enhance antibody affinity maturation in secondary immune responses in a mouse model

Section 1 Introduction

Successful vaccination for humoral immune responses depends on the B cells' ability to undergo antibody affinity maturation. Affinity maturation is an increase in the average binding strength for an organism's antibodies to an antigen and is achieved by mutation of weakly binding antibodies to bind more strongly. In immune responses to T cell-dependent (TD) antigen, affinity maturation occurs in germinal centers (GCs) that form in follicular regions of spleen and lymph nodes after exposure to antigen. GCs support rapid proliferation of B cells and activation-induced cytidine deaminase (AID)-dependent somatic hypermutation of the immunoglobulin (Ig) gene, which creates a diverse pool of closely related B cell clones. Selection of B cells expressing high affinity Ig in their B cell antigen receptors (BCRs) occurs via competition for survival signals that are generated by interactions with antigen-presenting follicular dendritic cells (fDCs) in the GC (reviewed by McHeyzer-Williams *et al.*, 2005) [13].

The use of adjuvants, non-antigen additives, as part of immunogen is known to enhance immune responses. However, whether adjuvants can induce or enhance the affinity maturation is unclear. Many adjuvants serve as 'danger-signals', causing strong localized innate immune responses (i.e. topical application of imiquimod near

intradermal injection site; [329, 331-333]) or weak systemic inflammation (i.e. peritoneal injection of alum/CpG oligodeoxynucleotides; [340, 348, 352, 353, 409]). The most successful adjuvants for mice include Freunds and Ribi adjuvants, which contain several proinflammatory ingredients. The major component of complete Freunds adjuvant is heat-killed mycobacteria in an oil-in-water emulsion [317]. Ribi adjuvant contains monophosphoryl lipid A (MPL; a TLR4 agonist) and trehalose dicorymycolate (TDM; a TLR2/4 agonist) also supplied in an oil-in-water emulsion [318, 410-412]. In humans, Freunds and Ribi adjuvants cause stronger inflammatory responses than in mice, sometimes even leading to septic shock in the recipient, and thus are not safe for human application [315]. Only two adjuvants are currently approved by the FDA for use in humans: aluminium salts (alum) and monophosphoryl lipid A (MPL) [313]. Alum is thought to precipitate the antigen, slowing its dilution and temporally extending its release (reviewed by Lindblad, 2004) [413]. A separate mechanism for alum function has recently been proposed to work through stimulating uric acid production, a host cell damage signal rather than a microbial antigen [409], and through inflammasome activation [414]. Alum adjuvant has been shown to trigger Th2 type immune responses [413], inhibiting the release of proinflammatory Th1 cytokines [415-417]. Nevertheless, alum is very weak in its immunostimulatory properties and only minimally improves vaccination [314, 418]. Therefore, a stronger yet safe adjuvant is needed to improve human vaccine efficacy. MPL is a highly detoxified derivative of bacterial LPS [318, 411]. MPL weakly activates dendritic cells and macrophages, causing weak inflammation [316, 411,

419]. Several different agonists of TLRs, including CpG ODN and gardiquimod, are also currently undergoing clinical trials.

Toll-like receptors (TLR) are a family of pattern recognition receptors that recognize common pathogen-associated molecular patterns (PAMP) of bacteria and viruses, such as peptidoglycan [246, 247], flagellin [252], lipopolysaccharides (LPS) [229, 230, 234, 237, 248, 249], single-stranded RNA [245, 253-255], and DNA with hypomethylated cytosine-guanine (CpG) motifs [161, 231, 256-259, 261]. TLR stimulation affects both innate and adaptive immune responses. TLRs are highly expressed in cells of the innate immune system, such as macrophages and dendritic cells [230, 270], where they promote inflammation through the maturation of dentritic cells and stimulation of cytokine production (reviewed by Akria *et al.*, 2006) [270]. TLRs also are expressed in subsets of T and B lymphocytes. Mouse resting B cells express TLRs 2, 4, 7, and 9 [269, 273-277]. TLR stimulation provides a second signals for the activation of B cells in response to T cell-independent (TI) antigens [344]. In humans, while resting follicular B cells do not express TLRs, antigenic stimulation induces TLR expression. Human memory B cells express TLRs 2, 6, 7, 9, and 10 [160, 263, 271, 272, 278]. Therefore, TLRs impact adaptive immune responses directly through TLR expressed by lymphocytes and indirectly through the functional interaction of innate cells and lymphocytes.

The signal transduction of TLRs primarily is mediated by the adaptor molecule myeloid differentiation primary response gene 88 (MyD88), leading to activation of

NF-κB and other transcription factors involved in immune cell activation [270]. MyD88$^{-/-}$ mice are hyporesponsive to IL-1R and most TLR stimulation. MyD88$^{-/-}$ mice not only show no response to LPS in their macrophages, which makes them completely resistant to endotoxic shock from LPS [420], and inhibition of DC priming for cellular immune responses [282], but also exhibit impaired humoral immune responses to T cell-dependent antigen [282]. Schnare *et al.* showed that MyD88$^{-/-}$ mice immunized with OVA and complete Freund's adjuvant had reduced titers of IgG_{2a} subtype, while IgG_1 and IgE responses were relatively normal [420]. It remains unclear, however, whether the defect is B cell intrinsic or due to lack of T cell help [289, 421].

The strong immune stimulatory properties of TLR agonists made them good canadates of adjuvant. Imiquimods and unmethylated CpG-containing oligodeoxynucleotides (CpG ODN) are two families of synthetic TLR agonists that show promising ability to increase antibody response in animals [299, 301, 336, 337, 340, 343-347, 349, 351, 422]. In addition, these two types of TLR agonists passed safety tests in phase 1 human clinical trials [329-333, 339, 354, 355, 357, 359-361]. The structures of the imiquimod family molecules mimic single-stranded RNA. They stimulate TLR7 and TLR8 to various degrees depending on the structures of the molecules (reviewed by Ferratini et al, 2008) [328]. Gardiquimod (1-(4-amino-2-ethylaminomethylimidazo-[4,5-c]quinolin-1-yl)-2-methylpropan-2-ol; made by InvivoGen, San Diego, CA) predominantly binds to TLR7 [253, 423] and is about 10 times more effective than imiquimod in NF-κB activation in HEK293 cells

overexpressing human or mouse TLR7 [253]. Synthesized CpG ODNs stimulate through TLR9 [161, 231, 256-259, 261]. Class A CpG ODN are most efficient at stimulating dendritic cells and IFNα production (reviewed by Krieg, 2002) [262]. Class B CpG ODNs are most efficient at stimulating B cell proliferation and differentiation into plasma cells [262]. Both class B CpG ODN and gardiquimod are able to increase antigen specific antibody titers, and typically increase the IgG_{2a}/IgG_{2c} and IgG_{2b} antibody isotypes associated with the more pro-inflammatory Th1 cytokines [253, 262, 299, 335, 340, 341, 423, 424]. However, little is known about these molecules' ability to induce antibody affinity maturation or immune memory.

In this study, we asked whether TLR agonists CpG ODN and gardiquimod would be able to increase antibody affinity *in vivo* using a mouse immunization model with T cell-dependent antigen. Comparison of Ribi adjuvant, CpG ODN, and gardiquimod by three different methods shows they are equally able to induce antibody affinity maturation. All three of these adjuvants reduced Th2 bias of the antigen. Analysis of resting memory showed that despite elevated titers, the size of the antigen-specific mB and ll-PC populations is not significantly different between mice immunized with or without these adjuvants. Our results suggest a role for TLR agonists in promoting affinity maturation.

Section 2 Materials and Methods

Mice and immunizations

For generating T cell-dependent humoral immune responses, 6-12 week-old C57BL/6 mice (Taconic, Inc., Gaithersburg, MD) were injected twice, 28 days apart, via intraperitoneal route with 100 μg/mouse NP_{19}-KLH (NP, 4-hydroxy-3-nitrophenylacetyl; KLH, keyhole limpet hemocyanin) (Biosearch Technologies, Novarto, CA) emulsified in 2.0% squalene oil (Sigma-Aldrich, St. Louis, MO) and 0.2% Tween 80 (Sigma-Aldrich, St. Louis, MO) in sterile PBS alone or with Ribi adjuvant (MPL+TDM Adjuvant System, Sigma-Aldrich, St. Louis, MO), with 50 μg/mouse completely phosphothyated unmethylated class B CpG –containing oligodeoxynucleotides (CpG ODN) (5'-TCCATGACGTTCCTGACGTT-3', phosphorthiolated at every base, Operon Biotechnologies, Inc., Huntsville, AL) or with 50 μg/mouse Gardiquimod (InvivoGen, San Diego, CA). Serum samples were collected from mice by tail vein bleed two and four weeks after first immunization and five days, two and four weeks, and 100 days after the second immunization. Sera were stored at -20^{o}C before analysis.

Antibody titration by ELISA

ELISA for detecting NP-specific antibody titer was performed as previously described [425]. Briefly, ELISA plates (Nalge Nunc International, Rochester, NY) were coated with 1 μg/mL of NP_{26}-BSA (Biosearch Technologies, Novarto, CA) in 50 mM NaHCO3 (pH 9.6) over night at 4^{o}C and blocked with 0.3% milk, 1% FBS, and 0.1% Tween 20 in PBS for 1 h at 37^{o}C. Mouse serum was serially diluted into

the wells and incubated at room temperature for 2 h. For plate-to-plate control, one row of wells was coated with 5 μg/mL of anti-mouse IgG+M antibody (Jackson ImmunoResearch, West Grove, PA) and incubated with mouse IgG standard (Jackson ImmunoResearch, West Grove, PA) during serum incubation step. After washing the plates three times with PBST (0.1% Tween-20 in PBS), the plates were incubated with a cocktail of 1:1000 dilutions of horseradish peroxidase (HRP)-conjugated anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3 antibodies (Southern Biotech, Birmingham, AL). After further washing the plates three times in PBST, the amount of plate-bound antibody was visualized by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, St. Louis, MO) and 0.03% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) in phosphate-citrate buffer, pH 5.0 (Sigma-Aldrich, St. Louis, MO). The reaction was stopped after 5 min with 0.5% SDS (Fisher Scientific, Pittsburgh, PA). The absorbance at 405 nm was measured using a Spectramax plus 384 plate reader (Molecular Devices, Sunnyvale, CA). The antigen-specific antibody serum titer was determined in ELISA units (EU) on a linear trend line (least squares regression method) for the steepest/most linear portion of each titer curve (R^2>0.9 data to linear fit), with definition of 100 EU correspond to an O.D. value of 1 (Figure 3.1). The serum concentration corresponding to 2.5 EU were in the linear range for all serum samples, even when the colorimetric detection was extended to 10 minutes to discern differences on this small scale (not shown).

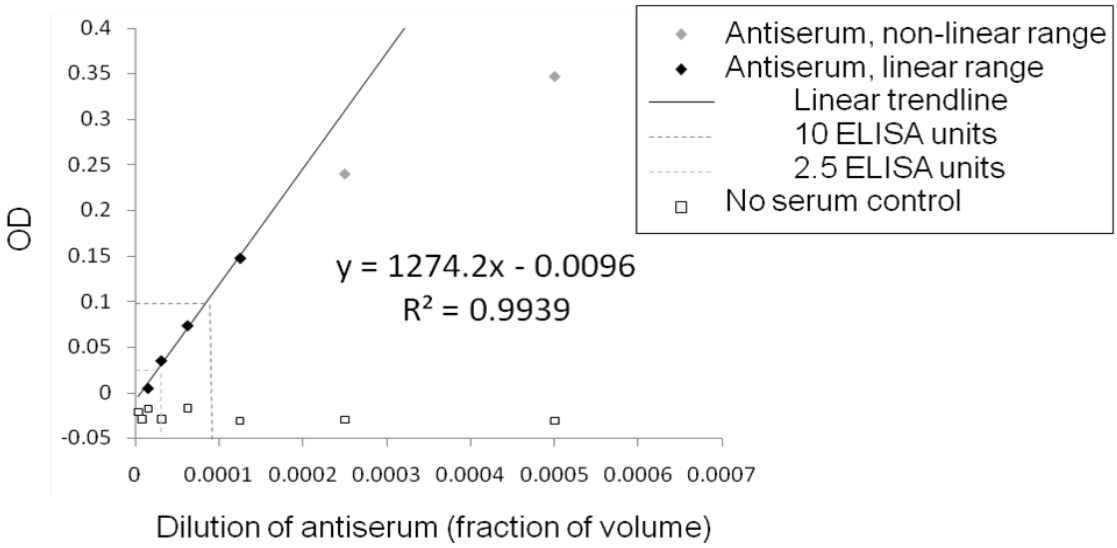


Figure 3.1. Example of ELISA unit determinations. Appropriate dilution was determined for each serum sample by intrapolation within the linear range. 10 EU was in the linear range for low titer anti-sera, but at time points following second immunization was at the cusp between the linear and non-linear range. 2.5 EU was in the linear range for all samples.

Antibody affinity analysis by descreasing densities of ELISA coating antigen

ELISA plates are coated with 1 μg/mL of NP_4-BSA (Biosearch Technologies, Novarto, CA) in a half of the wells to limit binding of low affinity antibodies, and coated with 1 μg/mL NP_{26}-BSA, or 5 μg/mL of anti-mouse IgG+M antibody in the other half of the plate. The ELISA analysis was performed as described above. The levels of NP-specific antibodies that bind to NP_4-BSA (high affinity) and NP_{26}-BSA (total) were determined independently. The fraction of high affinity antibodies in the total NP-specific antibodies was determined.

Antibody affinity analysis by chaotropic inhibition with urea

Competitive ELISAs using chaotropic inhibition was performed as previously described [426]. Serum titers (ELISA Units) were determined using ELISA that was performed as described above except that serum was incubated with the plate for 20 min rather than 2 h to match the optimal incubation time of the competitive assays. For chaotropic inhibition of ELISA, ELISA plates were coated with NP_{26}-BSA and blocked as described above, incubated with 100 μl of serum, pre-diluted to 2.5 EU, for 20 min at room temperature, and washed three times with PBST. Urea (Sigma-Aldrich, St. Louis, MO) at varying concentrations (0.0-10.0 M in PBS) was added at 100 μL/well and incubated for 10 min at room temperature. After washing with PBST, the amount of IgG bound to the plate was determined as described above, allowing 10 minutes for ABTS color change. The concentration of urea necessary for a 50% decrease in antibody binding (OD of wells without urea or the average OD of a high plateau (100%) minus the wells without serum or the average OD of a low

plateau (0%), divided by two) was determined using least squares regression trend line for the steepest/most linear section of each inhibition curve. Pipetting error was determined by calculating variance of the OD without urea wash (10 min PBS wash). Only assays with variance of less than 5% of the average OD at maximal binding were analysed. The analyses were carried out for 3-6 replicates.

Antibody affinity analysis by competitive ELISA with free antigen

Competitive ELISAs with free antigen competition was performed as previously described [427]. Briefly, all serum samples were pre-diluted for a final concentration of 2.5 EU as described above (3.3.4). Free antigen, NP-OH (Biosearch Technologies, Novarto, CA), was serially diluted in the blocking buffer. Serum was pre-incubated with 0-50 mM NP-OH for 45 min at room temperature. Plates that were coated and blocked as described above were incubated in triplicate with the mixture of serum and free antigen (100 µl) for 20 min at room temperature. Then plates were washed with PBST, and the total IgG bound to the plate was determined as described above. For each serum sample, the concentration of the free antigen that causes a 50% descrease in antibody binding to the antigen coated plate (IC50) was determined using a non-linear fit equation "log(inhibitor) vs. response, variable slope" (GraphPad Prism 5.02, San Diego, CA), where inhibitor is the concentration of free antigen and response is the percentage of maximal OD.

Determination of the IgG isotypes of NP-specific antibody

The isotypes of NP-specific IgG was determined by ELISA as described above with a few modifications. NP_4-BSA was used as the coating antigen, and HRP-conjugated anti-mouse IgG_1, IgG_{2a} + IgG_{2c}, IgG_{2b}, or IgG_3 antibodies (Southern Biotech, Birmingham, AL) were used as secondary antibodies individually to detect different isotypes of IgG. The ratio of IgG_1 to IgG_{2a} + IgG_{2c} was determined.

Statistical analysis

Statistical difference for serum titers (Figure 3.1) was carried out by one-way ANOVA and Tukey post-test, using 95% confidence interval. Statistical significance comparing between immunization groups (Figures 3.2 - 3.5) was determined by two-tailed Mann-Whitney t-test with cut off at p=0.05, and comparing the same animals at different time points by paired t-tests. All statistical tests were carried out in GraphPad Prism (GraphPad Prism Software version 5.02, San Diego, CA).

Section 3 Results

TLR7 and 9 agonists increase antigen-specific antibody titers but are less effective than Ribi adjuvant

To determine the abilities of TLR agonists to adjuvate antibody responses, we compared the titers of antigen-specific antibodies in mice immunized with NP_{19}-KLH as antigen alone, antigen plus CpG ODN (TLR9 agonist), Gardiquimod (TLR7 agonist), or Ribi adjuvant (an agonist predominantly for TLR4). We focus on CpG ODN and gardiquimod as adjuvants because these are likely to be much safer for

people than Ribi adjuvant. Mice were immunized twice, 28 days apart, and sera were collected every two weeks for the initial eight weeks of immunization, as well as on the fifth day and 100th day after second immunization (Figure 3.2A). The serum collecting schedule was established based on the general properties of antibody responses in mice: primary antibody responses peak at two weeks after an immunization, and secondary antibody responses can be observed before new primary antibody responses to booster immunization (five days) and sustain for long time (100 days), which reflects humoral memory (Figure 2.1) [41, 86, 98, 404-406]. Because affinity maturation is often associated with isotype switching (reviewed by McHeyzer-Williams and McHeyzer-Williams, 2005) [13, 54], we focused on IgG. Mice that received any of the adjuvants had higher titers than those immunized without adjuvant. However, CpG ODN and Gardiquimod did not increase the titer as much as Ribi adjuvant. Across all immunization groups, most mice had a relatively low antigen-specific IgG titer at two weeks after primary immunization in comparison with that after the second immunization, regardless of whether or not they received an adjuvant (Figure 3.2B). Two weeks after the first immunization, the serum titer of mice that received Ribi as adjuvant already was significantly higher than those of mice that received CpG ODN ($p=0.0486$) or gardiquimod ($p=0.0188$) as adjuvant, and those of mice that did not receive adjuvant ($p=0.0096$). However, CpG ODN and gardiquimod did not significantly increase antibody titers at two weeks post fist immunization (Figure 3.2B). By four weeks after first immunization, the antibody titer in mice that received immunization with adjuvant continued increasing, while the antibody titer in mice received immunization with antigen alone remained

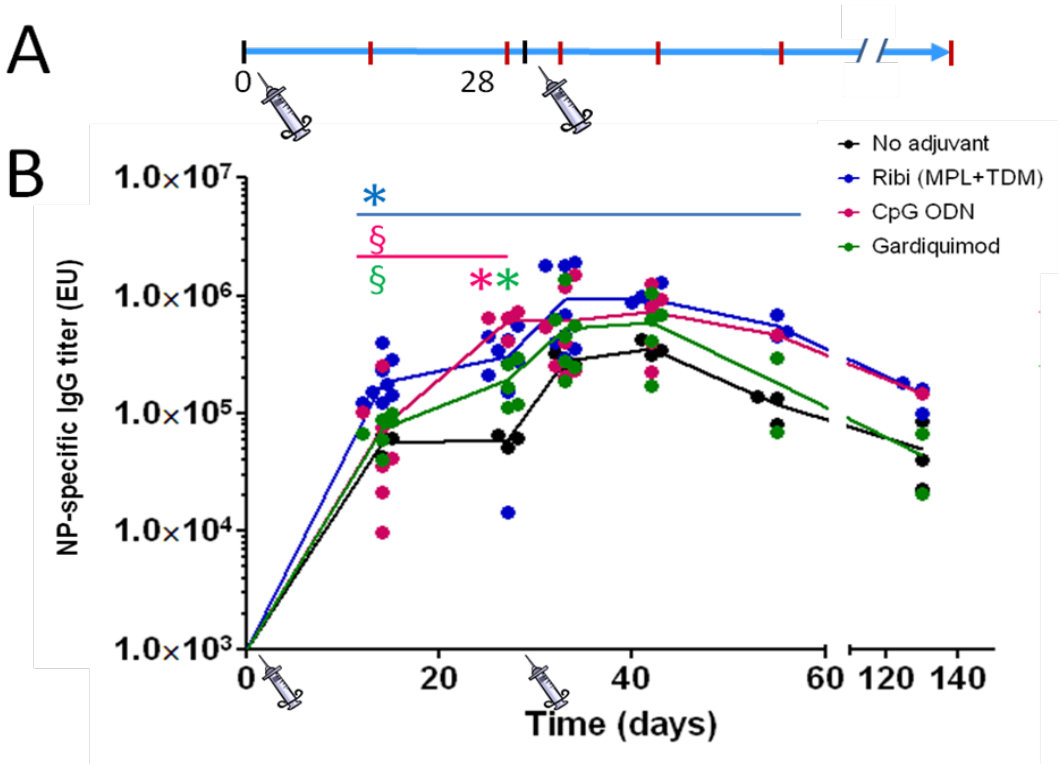


Figure 3.2. Immunizations with CpG ODN or Gardiquimod increase the antigen-specific IgG titer compared to immunization with antigen alone. A. This schematic of the immunization indicates time of injection (black) with NP_{19}-KLH with or without indicated adjuvants and time of serum collection (red) in days. B. Antigen-specific IgG titer over the first eight weeks after immunization. Each circle represents one mouse immunized with NP_{19}-KLH alone (black), NP_{19}-KLH + Ribi adjuvant (blue), NP_{19}-KLH + CpG ODN (magenta), or NP_{19}-KLH + gardiquimod (green). Lines connect group means; * $p<0.05$ compared to NP_{19}-KLH alone, § $p<0.05$ compared to NP_{19}-KLH + Ribi adjuvant.

the same as those two weeks post first immunization (Ribi p=0.0024, CpG ODN p=0.0039, gardiquimod p=0.0245 compared to no adjuvant). After four weeks, the antibody titer of mice that had been immunized with CpG ODN as adjuvant was significantly higher than those immunized with Ribi as adjuvant (p=0.0096 compared to Ribi adjuvant) (Figure 3.2B).

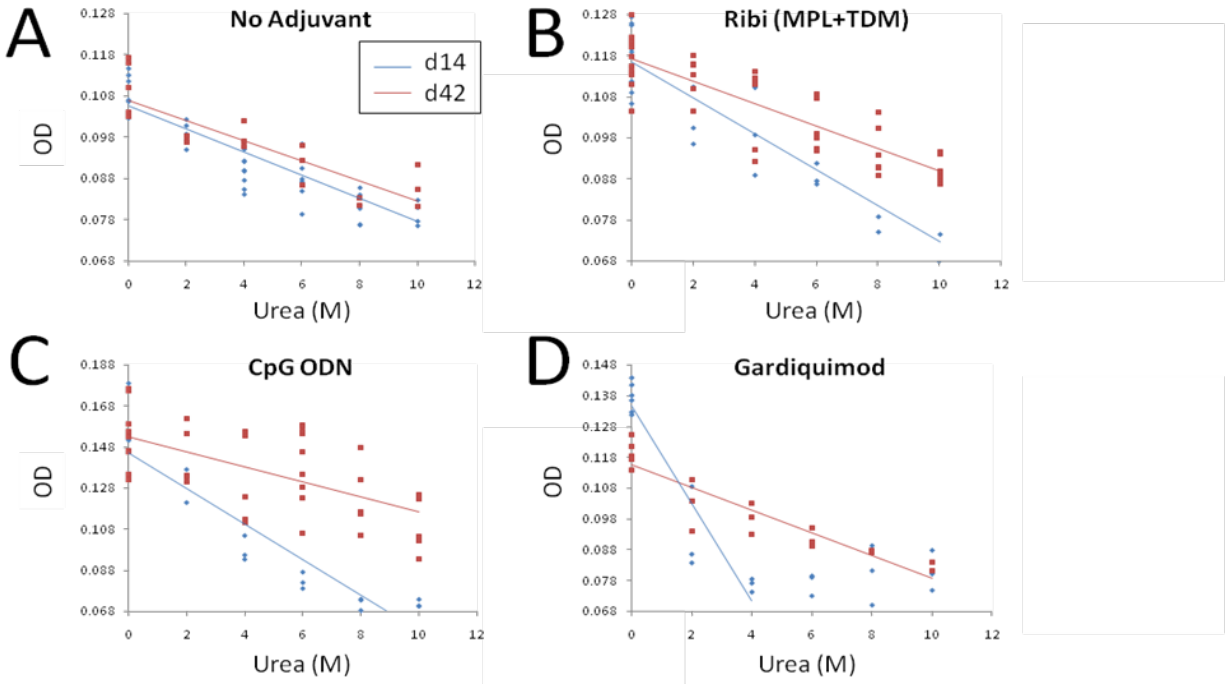
After second immunization, the NP-specific antibody IgG titers increased from 2-fold to 10-fold over those observed in primary response in all groups no matter if mice were immunized with or without adjuvant. Mice that had shown the most increase at the 2^{nd} and 4^{th} week after primary immunization showed the least increase after the second immunization, indicating that there may be a cap on NP-specific IgG secretion. The mice that received Ribi as adjuvant had significantly higher titers than mice that received antigen alone at 5 days (p=0.0258), 2 weeks (p=0.0015), and 4 weeks (p=0.0224) after the second immunization. The titers in mice immunized with CpG ODN or gardiquimod as adjuvant were higher than those in mice immunized with NP_{19}-KLH alone but lower than those in mice immunized with Ribi as adjuvant. However, the differences were not statistically significant (p>0.05) (Figure 3.2B). At four weeks after second immunization, antibody titers in all groups had started to decline, albeit they were still significantly higher than those after first immunization (Figure 3.2B). After 100 days resting, the NP-specific IgG titers were significantly lower than during peak secondary immune response, but were still approximately as high as during primary response in all groups (Figure 3.2B). Following the response of individual mice was only possible for a few mice that retained their identification

tag for the duration of the experiment and was not sufficient to establish mouse-to-mouse variance in the response. Taken together, our findings agree with previously published data that TLR7 and TLR 9 agonists are able to increase antibody titers but are somewhat less effective than TLR4-stimulating Ribi adjuvant (reviewed by Krieg, 2002) [262, 337].

TLR7 and 9 agonists as adjuvant increases antibody affinity

The efficacy of a vaccine is not only dependent on the amount of antibodies that the vaccine can induce, but also the affinity of antibodies to the antigen. To determine the effect of CpG ODN or gardiquimod as adjuvants on affinity maturation of B cells, we compared the affinity of antibodies generated with these two adjuvants with those induced by antigen alone and antigen plus Ribi adjuvant. We mainly focused on two weeks after first (day 14) and second (day 42) immunizations when the primary and secondary antibody responses nearly peak respectively. We first evaluated the relative affinity of antibody for NP using the urea resistance assay adapted from Dr. Kazutoya Miura's laboratory [426]. Urea is a chaotropic agent and can interrupt the antibody-antigen interaction; the higher the affinity of interaction, the higher a concentration of urea is required to interrupt the interaction. Control assays applying urea to antigen-coated plates before antibody binding show that the range of urea concentrations used for this assay does not affect the stability of the coating antigen (data not shown). To minimize the effect of antibody avidity and concentration on this assay, we focused on IgG and diluted all serum samples to equal IgG titer, 2.5 ELISA units. We chose this level of IgG because it is within the linear range of

detection. After serum incubation, the plate was washed with a range of 0-10 M urea, followed by incubation with a mixture of HRP-conjugated anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3 secondary antibodies for detection. The amount of IgG remained bound to the antigen-coated plate after urea washes was determined. Plates that were incubated with PBS instead of urea were used as controls, as PBS was not able to decrease the amount of antibody binding to antigen coated plate significantly. Using these data, we determine the concentration of urea that is able to remove lower the OD by 50%. Urea with increasing concentrations was able to remove up to 100% of antibody binding (Figure 3.3, A-D). A relatively higher concentration of urea necessary to drop the OD by 50% indicates a higher binding affinity of antibodies to NP. The sera from all four groups showed similar resistances to urea wash two weeks after the first immunization. All three types of adjuvant significantly increased resistance to urea wash following the second immunization, indicating increases in antibody affinity (Figure 3.3). However, the second immunization with antigen alone did not increase the antibody binding strength significantly (Figure 3.3, A and E). Importantly, although the titers of mice immunized with CpG ODN and gardiquimod as adjuvants were slightly lower than those with Ribi adjuvant (Figure 3.2B), their urea resistance levels were similar when same amount of antibodies were present, indicating similar increases in antibody affinity in all three groups with adjuvant (Figure 3.3, B-E). These results indicate that these adjuvants do increase antibody affinity, and promote B cell affinity maturation.


A
No Adjuvant
d14
d42
OD
Urea (M)
B
Ribi (MPL+TDM)
OD
Urea (M)
C
CpG ODN
OD
Urea (M)
D
Gardiquimod
OD
Urea (M)

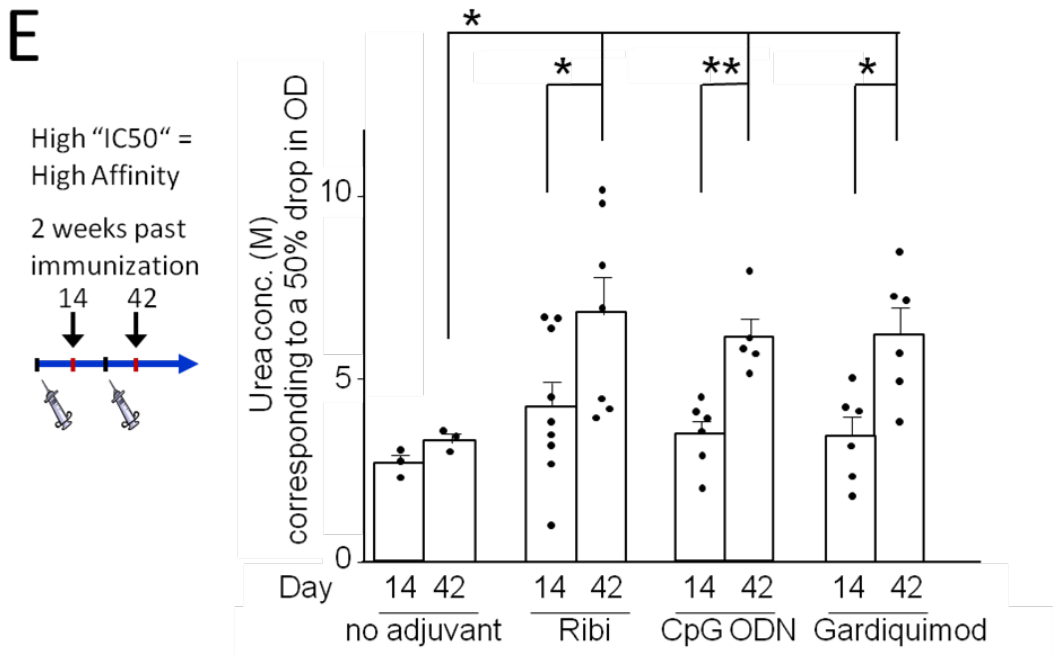

E
High "IC50" =
High Affinity
2 weeks past
immunization
14
42
Urea conc. (M)
corresponding to a 50% drop in OD
10
5
0
*
*
**
*
Day
14 42
14 42
14 42
14 42
no adjuvant
Ribi
CpG ODN
Gardiquimod

Figure 3.3. Immunization with CpG ODN and Gardiquimod increases the antibody binding strength as much as Ribi adjuvant in a chaotropic inhibition (urea wash) ELISA. A-D. representative inhibition curves over increasing concentrations of urea comparing anti-sera at two weeks past 1st immunization (d14, blue) and two weeks past 2nd immunization (d42, red) symbols show technical replicates. E. Quantification of antibody affinity by concentration of urea resulting in a 50% drop in OD. Because the affinities of antisera on day 130 were significantly higher, they were analyzed separately. Each circle represents one mouse, bars show mean and SEM; * $p<0.05$, ** $p<0.001$.

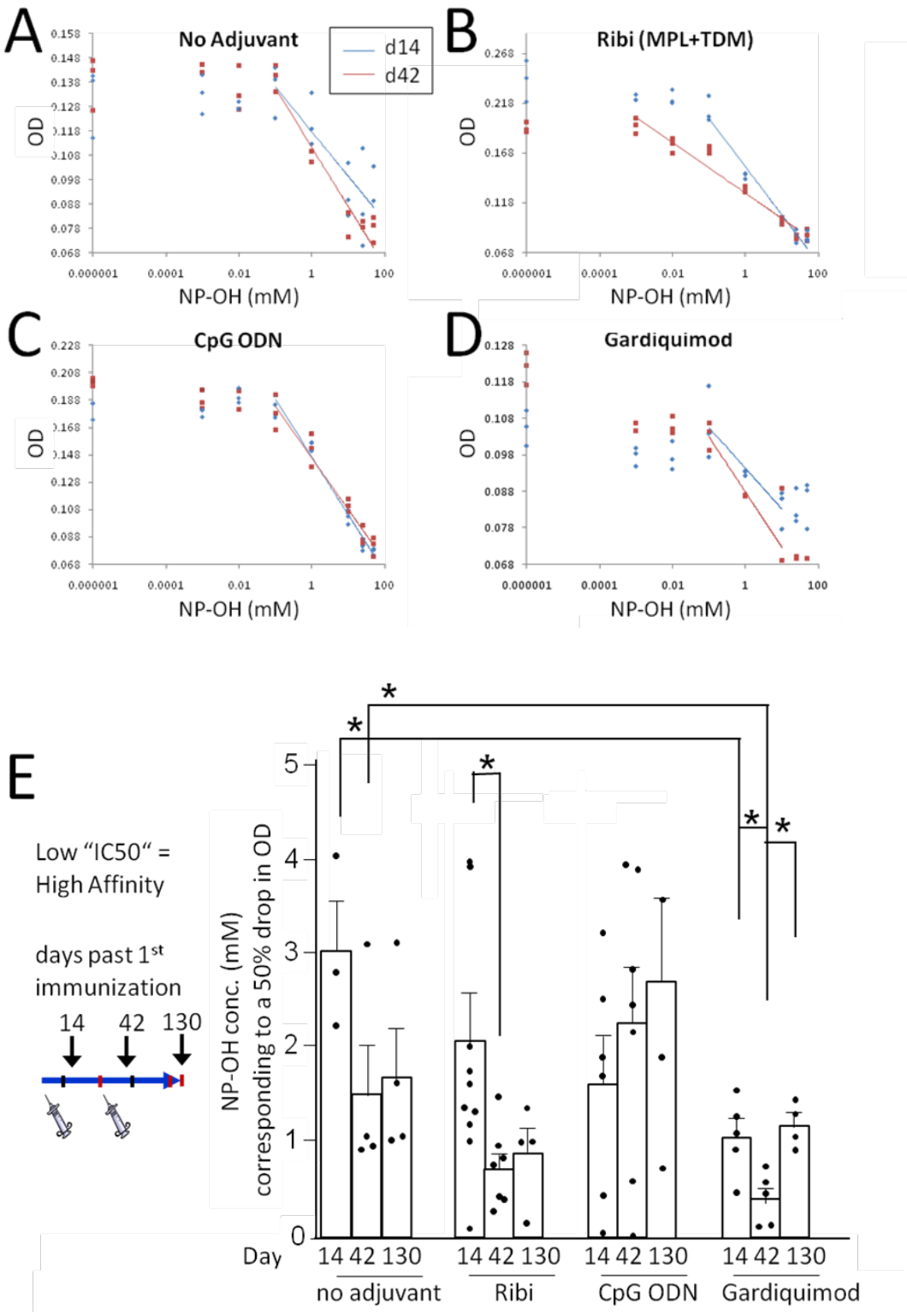

A
No Adjuvant
d14
d42
OD
NP-OH (mM)
B
Ribi (MPL+TDM)
C
CpG ODN
D
Gardiquimod
E
Low "IC50" =
High Affinity
days past 1st
immunization
14 42 130
NP-OH conc. (mM)
corresponding to a 50% drop in OD
Day
14 42 130
no adjuvant
Ribi
CpG ODN
Gardiquimod

Figure 3.4. Immunization with Gardiquimod but not CpG ODN increases the antibody affinity as much as Ribi adjuvant in a competitive ELISA with free antigen. A-D. Representative inhibition curves over increasing concentrations of NP-OH comparing sera at two weeks past 1st immunization (d14, blue) and two weeks past 2nd immunization (d42, red) symbols show technical replicates. E. Quantification of antibody affinity by concentration of NP-OH resulting in a 50% drop in OD. Each circle represents one mouse, bars show mean and SEM; * $p<0.05$.

The low titer antibodies that remained at 100 days after booster immunization (dpb) demonstrated very high affinities that were outside of the measurable range of this assay. Modifications of the assay allowed us to compare affinities among the different immunization groups at this time point, but no conditions that allowed for the simultaneous execution of all time points were found. Separate analysis of the sera at 100 dpb showed no significant difference among any of the immunization groups (not shown).

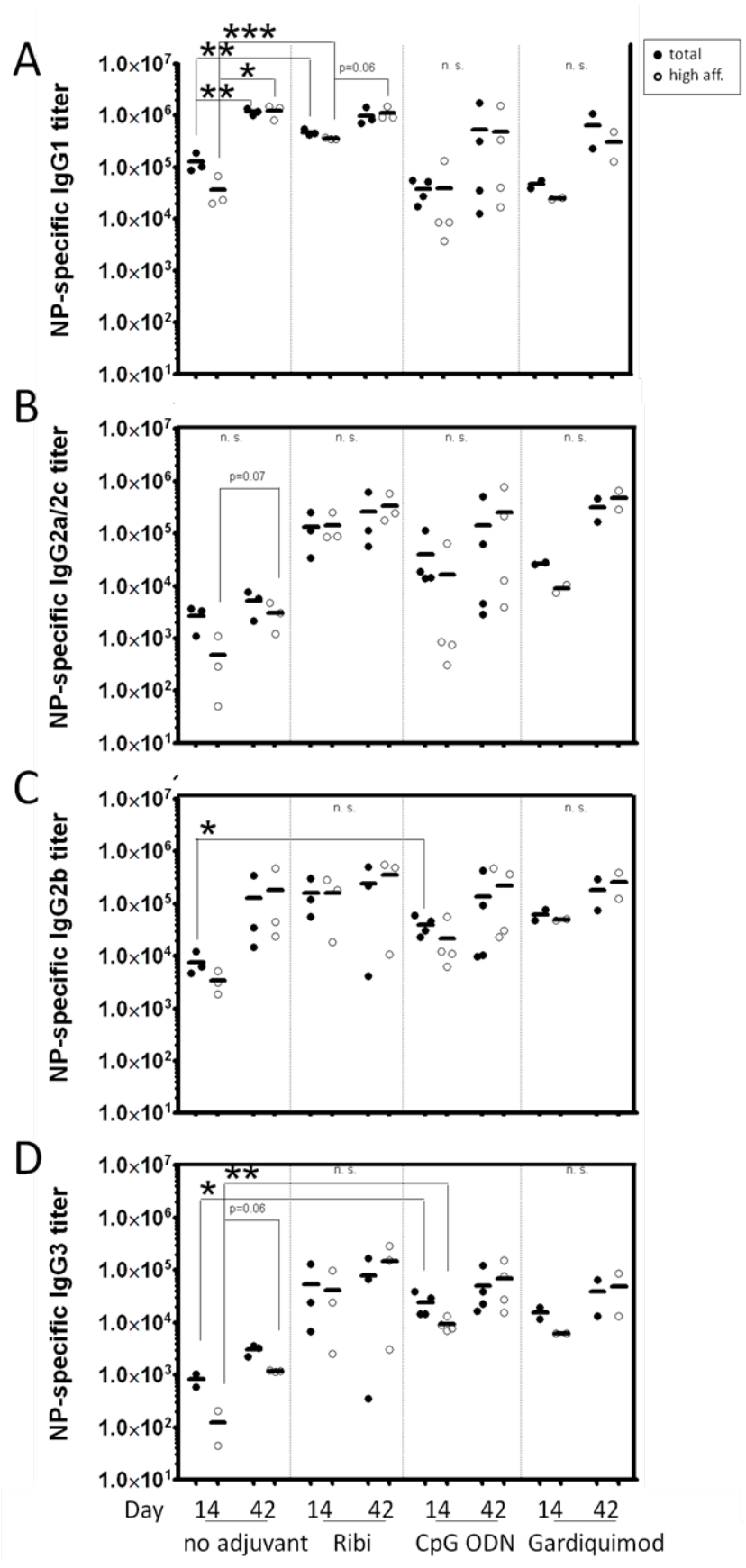
We employed a free antigen competition assay adapted from Nieto *et al*. [427] as a second method to determine the relative affinity of NP-specific serum IgG from mice immunized with different adjuvants. We used free, soluble NP-OH to compete with NP_{26}-BSA coated on the plate to bind antibodies. Anti-sera with equal titers (2.5 ELISA units) were first incubated with different concentrations of free NP-OH hapten to allow NP-OH to bind antibodies. Then the antigen-coated plates were incubated with the sera-NP-OH mixtures for 20 min. The concentration range of NP-OH used for this analysis was 100 nM to 50 mM. The amount of antibody binding to the NP_{26}-BSA coated plate was measured. Using these data, we determined the concentration of NP-OH that was required for a 50% reduction in the amount of antibodies binding to antigen coated plates. The stronger the interaction between antibodies and free antigen is, the lower the NP-OH concentration is required for the 50% reduction, thereby indicating a relative higher binding affinity of the antibody to NP. Our results show that immunization with antigen alone only slightly increased antibody affinity for NP after second immunization (Figure 3.4, *A* and *E*, $p>0.05$).

Immunization with Ribi or gardiquimod as adjuvant significantly increased the affinity of NP-specific antibodies following second immunization (Figure 3.4, *B* and *E*). Immunization using CpG ODN as adjuvant, however, showed wide range of changes in antibody affinity from individual mice (Figure 3.4, *C* and *E*). Immunization with gardiquimod as adjuvant showed the highest affinity among all four groups after both the first and second immunization (Figure 3.4, *D* and *E*). These results suggest that gardiquimod is a more effective adjuvant among three we tested in this immunization mouse model.

Effect of TLR4, 7, and 9 agonists on Ig isotype switching

While the primary antibody response is dominated by IgM, the secondary antibody response primarily consists of IgG. Antigenic stimulation and helper T cell engagement induce isotype switching of B cells. The isotype switch is also influenced by the immune environment of B cells. TLR agonists are known to promote isotype switch and to change the type of cytokine environment from Th2 to Th1 profile (reviewed by McHeyzer-Williams and McHeyzer-Williams, 2005) [13]. The Th1 cytokines like IFNγ and Th2 cytokines like IL-4 induce isotype switching to IgG_{2a} and IgG_1, respectively [66, 70]. To investigate how adjuvant impacts the type of humoral responses, we determined the IgG isotype induced by immunization with CpG ODN or gardiquimod as adjuvant. We determined titers for each of the four mouse IgG isotypes: IgG_1, IgG_{2c} (the alternate allele for IgG_{2a} expressed in C57BL/6), IgG_{2b}, and IgG_3. In addition, we evaluated the relative amount of high affinity antibody and the total amount of antigen-specific antibody in each isotype of IgG using the low (NP_4-BSA) and high (NP_{30}-BSA) density coating antigens. No matter if

mice were immunized with or without adjuvant, in general, the second immunization increased the titer of all the IgG isotypes and the portion of high affinity antibody in all the IgG isotype (Fig. 3.5, A-D). IgG_1 remained the dominant isotype in all immunized mice. Compared to the titer of mice immunized with antigen alone, all three types of adjuvant increased the titers of IgG_{2c} and IgG_3 by at least 10-fold, but did not change the titers of IgG_1 and IgG_{2b} after the second immunization (Figure 3.5, A-D). Ribi adjuvant generated higheast titers of all the IgG isotypes after the first immunization, but the second immunization with Ribi adjuvant has less impact on the IgG titers than other adjuvants (Figure 3.5, A-D). Although some of the observed differences were not statistically significant, a combination of the rise of IgG_{2c}, IgG_{2b}, and IgG_3 titers is responsible for the increased total IgG titer shown in Figure 3.2.
To investigate the impact of adjuvant on the Th1 and Th2 bias of humoral immune responses, we calculated the ratio of IgG_1:IgG_{2a} titers [428]. In absence of adjuvant, the antibody response exhibited a strong Th2 bias. Immunization with Ribi, CpG ODN or gardiquimod as adjuvants significantly dampened this Th2 bias (Figure 3.5E). These data are in agreement with previous studies that TLR agonists promote a Th1 response. However, in this mouse immunization model, TLR agonists are not sufficient to switch this Th2 dominant response to a Th1 dominant response.


A
B
C
D
NP-specific IgG1 titer
NP-specific IgG2a/2c titer
NP-specific IgG2b titer
NP-specific IgG3 titer
1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
1.0×10^3
1.0×10^2
1.0×10^1
total
high aff.
***
**
*
**
p=0.06
n. s.
p=0.07
p=0.06
Day 14 42 14 42 14 42 14 42
no adjuvant
Ribi
CpG ODN
Gardiquimod

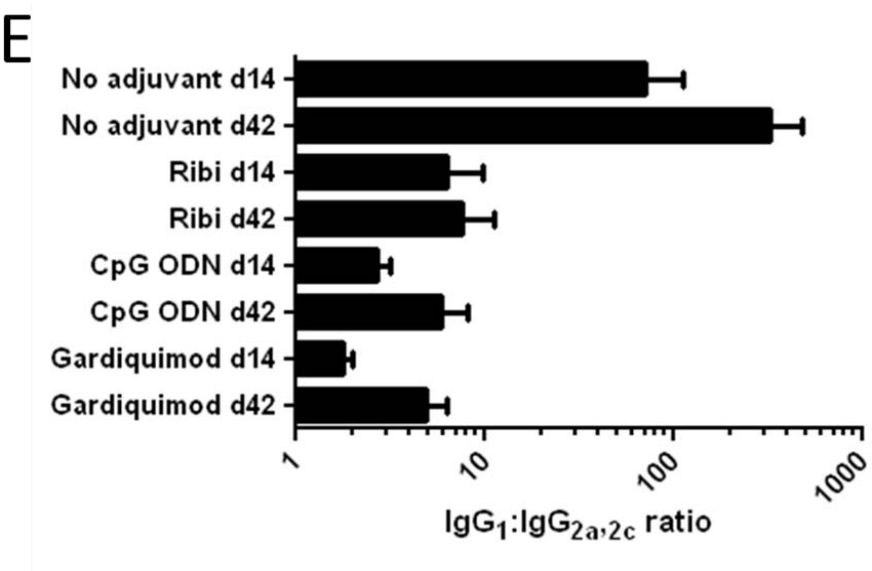


Figure 3.5. Immunization with Gardiquimod and CpG ODN increases diversity of Ig isotypes produced and lowers Th2 bias of the humoral immune response. A-D. Titers of mice immunized with NP_{19}-KLH alone or with Ribi, CpG ODN, or gardiquimod adjuvants at two weeks past 1st (14dpi) and two weeks past 2nd (42 dpi) immunization on high density antigen-coated (total, filled circles) and low density antigen coated (high affinity, open circles) plates, using isotype-specific secondary antibodies (A: IgG_1, B: IgG_{2a}+IgG_{2c}, C: IgG_{2b}, and D: IgG_3) for detection. E. Ratio of IgG_1 to $IgG_{2a/2c}$. *p<0.05, **p<0.01, ***p<0.001.

Section 4 Discussion

This study addresses whether TLR agonists could impact antibody affinity maturation and memory development. Ribi, CpG ODN, and gardiquimod used as adjuvants were each able to enhance antibody titers, which was mostly due to increased secretion of IgG_{2c} and IgG_3 in CpG ODN and gardiquimod immunized mice. Ribi adjuvant additionally enhanced the titers of IgG1 and was overall a stronger enhancer of IgG titers than CpG ODN or gardiquimod. On the other hand, affinity maturation is shown to be increased to a similar extent by all three adjuvants. In some assays measuring affinity maturation, gardiquimod and Ribi adjuvants showed greater capacity to increase affinity maturation than CpG ODN.

Our choice of specific TLR agonists to study was based on the expression of TLRs on B cells. Mouse TLRs 2, 4, 7, and 9 are expressed on FO B cells [269, 273-277], and human TLRs 7 and 9 are expressed only after B cell activation while TLR2 and 4 expression remains low or undetectable [160, 263, 271, 272, 278]. Therefore, this study focused on a TLR9 agonist, Class B CpG ODN, and a TLR7 agonist, gardiquimod. TLR2 agonists were excluded because clinical trials showed severe systemic side effects including asthenia and fever [429] and are thus of limited use in human vaccines. Most TLR4 agonists can precipitate septic shock in humans making them unsafe for vaccine adjuvants as well. However, since the roles of LPS and MPL, two TLR4 agonists, as adjuvants have been extensively studied in mice [319-326], we included it for comparison. In contrast to LPS (TLR4 agonist), TLR7 and TLR9 agonists were shown to be safe and effective in human phase 1 clinical trials

[329-333, 339, 354, 355, 357, 359-361]. Imidazoquinolines are frequently used topically near the injection site of intradermal vaccine administrations for the recruitment of antigen-presenting cells [230, 253, 270, 299, 329-335]. Gardiquimod has also been shown to be safe and effective when injected as adjuvant in anti-tumor therapeutic vaccines [334]. CpG is being tested as an adjuvant in a variety of vaccines including vaccines to prevent viral infections such as influenza [355] and hepatitis B [361], and vaccines for parasite mediated tropical diseases such as malaria [356, 357, 359, 389, 430-432] and leishmaniasis [341] and for immunotherapies including sublingual therapy for food allergens [358] and cancer immunotherapy [339]. Three out of four stage 1 clinical trials showed that CpG ODN is safe to use as human vaccine adjuvant ([357, 359, 361, 432]. Most recipients of the vaccine with class B CpG ODN had only mild side effects such as transient neutropenia [357].

Previous studies have shown that the TLR9 ligand CpG ODN and the TLR7 ligand gardiquimod are both able to enhance IgG titers [336, 342, 343, 346-349]. IgG titers in response to intraperitoneal immunizations with the T cell-dependent antigen OVA had been shown to increase 10-20 –fold with CpG ODN, compared to immunization with the same immunogen minus CpG ODN [346]. Viral particles that had been stripped of all TLR agonists and had only single-stranded RNA added back in also were able to increase IgG titers approximately 10-fold, compared to the virus-like particles alone [342]. Here, we only detect a two- three fold increase in IgG titers. While multivalent antigens, such as NP_{19}-KLH, are predicted to have high responses even in the absence of adjuvant, this trend in our data may also be attributed to a

potential adjuvant effect of oil-in-water emulsion or potential LPS contamination used as the vehicle of the antigen, and thereby generating relatively high titers in the absence of TLR agonists (Figure 3.1). Previous studies in humans showed that immunization with CpG ODN as adjuvant reduced the number of booster vaccines necessary to attain protective levels of antibodies for malaria vaccine [357] and hepatitis B vaccine [354], compared to preparations of the vaccines without CpG ODN. This study shows sustained high titers following TLR adjuvanted vaccination that was especially strong with CpG ODN (Figure 3.1), which provides an explanation for a reduced number of required boosters. Gardiquimod is a relatively new TLR7 agonist, and therefore little is known about its ability to increase antibody titers. A recent study found that gardiquimod stimulation doubled the number of *in vitro* generated plasma cells from mice [299]. Intradermal and subcutaneous injections of a *Mycobacterium tuberculosis* fusion protein in mice showed enhanced IgG_1 and IgG_{2a}/IgG_{2c} titers with the addition of gardiquimod [336]. Increased Ig titers with TLR stimulation may also partially be explained by increased ability to produce Abs in TLR stimulated plasma cells [279]. However, the effect of these TLR agonists as adjuvants on antibody affinity maturation had not been addressed. We show here that TLR7 and 9 agonists are just as effective as TLR4 agonists in promoting antibody affinity maturation (Figures 3.2-3.4), despite their relatively low ability to increase antibody titers, compared to TLR4 agonists (Figure 3.1).

We used three different methods to assess the affinity of antibodies: the ability of antibodies to bind low density antigen, the resistance of antibody-antigen interaction

to urea washes, and specific competition of antibody binding to coated antigen against free antigen. The low density antigen assay has been widely used for a rough estimate of the portion of relatively high affinity antibodies [327, 433, 434]. Using this assay, we show that antibody affinity maturation increases in all groups after second immunization (Figure 3.4). This measure of relative affinity is quickest and technically easiest to perform, but is not quantitative.

The urea wash assay can quantitatively measure antibody binding strength. Urea, a chaotropic solvent, non-specifically interferes with antibody-antigen interaction. Contrary to exposure to fully denaturing conditions, the antibody can re-fold once urea is removed which allows detection by secondary antibodies [435]. Weakly binding antibodies can be eluted at lower concentrations of urea[436, 437]. However, the concentration of urea cannot be higher than 10 M because of its solubility. Therefore, we were unable to use this assay to distinguish differences among the very high affinity antibodies.

The free antigen competition assay is also a quantitative assay to measure antibody affinity, with free antigen serving as a specific competitor for coated antigen. On and off-rate of antibody-antigen binding contributes to the affinity measurement of this assay. The higher the affinity of the antibody for the antigen is, the more time it will be interacting with the antigen, either through longer interactions (slower off-rate) or more frequent contact (higher on-rate). Therefore, high affinity antibodies require less free antigen to prevent them from binding to the antigen coated on the plates.

Because of the specific nature of the competition, it yields a true inhibitory concentration (IC50) for the antibody-antigen binding. In addition, this quantitative assay works over a wide range of antibody affinities and has very low internal variability. However, NP-specific antibodies of undetected isotypes (i.e. IgM, IgA, IgE) may compete for the free antigen, resulting in an artifactually increased IC50 (lower affinity) reading. Broad diversity of responses among mice immunized with NP_{19}-KLH + CpG ODN may indicate the induction of one of these untested isotypes in a subset of the mice, probably IgM. Differences in affinity measurements in the urea wash assay and the competitive ELISA with free antigen of the samples obtained after 100 days rest may also be a result of an untested isotype, probably IgE. As these artifacts all exclusively lower the affinity estimates of the assay, the affinity is at least as high as indicated by the free antigen competition assay.

Using these three different approaches, we were able to evaluate fully the affinity of antibody induced by immunization with different adjuvants. Our results show that both CpG ODN and gardiquimod as adjuvants increase IgG affinity maturation in mice immunized with NP_{19}-KLH. Whether these TLR agonists have a similar ability to promote antibody affinity maturation in humans remains to be determined. While the general process of antibody affinity maturation is similar in both species, human B cells do not express TLRs prior to activation [160, 271]. This may delay the direct effects of TLR agonists on B cells in the human immune response. B cells in the germinal center, where affinity maturation occurs, show enhanced expression of TLR9 [271], suggesting a possible direct role of TLR9 in B cell affinity maturation.

A study reported a mild decrease in the expression of TLR7 in human GC B cells [271], which argues against a role of TLR7 in affinity maturation process in human. However, human mB were shown to re-express TLR7 on the mRNA level [160]. Along with our finding that TLR7 stimulation induced more affinity maturation than TLR4 or TLR9 stimulation, the TLR7 agonists show protential for overcoming one of the anomalies of CpG ODN adjuvants. Human trial vaccines containing CpG ODN as adjuvant are generally effective at generating high Ab titers in North American populations [356, 361], but could not significantly boost immune responses of populations in endemic areas [431]. First contact with antigen leads to recruitment of a diverse set of antigen-specific B cells to fibroblastic conduits, which is taken as evidence for interclonal competition among B cells for antigen [86]. B cells with the highest affinity BCRs at this state were later excluded from germinal centers and instead differentiated into plasma cells extrafollicularly [403]. This interclonal competition may prevent most memory B cells from undergoing further affinity maturation upon re-exposure to antigen. In our study, gardiquimod was able to increase antibody affinity significantly after both the first and second immunization (Figure 3.3). This suggests that the block of further affinity maturation with pre-existing high affinity B cells can be overcome under some TLR adjuvanted conditions.

The mechanism by which TLR activation increases affinity maturation is unknown. The stimulation through TLRs could promote B cell proliferation, which is required for affinity maturation as well as differentiation into plasma cells [49, 64].

Lymphocyte proliferation is reduced in MyD88 and various TLR knock-out mice (ref) [231, 253, 420]. B cells are able to secrete cytokines to exert autocrine and paracrine control. B cells can secrete TNFα, IL-6, IL-10 for their own activation (reviewed by Pistoia and Corcione, 1995) [438] and sufficient amounts of IL-2, IFNγ, IL-12, and IL-4 to allow T cell polarization [14]. Barr and colleagues recently showed MyD88-dependent signals control the production of IL-6, IFNγ, and to some extent IL-10 by B cells, and that these are required by B cells for primary Th1 responses [439]. While LPS is known to increase the number of B cells that isotype switch in response to cytokines, in the absence of cytokines LPS activates B cells without inducing isotype switching [69]. Therefore it is unlikely that the TLR signaling mechanism directly impacts the activation of activation induced cytosine deaminase (AID), a critical enzyme involved in both isotype switching and somatic hypermutation [54]. However, IgG_{2a} production in mice immunized with virus-like particles and CpG ODN was completely TLR9-dependent [342]. IgG_3 was partially dependent on TLR9 as well, but IgG_1 titers were increased in the $TLR9^{-/-}$ mice [342]. And in mice immunized with OVA in complete Freund's adjuvant, IgG_{2a} production, but not IgG1 or IgE production, was found to be MyD88-dependent as well [282]. Immunization of $MyD88^{-/-}$ with LPS-conjugated OVA emulsified in incomplete Freund's adjuvant reduced the titers of IgM and IgG_1, and completely eliminated IgG_{2a}/IgG_{2c} titers. When alum adjuvant was used, antibody production in $MyD88^{-/-}$ mice was similar to wild type [288]. However, in a mouse model that eliminates both the MyD88 and the TRIF adaptors, $MyD88^{-/-}Trif^{Lps2/Lps2}$ mice, eliminating all known TLR signaling paths, there was an IgG_{2a}/IgG_{2c} response against the T cell-dependent

antigen TNP-hemocyanin elicited IgG_{2a}/IgG_{2c} titers that were as high as in responses to T cell-inedpendent antigen, despite lower pre-immune titers of IgG_{2a}/IgG_{2c} and some other subtypes [289]. This indicates that MyD88-dependent defect in signaling can be ascribed to lack of T cell help. More potential pathways of indirect activity of TLR agonists on isotype swithching to IgG_{2a}/IgG_{2c} and affinity maturation includes TLR activation of follicular dendritic cells (fDCs). Mice treated with TLR4 blocking antibodies decrased the number of germinal centers, the number of B cells with a mutated Ig sequence, and lower high-affinity antiserum titers in mice immunized with OVA and LPS [327]. This suggests that TLR4 agonists play a role in germinal center formation. This study further shows that chimeric mice with $TLR4^-$ fDCs display a similar defect, as well as causing reduced expression of AID in B cells [327]. In addition, DCs are known to be activated by TLR agonists, such as CpG ODN, leading to stimulation of Th1 maturation in T cells [440-443]. T cells can then activate B cells via CD154-CD40 interactions. In humans, mutations in either CD40 or CD40L cause hyper-IgM syndrome 1 (HIGM1) [71, 72], a disease associated with lack of isotype switching and poor immunological memory.

Our results show that both CpG ODN and gardiquimod increase Th1 antibody responses even though they were used as adjuvant with a Th2 antigen. Previous studies had shown similar antibody responses agains CpG ODN adjuvanted OVA, virus-like particles, Hepatitis B, and *Mycobacteria* vaccines [336, 342, 346, 349]. In addition to adjuvant, the immunization routes have been shown to impact the Th1/Th2 polarity of antibody responses. For example, Baldwin and colleagues

showed gardiquimod was able to dampen the strong Th2 bias of *Mycobacterium tuberculosis* fusion protein ID83 toward Th1 in intradermal vaccine. However, subcutaneous vaccine with the same concentration of ID83 and gardiquimod maintained a similar strong Th2 bias as un-adjuvanted response. CpG ODN adjuvant in intradermal vaccine completely changed the ID83 bias to a strong Th1 response, while in subcutaneous injection it only weakly induced Th1 responses [336]. The ability of TLRs to promote Th1 antibody responses is mediated through induction of Th1 cytokine in marophages and dendritic cells and of Th1 T cell differentiation [231, 348, 420, 444, 445]. Leaning towards Th1 responses could make vaccines more effective against intracellular pathogens, such as influenza or HIV [14, 428].

Chapter 4: General Discussion and Future Directions

B cell activation and affinity maturation are critical events in the generation of immunological memory following vaccination as well as in the development of auto-immune diseases. This thesis asked how TLR stimulation affects B cell activation and affinity maturation to generate antibody responses in mice.

Currently, there are two ways for TLR to contribute to B cell activation, indirect and direct activation, which could co-exist. In the widely accepted indirect activation model, TLR agonists are thought to stimulate maturation of DCs and thereby improve the availability of T cell help and cytokines to amplify primary B cell activation (reviewed by Banchereau and Steinman 1998; Lee and Iwasaki 2007, Akira et al 2006, Reis e Sousa 2006) [270, 378, 381, 446]. TLR-dependent maturation of DCs also affects the cytokine profile of the immune response, with wide-ranging effects on the activation of lymphocytes. In contrast, models of direct B cell activation through TLR signaling are less well established. They suggest independent signaling cascades for BCR and TLR dependent activation of transcription factors associated with proliferation and differentiation into plasma cells. Activating B cells through LPS *in vitro* can increase proliferation and survival of B cells [322, 324, 325] and underline positive effects transmitted through T cell help in activation of B cells to a T cell dependent antigen (reviewed by Ruprecht and Lanzavecchia, 2006) [287]. When *in vitro* LPS-stimulated B cells were adoptively transferred into mice, they preferentially migrated to GC dark zones where they also proliferated and enhanced

ongoing immune responses [323]. Our finding that memory B cells differentiate into high affinity IgG secreting cells *ex vivo* with CpG ODN or LPS stimulation in absence of antigen stimulation or B cell receptor cross-linking support the existence of a B-cell intrinsic TLR activation pathway (Chapter 2).

When TLR agonists are used as vaccine adjuvants, the kinetics of the secondary immune response were altered. All TLR agonists appeared to slow the release of IgG in secondary responses, seen in lower NP-specific IgG titers in response to adjuvant-containing immunogen compared to response to antigen immunization alone by 5 days post-boost (Chapter 2) in parallel to increasing the frequency of plasma cells. The difference in the timing of plasma cell emergence from the spleen and IgG secretion should result in fewer IgG-containing immune complexes to be formed. We would predict that these kinetic differences protect the newly differentiated plasma cells from Fc receptor cross-linking and the associated apoptosis [447].

We also find that there is a difference in the extent of B cell activation depending on which TLR is engaged (Chapters 2 and 3), with TLR9 stimulation allowing maximal *in vitro* differentiation into antibody secreting cells from memory B cells, but only moderate effect on IgM-expressing B cells. Thus, there is a bias toward memory B cell activation (memory bias) by CpG ODNs *in vitro* (Chapter 2). However, it is unclear whether CpG ODN can stimulate affinity maturation due to differences in the results between nonspecific inhibition and specific competition assays (Chapter 3). Analysis of antisera for induction of other immunoglobulin isotypes may allow us to

explain the difference. TLR9 stimulation also appears to have a strong temporal elongation of primary immune responses (Chapter 3). Mice immunized with CpG ODN as adjuvant had significantly higher NP-specific IgG titers than mice immunized with Ribi as adjuvant (highest titers at all other time points) at the 4th week after immunization. At this time point the newly generated memory B cells should have emerged from the germinal centers [86]. A similar extension of responses was observed in one of the clinical trials with CpG ODN as adjuvant [357]. CpG ODN allowed the gradual increase of protective titers, and thus required fewer booster vaccines. To verify that this phenomenon uniquely affects post-germinal center B cells, IgM titers should be compared in mice immunized with CpG ODN or Ribi as adjuvant. The underlying mechanism of the memory bias by CpG ODNs is unknown, but a more focused study on both of these effects will reveal the full duration and final outcomes. In our hands, the TLR7 ligand gardiquimod induced the most affinity maturation of the three TLR agonists tested (Chapter 3).

Here, we found that memory B cells down regulate TLR4, but not TLR9 expression, which can contribute to this memory bias of CpG ODN (Chapter 2). The additional explanation may lay on differences in the activation mechanism of TLRs. TLR4, TLR7, and TLR9 use MyD88 signaling pathway. TLR4 is also able to associate with the TRIF adaptor, which may allow different contribution to the B activation (reviewed by Akira, *et al*, 2006) [270]. TRIF recruitment to TLR4 only occurs after TLR4 internalization. Whether TLR4 is internalized in B cells and whether TLR4 is internalized at a same rate in naïve and memory subsets of B cells are unclear.

In human B cells, TLR stimulation has been implicated for polyclonal activation of memory B cells to maintain memory B cell and long-lived plasma cell populations. Bernasconi and colleagues noticed a subtle increase in circulating plasma cells secreting IgG antibodies with other specificities during the response to tetanus booster vaccines in human volunteers [112]. It was unclear whether these plasma cells were newly generated from non-specific memory B cell turnover, or were older long-lived plasma cells displaced from the bone marrow (reviewed by Radbruch, 2006) [134]. We tested for activation of memory B cells in mice by administering TLR4 and TLR9 agonists in the absence of antigen and were unable to see an immune response (Chapter 2), indicating that TLR stimulation is not sufficient for memory B cell activation in this model. While TLR stimulation alone was unable to activate memory B cells polyclonally to generate secondary immune responses *in vivo*, they did enhance antibody affinity maturation, particularly after the booster vaccination, compared with immunization with antigen alone (Chapter 3). These data indicate that TLR stimulation may play a role in the generation of memory B cells. Current paradigms about the contribution of TLR stimulation to B cell affinity maturation hold that TLR stimulation leads to a stronger germinal center (GC) response. Stimulation with LPS had been shown to activate innate immune cells to establish new B cell follicles and GCs in lymph nodes [321]. Recently, TLR4 stimulation has also been shown to enhance maturation of follicular dendritic cell (fDC) networks [327]. Since fDCs are essential to the establishment of germinal centers as well as selection of high affinity B cell clones (reviewed by Muller and

Germain, 2009; Carter and Myers, 2008) [11, 448], these findings also support an indirect role for TLRs to support B cell affinity maturation. TLR4 stimulation and other signals allow the maturation of fDCs in locations outside of secondary lymphoid oragans, allowing the formation of tertiary lymphoid tissues that can form germinal centers and support B cell affinity maturation [11]. TLR4 deficiency in fDCs results in fewer and smaller GCs as well as fewer mutations in B cells and lower high-affinity titers [327]. A small but significant indirect effect on B cell expression of AID was also described [327]. Furthermore, LPS stimulation of B cells triggered class-switching *in vitro* but was insufficient for somatic hyper-mutation [54]. MyD88-/- mice are significantly slower at isotype switching [282, 449]. Gargano and colleagues showed that in $MyD88^{-/-}$ mice only few B cells were able to enter germinal centers [449], so one possible mechanism for TLRs to enhance antibody affinity maturation further may be allowing naïve and memory B cells entry into germinal centers. However, a recent study suggests that MyD88 is also involved in innate stimuli of B cells via BAFF and APRIL signaling pathways to induce isotype class switching, independent of TLR stimulation of B cells as MyD88 directly binds to the BAFF/APRIL receptor TACI [450]. Therefore, it is possible that TLR stimulation leads to signaling cross-talk downstream of TACI. This hypothesis is further supported by a previous study showing auto-antibody production depends on MyD88 signaling in a BAFF transgenic mouse model [451]. On the other hand, it is likely that previous studies using $MyD88^{-/-}$ to study the effects of TLR stimulation on B cells also blocked the TACI pathway in B cells. This may account for the few differences between $MyD88^{-/-}$ and the specific $TLR^{-/-}$ in response to its agonist

stimulation. Furthermore, maximum IgG titers in MyD88$^{-/-}$ mice also were shown to be similar to those of WT mice, although with delayed kinetics [449], which argues against a role of TLR in B cell affinity maturation. Thus, the roles of TLRs on B cell affinity maturation remain poorly understood. This study demonstrates that TLR4, 7, and 9 stimulations in the presence of antigen were able to increase antibody affinity following second immunization (Chapter 3). This is the first study on the effects of TLR agonists other than LPS on antibody affinity. Our study also confirmed previous reports that CpG and gardiquimod increased isotype switching *in vivo* [301, 351, 361, 452] (Chapter 3).

TLR activation of B cells has been implicated in the activation of autoreactive B cells and production of autoantibodies that leads to autoimmune diseases in mice and humans (reviewed by Marshak-Rothstein, 2006; Krieg and Vollmer, 2007; Lanzavecchia and Sallusto, 2007; Deane and Bolland, 2006) [286, 379, 380, 382]. How TLRs contribute to the generation and activation of autoreactive B cells is unknown. TLRs could stimulate chemokine production in stromal cells and lymphocytes, which enhance the formation of tertiary lymphoid tissues that further amplify immune and autoimmune responses [453-456]. Polyclonal activation capability of TLRs could break tolerance of auto-reactive B cells [457, 458]. On the other hand, some pathogens induce a form of tolerance [356, 389, 430, 459]. Therefore, understanding the mechanisms by which TLRs directly and indirectly activate B cells is important for the application of TLR targeted adjuvant for vaccines and TLR targeted therapies for autoimmune diseases.

Section 1 Future Directions

Some of the important questions that remain are (1.) to establish the molecular basis for differences in *in vitro* stimulation with different TLR agonists, (2.) to establish molecular mechanism for the intrinsically higher responsiveness to *in vitro* TLR stimulation by memory B cells compared to unswitched B cells (3.) to establish the molecular mechanisms of TLR-induced enhancement of B cell affinity maturation, and (4.) to test if the enhanced affinity allows more memory B cells and long-lived plasma cells to be maintained and enhance protection in antigen challenge in mice immunized with TLR-based adjuvants.

To understand the molecular basis for the difference between TLR4 and TLR9 activation of B cells, we should first test whether the greater enhancement of Ribi adjuvant to antibody titers is TRIF-dependent. TRIF-/- mice will be used to carry out the immunization study to determine whether TRIF deficiency will abolish the difference in antibody responses induced by TLR4 and TLR9 agonists as adjuvant.

To determine whether TLR9 in mB and naïve B cells mediates signaling through similar mechanism, we will analyze the cellular distribution of TLR9. In naïve B cells, TLR9 stimulation alone typically results in MyD88 re-localization to late endosomes [206]. However, when the BCR is stimulated either alone or together with CpG ODN on naïve B cells, the TLR9 is recruited to a large multi-lammellar

autophagosome-like compartment. We can compare the subcellular distribution of TLR9 in mB and naïve B cells in the presence of TLR9 and/or BCR ligands.

Data from chapter 3 indicate a possibility that immunization with adjuvant altered the kinetics of antibody secretion in both the primary and secondary anti-NP immune responses. Previous reports suggest that the kinetics of the primary anti-NP immune response should be completed by the fourth week (GC forming within 1 week of exposure and dissolving approximately 2 weeks later), with peak antibody secretion of the primary immune response at two weeks past immunization, while secondary responses show higher IgG titers without the lag period for germinal center formation [41, 86, 98, 404-406]. In our hands, the responses to TD antigen did not follow the model exactly. Notably antibody titers failed to decline between the 2^{nd} and 4^{th} week after primay immunization, and we observed a continuous increase in IgG titers between the 2^{nd} and 4^{th} week in most mice that were immunized with an adjuvant. And while secondary immune responses are reported to retain high titers for at least six weeks after immunization, we observed that IgG titers already decline between 2 and 4 weeks after the second immunization. However, by 100 days after immunization, the latest time point in this study, IgG titers were still almost as high as two weeks into the primary immune response (Chapter 3). Some changes to this preliminary study should be undertaken for studying the effect of TLRs on kinetics of humoral responses. In order to simulate reported kinetics more closely in the no adjuvant control, a lower dose of antigen should be administered. In the literature, concentration of NP antigen conjugated to protein carriers for successful

immunization ranges from 10 - 400 μg/mouse [28, 86, 397-400, 402, 460, 461]. In order to evaluate the extension of responses by adjuvants fully, delivery of the second injection will be postponed until the primary antibody levels have declined. This may also result in a greater boosting effect. To consider whether TLR agonists actually reach memory B cells *in vivo*, this experiment will be carried out in two sets of mice receiving the immunogen either by the intraperitoneal route, as before, or by intravenous route to increase likelihood of lymphocyte exposure to the agonists.

To address the question of B cell-intrinsic differences in the activation of memory and follicular B cells that enable memory B cells to produce a more robust response, we propose to follow expression level and phosphorylation kinetics of B cell activation-related molecules. Our first goal is to determine the activation threshold for memory B cells. For this experiment CFSE-labeled B cells will be incubated in serial dilutions of antigen and suboptimal concentrations of TLR agonists, and the ability to proliferate and differentiate into plasma cells will be analyzed.

Second, we would like to assess differences in the activation kinetics of naïve and memory B cells. Our preliminary data show diminished expression of CD21, complement receptor 2, on memory B cells compared to naïve follicular B cells (see appendix). We will therefore focus on known CD21 interaction partners. CD21 is a component in the B cell co-receptor complex that signals through CD19. The expression level of CD19 is similar in memory and naïve B cells. We will compare the distribution of CD19 relative to the BCR by microscopy and follow CD19

intracellular tail phosphorylation kinetics by flow cytometry. Phosphorylated CD19 recruits PI3K, and we will also assess kinetics of local changes in the PI3K product $PI(3,4,5)P_3$ (PIP_3) as well as kinetics of signal molecules downstream of the BCR and calcium responses to TLR and antigen stimulation. CD21 is also known to interact with the low affinity IgE receptor, CD23, which has been shown to regulate IgE and IgG_1 responses [462-464]. Preliminary data show that in mouse mB the CD23 expression level is also reduced. CD23 signaling uses the transcription factor Notch, and we will follow the nuclear translocation of the active Notch intracellular domain following TLR and antigen stimulation by microscopy.

To test whether TLR-based adjuvants affect the generation of memory B cells or their maintenance, we plan to quantify the frequency of antigen-specific memory B cells four weeks after each immunization. To follow up on the alternate kinetics of humoral responses, we will track anti-sera of all Ig isotypes following one single immunization with CpG ODN, gardiquimod, Ribi, and alum as adjuvant or PBS weekly until a significant decline in NP-specific titers is evident. After this, the booster immunization can be administered, and we will follow the secondary immune response, paying special attention to further isotype switching, such as to IgE. In mice the sequence of Ig heavy chain gene segments is IgM/D – IgG3 – IgG1 – IgG2b – IgG2a/2c – IgE – IgA. Preliminary data are shown in the appendix.

Appendices

Different co-receptor expression profile on memory and follicular B cells

Introduction

The rapid and robust secondary antibody response suggests a lower signaling threshold and faster kinetics of mB activation. However, the signaling does not happen through the BCR alone. An extensive network of both positive and negative co-regulators modulates BCR signaling. We here focus on CD19 (B cell co-receptor), CD21 (complement receptor 2), CD23 (Fc epsilon receptor II).

CD19 is a major co-stimulatory molecule to the BCR. Its expression is unique to B cells and it serves as a marker for these from early B cell development (CD19 expression commences in the pre-B cell stage after DJ rearrangement of the Ig heavy chains has occurred, and is triggered by IL-7 *in vitro*.) up to plasma cell differentiation in humans and mice. CD19 can be expressed in the membrane as an individual transmembrane protein, but in mature B cells CD19 usually forms signaling complexes with complement receptor 2 (CR2; CD21), and with tetraspan family of transmembrane proteins member CD81 (reviewed by Tedder *et al*, 1997) [223, 465]. When coligated with the BCR, CD19 enhances proliferation induced by high affinity-BCR interactions with its antigen and to a lesser extent enables the proliferation after low affinity-BCR interactions with its antigen [223]. CD19/CD21 interaction with the BCR extends the transient BCR association with the sphingolipid-rich and cholesterol-rich membrane domains that are also called lipid

rafts [466]. CD19 also serves as adaptor recruiting several signaling molecules. CD19 is phosphorylated, and thereby activated, by Lyn. Phosphorylated CD19 provides a binding site for PI3K [467]. PI3K, in turn, phosphorylates PIP_2 to produce the second messenger PIP_3. PIP_3 recruits Btk to the plasma membrane, which leads to Btk activation. It has also been observed that CD19 co-ligation with BCR increases the percentage of the surface BCR in lipid rafts and retains the BCR there longer than what is seen in antigenic activation [186, 466] (reviewed by Cheng *et al*. [468] and by Pierce [469]). Thus CD19 functions in both phosphorylation signaling and in clustering of the BCR in FO B cells. While CD19 over-expression results in 2-3 fold threshold reduction of BCR signaling, CD19 co-ligation with BCR results in a 1000-10,000-fold threshold reduction for antigen stimulation [223, 470, 471].

CD21 is complement receptor 2 (CR2). CD21 is expressed predominantly on B cells [472]. FDCs express a slightly longer isoform of CD21 that has one more of the consensus repeats [472]. Some activated T cells and epithelial cells also express the short isoform of CD21 [473]. CD21 has several known ligands including C3 fragments [474-476], IFNα, and Epstein-Barr Virus [477]. In the absence of coligation with the BCR, CD21 can also associate with CD35, the complement receptor 1 (CR1) that aids in the degradation of C3b to make the C3d substrate for the CD21 receptor [223]. Interestingly, in human, CD21 also binds CD23 (IgE receptor).

C3-depletion by cobra venom factor inhibits humoral immune responses [478], pointing to involvement of CR1 and CR2 in B cell responses. *Cr2*-/- mice have

inhibited humoral response to challenges with T cell-dependent and T cell-independent antigens [479, 480]. These mice also have low levels of natural antibody and are sensitive to infections. The germinal centers in these mice are few and underdeveloped, probably due to apoptosis of germinal center B cells (reviewed by Carroll, 2004) [21]. Uptake of immune complexes by FDCs is inhibited (CD21/CD35 is involved in transferring ICs from the MZ B cells to the FDCs) [481-484]. Defects in the germinal center appear to be dominated by missing complement receptors in the B cells, because chimeric mice with WT bone marrow and B cells in a *Cr2*-/- background (FDCs are KO) appear to be fine, except for long-term maintainance of antibody and memory B cells [479, 485] (The antigen requirement for maintainance of memory B cells is controversial.) Most recent evidence suggests that the symptoms of *Cr2*-/- mice are effects of increased inflammation in the spleen caused by too much C3a release as CD21 limits complement convertase activity, and depletion of C3 restores IgG_3 production and gene expression patterns the *Cr2*-/- mice [486].

Bachmann and collegues observed that IgG responses were more affected than early IgM responses by the absence of CD21 in $Cr2^{-/-}$ mice; a higher epitope density was required for producing robust IgG titers [487], indicating that the CD19/CD21/CD81 complex may play a larger role in mB than in nB. CD19 deficiency hinders memory B-cell development [488]. In CD21-deficient mice, mB fail to differentiate into plasma cells, while primary antibody responses and development of mB are unaffected [489]. In addition, CD19 over-expression in mB, but not in nB, has been

found in some patients with autoimmune diseases [490]. It is therefore reasonable to hypothesize that the CD19/CD21/CD81 complex plays an important role in mB activation.

CD23 (FcεRII) is a C-type lectin. CD23 is a low affinity receptor for IgE antibodies. On B cells, CD23 interaction with IgE containing immune complexes negatively regulates B cell activation, thus limiting the synthesis of new IgE [462-464]. Curiously, over-expression of CD23 in mice also significantly decreased IgG_1 secretion [463, 464]. That the transcription of IgG_1 and IgE sites of the Ig gene may have similar regulation is not completely unexpected: Mouse B cells stimulated with IL-4 undergo class-switching to both IgG1 and IgE [66]. CD23 signaling pathways are largely unknown, but the same metalloproteinase that cleaves CD23, ADAM10, also activates the Notch1 signaling pathway [491, 492]. Notch2 has also recently been shown to play a role in allowing CD23 to be cleaved *in vivo* [493]. Notch family proteins are a unique class of signal regulators, acting both as membrane sensors and transcription factors. After cleavage, the intracellular domain (NICD) directly translocates to the nucleus, without amplification through any signaling cascades (reviewed by Fürthauer and Gonzalez-Gaitán, 2009) [494].

One other possibility of how CD23 regulates B cell signaling is through interacting with CD21 to recruit CD21 away from CD19 co-receptor, making CD21 less available for immune complex mediated cross-linking of BCR and CD19 [495]. CD23 has been shown to interact with human B cells via the CD21 receptor [496,

497]. Whether this binding recruits CD21 away from CD19 and its role in the co-receptor complex is unknown. It is also uncertain whether CD23 and CD21 interaction is limited to cell-cell contacts such as between a mast cell and a B cell, or whether they are able to assume a configuration to interact with each other on the same cell. In addition to receptor internalization, CD23 can be cleaved by metalloproteinases resulting in CD23 shedding from cell surfaces [491]. The interaction between CD23 and CD21 is strong enough that after the shedding of CD23 *in vitro*, the extracellular domain is often found in association with CD21 in the cell culture media and serum [498, 499]. In cells that shed CD23, the synthesis of CD23 is usually thought to be up-regulated to compensate for the loss and maintain membrane homeostasis of CD23. CD23 and CD21 are frequently co-expressed owing to similar regulatory elements in their respective genes [500-502]. To date, nothing is known about CD23 on mouse memory B cells.

Materials and Methods

B cell enrichment

Mice were immunized twice, 28 days apart, with NP_{19}-KLH + Ribi adjuvant. B cells were isolated from the spleen and bone marrow of mice at 5 days (activated) or 6 weeks (resting) after second immunization. Spleens were mechanically ground to create single cell suspensions in the complete medium (RPMI 1640 with 2 mM glutamine (Gibco/Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS), 55 μM β-Mercaptoethanol, 100 μM non-essential amino acids, 1 mM sodium pyruvate, and penicillin/ streptomycin, 10% CM). Bone marrow was resuspended in 10% CM. Red

blood cells were lysed with ACK lysis buffer (Lonza, Basel, Switzerland). After washing, T cells were removed using complement-mediated T cell lysis by targeting the T cells with anti-CD90.2 (Thy1.2) antibody (BD Biosciences, San Diego, CA) in 10% guinea pig complement (Rockland Immunochemicals, Gilbertsville, PA) in Hank's buffered salts solution, pH 7.4 (HBSS) (Sigma-Aldrich, St. Louis, MO) at 37^{o}C for 30 min. Remaining complement was washed out with HBSS. Adherent cells were removed by panning in 10% CM at 37^{o}C, 5% CO_2 for 1 h.

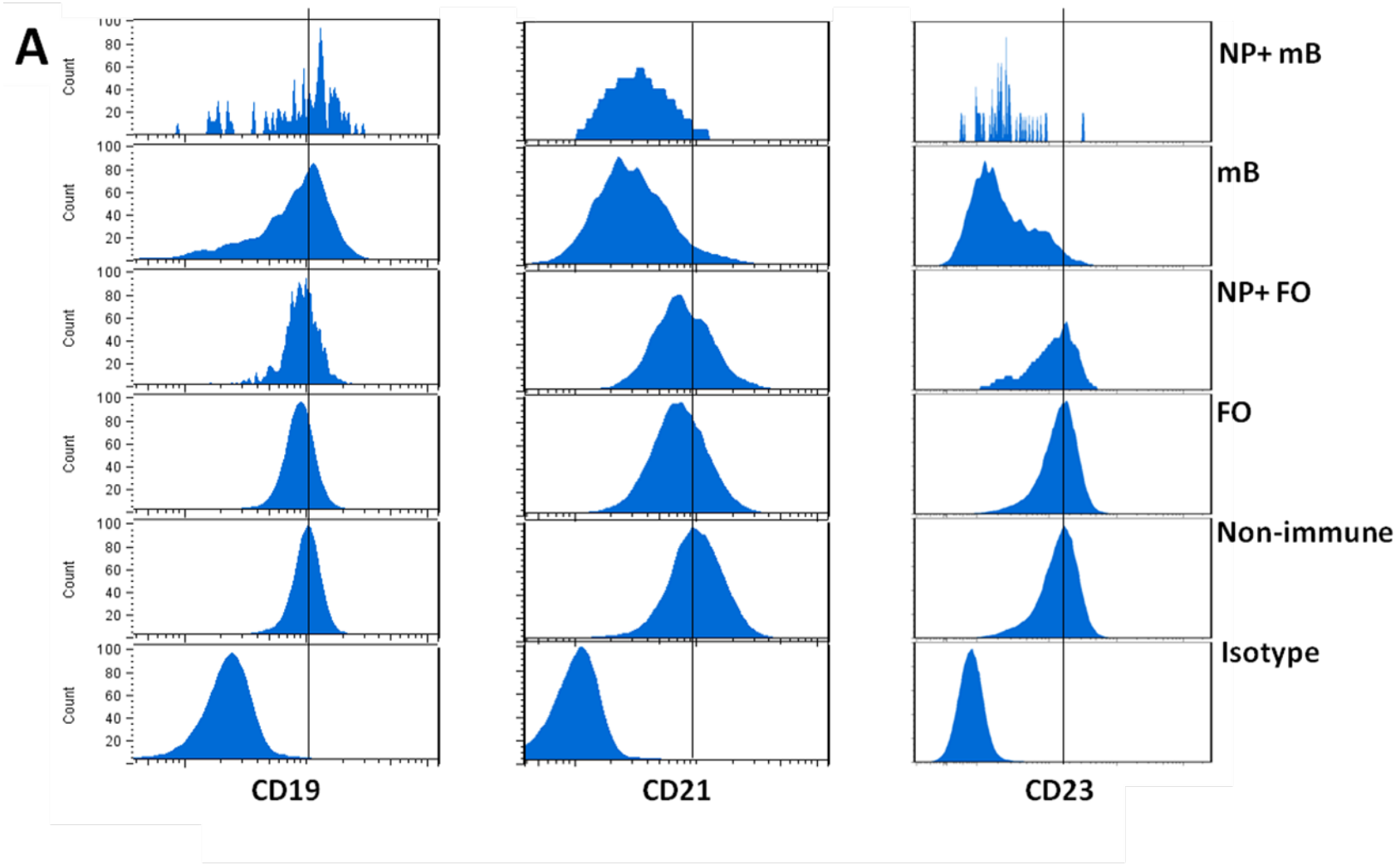
Flow cytometry analysis

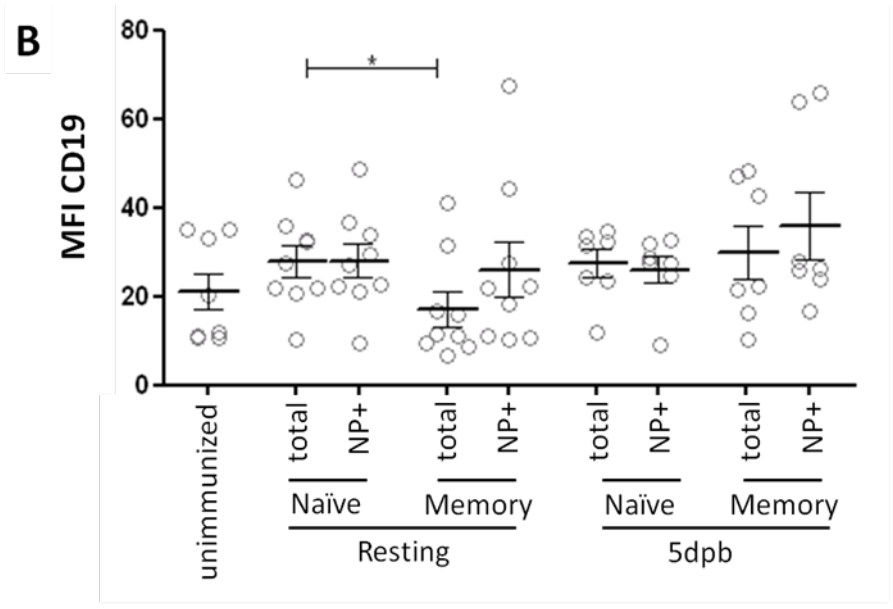
The protein expression level of CD19, CD21, and CD23 on memory B cells in spleen was analyzed by flow cytometric analysis. All labeling steps were performed on ice. B cells were pre-incubated with anti-mouse CD16/CD32 (BD Biosciences, San Diego, CA) in PBS containing 2% FBS for 10 min to block FcγR from binding to antibody, and labeled for 30 min with the following fluorescently conjugated antibodies and reagents: PerCP-Cy5.5-anti-B220, PE-anti-CD138, PE-Cy7-anti-IgM (all from BD Biosciences, San Diego, CA), biotin-anti-IgD (Southern Biotech, Birmingham, AL), and APC-NP_{12} for the identification of naïve follicular and memory B cell subsets, and either FITC-anti-CD19, FITC-anti-CD21, or PE-anti-CD21 and FITC-anti-CD23 (all from BD Biosciences, San Diego, CA) for surface expression of these receptors. APC-NP was generated by conjugating NP-OSu (Biosearch Technologies, Novato, CA) to APC. After washing three times with 2% FBS/PBS, cells were incubated with AF430-conjugated streptavidin for 20 min. Stained cells were washed three times with 2% FBS/PBS and once in PBS before

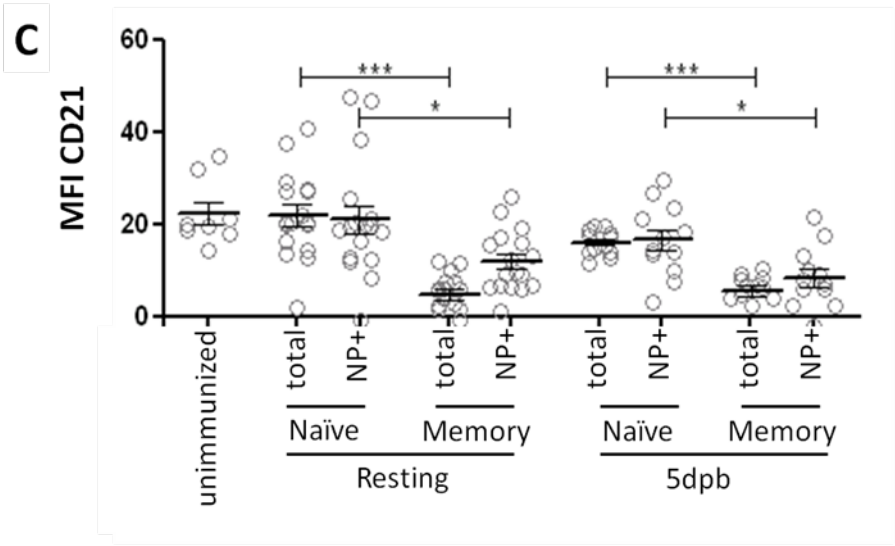
fixing with 2% paraformaldehyde in PBS for 10 min, followed by an additional wash with PBS prior to be analyzed on a flow cytometer (BD Canto II, BD Biosciences, San Jose, CA). Data analysis was performed using FACSDiva (BD Biosciences, San Jose, CA) and FlowJo (Treestar Inc., Ashland, OR) software. The frequency of memory B cells, defined as isotype-switched antigen-binding B cells ($B220^{+}CD138^{-}IgM^{-}IgD^{-}NP^{+}$) [109, 110], was determined.

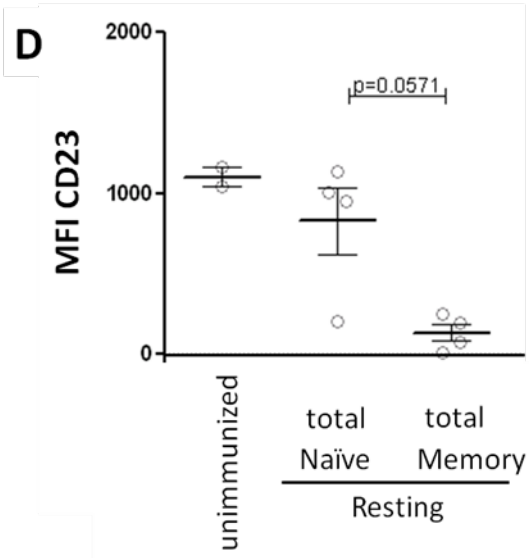
Results

To explore the underlying mechanism for more robust activation of memory B cells compared to activation of naïve B cells, we tested the expression profile of memory B cells of activation co-receptors. B cell-enriched splenocytes were stained for the identification of memory and naïve B cell subsets as well as antibodies against CD19, CD21 (complement receptor 2; CR2), and CD23 (FcεRII). CD19 levels were similar in all of the mature B cell subsets (Figure A.1, A, B). Memory B cells expressed significantly lower levels of CD21 than follicular B cells (Figure A.1, A, C). CD23 surface expression level also was lower in memory B cells (Figure A.1, A, D). Cells with the lowest expression of CD21 were also dimmest for CD23 (Figure A.1 E). Similar trends were observed in follicular and memory B cell subsets isolated from peripheral blood (not shown).


A
Count
100
80
60
40
20
CD19
CD21
CD23
NP+ mB
mB
NP+ FO
FO
Non-immune
Isotype


B
MFI CD19
80
60
40
20
0
*
unimmunized
total
NP+
total
NP+
total
NP+
total
NP+
Naïve
Memory
Naïve
Memory
Resting
5dpb


C
MFI CD21
60
40
20
0
***
*
***
*
unimmunized
total
NP+
total
NP+
total
NP+
total
NP+
Naïve
Memory
Naïve
Memory
Resting
5dpb


D
MFI CD23
2000
1000
0
p=0.0571
unimmunized
total
Naïve
total
Memory
Resting

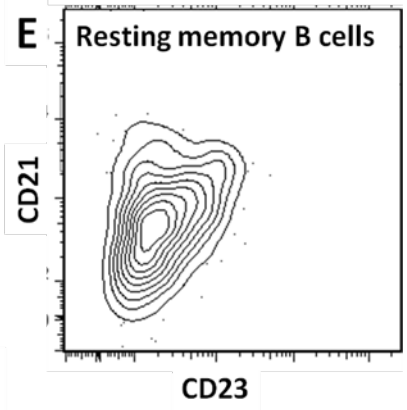

E
Resting memory B cells
CD21
CD23

Figure A.1. The expression levels of CD21 and CD23, but not CD19, are reduced in memory B cells compared to isotype unswitched B cells. A. Expression profile of indicated B cell subsets from a representative mouse 5dpb. The following subsets were identified by gating on surface markers: NP-binding memory B cells (NP+ mB) $B220^{+}$ $CD138^{-}$ IgM^{-} IgD^{-} NP^{+}, memory B cells (mB) $B220^{+}$ $CD138^{-}$ IgM^{-} IgD^{-} $NP^{+/-}$, NP-binding unswitched B cells (NP+ FO) $B220^{+}$ $CD138^{-}$ IgM^{int} IgD^{+} NP^{+}, naïve follicular B cells (FO) $B220^{+}$ $CD138^{-}$ IgM^{int} IgD^{+} NP^{-}. B-D. Quantification of CD19 (B), CD21 (C), and CD23 (D) expression levels. Each circle represents one mouse, bars show mean and SEM. (E) 2-dimensional histogram of CD21 and CD23 fluorescence intensity shows partial correlation of CD21 and CD23 down-regulation. This graph was gated on resting isotype-switched B cells (6 weeks past second immunization, gated on $B220^{+}$ $CD138^{-}$ IgM^{-} IgD^{-}).

Long-term memory induced by TLR4, 7, and 9 agonists

Introduction

Long-term humoral immunity is maintained by two cell types, memory B cells and long-lived plasma cells. While long-lived plasma cells continually secrete protective antibodies, memory B cells are resting until re-exposure to antigen prompts their differentiation into new plasma cells. We had previously tested the ability of different TLR agonists to induce affinity maturation, an essential step in memory development (Chapter 3). We then asked whether TLR agonists as adjuvants can influence the maintainance of memory B cells and long-lived plasma cells.

Materials and Methods

Mice were immunized twice, 28 days apart, with NP_{19}-KLH alone or with Ribi, gardiquimod, or CpG ODN as adjuvant. B cells were isolated from the spleen and bone marrow of mice at 100 days after second immunization and enriched as described above. The frequency of antigen-specific memory B cells in spleen and bone marrow B cells was analyzed by flow cytometric analysis as described above.

ELISpot analysis

The frequency of bone marrow cells that secrete specific antibodies (ASC) was determined by ELISpot assay as previously described [162] at 100 days after second immunization. ELISpot plates (MHAB pore size 0.45 μm; Millipore, Billerica, MA) were coated overnight at 4^{o}C with 10 μg/mL NP_{26}-BSA in PBS for ASC secreting

high and low affinity antibodies, NP_4-BSA for ASC secreting antibodies with relatively high affinity, or donkey-anti-mouse IgG+M (Jackson ImmunoResearch, West Grove, PA) for all ASC. Plates were blocked with 10% FBS/PBS for 1 h. Bone marrow cells, after T cell deletion and panning, were serially diluted and cultured in the plate for 5 h. The plates were washed three times with PBS and three times with PBST. A cocktail of biotinylated goat-anti-mouse IgG antibodies (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 from Southern Biotech, Birmingham, AL) in 1% FBS/PBST was applied for 1 h at room temperature. Plates were washed in PBST and visualized using streptavidin-HRP and True Blue reagents (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Plates were washed with distilled water and dried overnight with in the dark before scanning with a C.T.L. plate scanner using ImmunoCapture software (Cellular Technology Ltd., Shaker Heights, OH). Spots were counted using C.T.L. ImmunoSpot counting software and verified by manually counting randomly selected wells from each plate.

Results

To test whether immunization with CpG ODN or gardiquimod as adjuvants can increase long term immunity in the NP-immunized mouse model, we determined the Ig titer and affinity and the frequencies of NP-specific memory B cells in the spleen and NP-specific antibody secreting cells in the bone marrow in more than 14 weeks after second immunization. The NP-specific serum titers (Figure 3.1B) were relatively low, compared to those shortly after immunization in most mice. The affinity of serum IgG for the NP antigen was measured both by an urea wash assay

(Figure 3.2E and not shown) and by specific competition with antigen (Figure 3.3E). The affinity of the antibodies remained high, although the titers had decreased since the second immunization. We determined the frequency of NP-specific memory B cells using flow cytometry. The memory B cells were definded as $B220^{+}$ $CD138^{-}$ IgM^{low} IgD^{-} NP^{+} (Figure A.2, A and B). We used the frequency along with the number of cells isolated from each spleen prior to staining to determine the number of NP-specific memory B cells in each mouse's spleen (Figure A.2C). Compared with immunization without adjuvant, mice immunized with Ribi and gardiquimod as adjuvant on average had retained 8-fold more NP-specific memory B cells, but CpG ODN adjuvant did not increase the number of NP-specific memory B cells that were retained in the spleen (Figure A.2, A-C). We determined the frequency of NP-specific ASC in the bone marrow using ELISpot assay, and estimate their relative affinity using low density coating antigen (NP_4-BSA) (Figure A.1D). There were significant numbers of NP-specific IgG ASCs in the bone marrow of all the immunization groups, indicating the presence of long lived plasma cells. The number of antigen-specific long-lived plasma cells did not differ between mice immunized with antigen alone or antigen plus adjuvant. It should be noted that due to low sample number in each immunization group, the differences we observed between mice immunized with antigen alone or with any of the adjuvants were not statistically significant at the $p=0.05$ cut-off. More mice are currently in the pipeline for analyses.

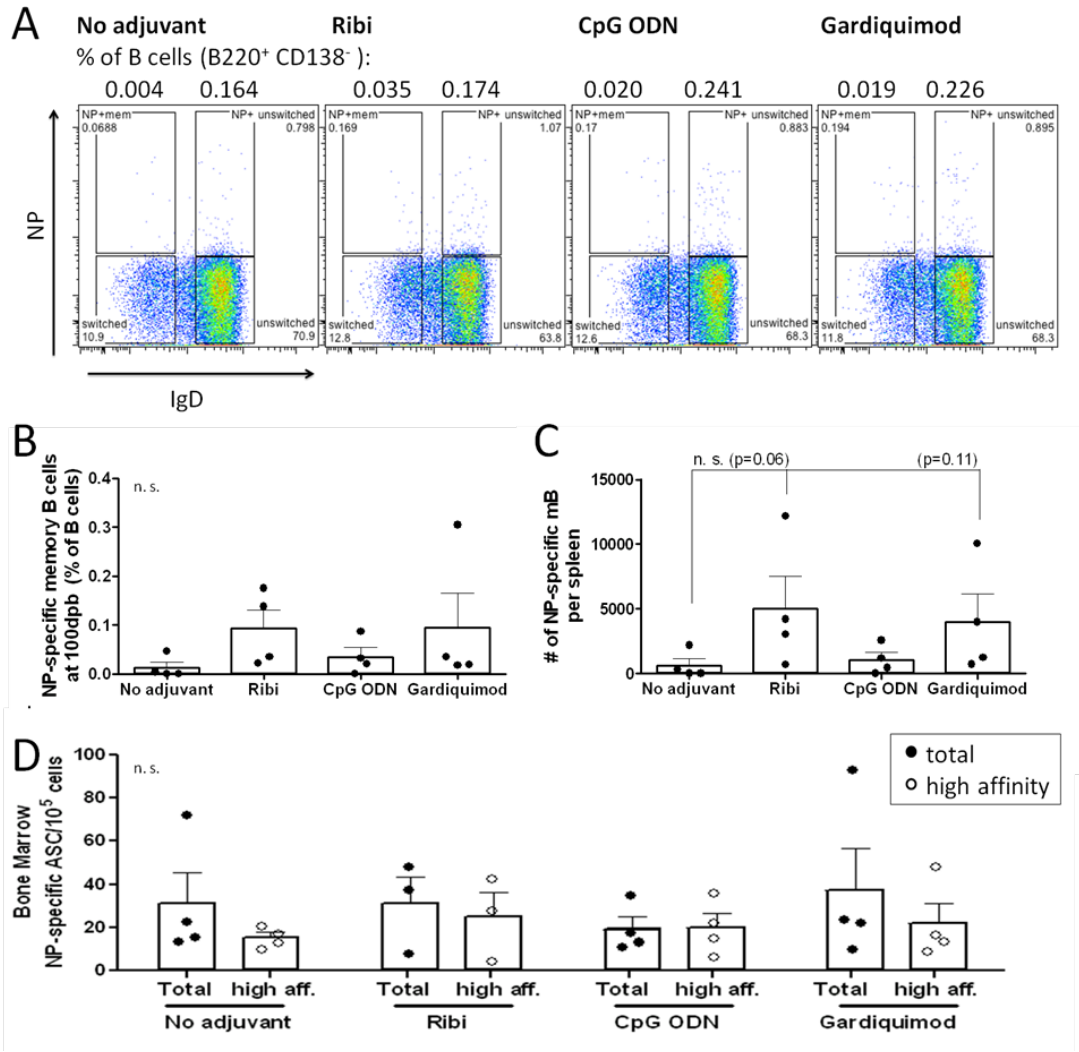

A
No adjuvant
Ribi
CpG ODN
Gardiquimod
% of B cells (B220+ CD138-):
0.004
0.164
0.035
0.174
0.020
0.241
0.019
0.226
NP+mem
NP+ unswitched
switched
unswitched
NP
IgD
B
NP-specific memory B cells at 100dpb (% of B cells)
n. s.
No adjuvant
Ribi
CpG ODN
Gardiquimod
C
# of NP-specific mB per spleen
n. s. (p=0.06)
(p=0.11)
D
Bone Marrow NP-specific ASC/10^5 cells
total
high affinity
Total
high aff.
No adjuvant
Ribi
CpG ODN
Gardiquimod

Figure A.2. No significant difference between the different adjuvants or without adjuvant on long-term immunity. A-C. The frequency of NP-sepecifc memory B cells were analyzed in spleens 100 days after second immunization with NP_{19}-KLH and the indicated adjuvant. B cell-enriched spenocytes were stained for B220, CD138, IgM and IgD, and incubated with allophytocyanin-conjugated NP without allowing internalization to assay antigen-binding ability. Representative histograms for flow cytometric analysis of memory B cells are shown in A. Histograms are gated on singlets, small lymphocytes by light scatter profile, $B220^+$ $CD138^-$ and IgM^-. IgD^- NP^+ cells (top left) are antigen-specific memory B cells. Unswitched (IgD^+) NP-binding cells are included as control population (top right). The frequency of these cells as a percentage of all $B220^+$ $CD138^-$ B cells is indicated above each histogram (A) and quantification is shown in (B). The frequency of NP-binding memory B cells was multiplied by the number of cells isolated from each spleen to obtain memory B cell counts per spleen (C). D. NP-specific bone marrow antibody secreting cells (ASC), representing long-lived plasma cells, were quantified in the same mice by ELISpot analysis on high density antigen-coated (total, filled circles) and low density antigen coated (high affinity, open circles) directly after B cell enrichment. Each circle represents one mouse, bars show mean and SEM.

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