TLR7 REGULATES SELECTION OF GERMINAL CENTER B CELLS AND AUGMENTS THE GENERATION OF MUTATED B MEMORY DURING THE PRIMARY RESPONSE

by
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Abstract

Recent work has demonstrated the importance of Toll-Like Receptor 7 (TLR7) in generating protective antibodies against viral infections. However, how TLR7 stimulation modulates mechanisms like somatic hypermutation and selection of B cells- so crucial for the generation of high affinity antibodies and memory- still need to be studied. Here, we demonstrate a role for TLR7 in the regulation of germinal center B cell selection and memory generation. We used a reductionist system to determine the effect of one TLR ligand on the germinal center reaction by immunizing mice intraperitoneal with nitrophenyl-chicken gamma globulin (NP-CGG) in the presence or absence of R837, a TLR7 agonist.

To test the effect on somatic hypermutation, germinal center B cells were isolated, cDNA encoding NP-specific IgH variable genes was sequenced, and activation-induced deaminase (AID) mRNA was measured. While AID expression was the same in both sets of mice, the mutation frequency and proportion of high affinity clones was significantly higher in mice that received TLR7 agonist. This observed phenomenon suggested that TLR7 played a role in selection of antigen-specific germinal center B cells. To further examine selection, the apoptotic profile and distribution of B cells in the light zone of the germinal center was examined. The total number of apoptotic B cells in the light zone was four fold lower in mice who received TLR7 agonist. However, the distribution of B cells in the light zone remained the same in both sets of mice, indicating that selection into the memory compartment had taken place. Mice that received TLR7 agonist had an enhanced ability to generate IgM B cell memory. When total naïve wild type or TLR7 knockout B cells were transferred into μMT mice- lacking mature B cells- an enhanced ability to generate NP-specific mutated memory was observed in mice receiving TLR7 agonist. However, when TLR7 knockout B cells were transferred, this enhanced ability was ablated indicating that B

cell intrinsic signaling of TLR7 was required for augmented memory generation. Together this data demonstrates that TLR7 intrinsically regulates germinal center B cell selection and memory generation during the primary response.

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CHAPTER I: Germinal Center B Cell Responses and Toll-Like Receptors

Germinal Centers

Germinal centers are clusters of rapidly proliferating germinal center (GC) B cells that are found in the lymph nodes (LN) and spleen after antigen encounter [1, 2]. GC B cells are derived from the follicular B cell pool and are identified by high surface expression levels of Fas, peanut agglutinin (PNA), and n-glycolyneuraminic acid (the ligand for GL7 antibody)[1]. These cells express the transcriptional factor BCL6 [3, 4] which is involved in suppressing anti-apoptotic molecules enabling proper selection to occur[5, 6]; it also suppresses activation of DNA damage sensing kinases allowing B cells to tolerate mutation activity[7, 8]; and down regulates plasma cell transcription factors, thereby contributing to GC maintenance [9].

These specialized GC B cell clusters are divided into two regions: the dark zone and the light zone [Fig 1]. B cells in the dark zone undergo multiple rounds of proliferation and diversify their B cell receptor (BCR) via a process called somatic hypermutation (SHM)[10-13]. The mutation process is controlled by activation–induced deaminase (AID), an enzyme which deaminates cytosine; and, can lead to mutations in the VDJ and switched regions of the BCR. VDJ mutations are responsible for generating a BCR with higher or lower affinity; while switch region mutations lead to switching of the constant region of the BCR and thereby provide the appropriate effector function[14-17].

B cells then travel into the light zone via their chemokine receptor CXCR5 and undergo selection for the highest affinity BCR[18-20]. During this selective process, two main cell players are involved follicular dendritic cells (FDCs) and T follicular helper T cells (T_{fh})[1, 2]. FDCs are radioresistant cells that form a network and are primarily responsible for presenting antigen (Ag) during the selective process as antigen-antibody complexes bound on their dendrites[21, 22]. Depletion of FDCs or their antigen-antibody binding complex receptor CR1/ CR2 results in decrease of germinal center maintenance and SHM[22-25]. The

current dogma regarding the selective affinity maturation process is that FDCs provide an Ag reservoir for competition. Here, higher affinity binding B cells out compete lower affinity binding B cells for antigen; this results in apoptosis of the low affinity binding B cells and survival of the high affinity binding ones [26, 27].

The next step in the process is to encounter the T_{fh}. These are $CD4^+$ T cells which express the transcription factor BCL6, are $CXCR5^+$ allowing for their entry into the B cell follicle; and are $PD\text{-}1^+$ $ICOS^+$ which enable negative or positive feedback signaling to the B cell[28]. Depoli et al.., [29]29] used an in vitro system to conjugate T cells with Epstein-Barr Virus-B cells which offered different levels of Ag presentation. While T cells synapsed with both low and high expressing peptide B cells, they polarized towards the B cell with the highest amount of peptide. In conjunction with this study, Reinhardt et al., [30] demonstrated in vivo that T helper cells secreting IL-4 were more likely to synapse with higher affinity GC B cells. T cell mediated selection was further demonstrated by Victora et al., [31] where GC DEC-205- expressing or deficient B cells of equal affinity were targeted with Ag using an anti-DEC antibody. Only the DEC-205 expressing B cells would uptake increased amounts of Ag and thereby have increased peptide-MHC (pMHC) loads. Data revealed that the DEC-205 expressing B cells expanded, while the DEC-205 deficient B cells were eliminated. These studies suggest that T_{fh} cells are able to differentiate B cells on the basis of pMHC displayed on the cell surface. Therefore, once the B cell binds Ag, it must process it into smaller antigenic peptides which can be loaded onto MHC class II molecules and presented on the cell surface as pMHC. T_{fh} then will form synapses with B cells of highest pMHC densities, enabling positive signaling. These signals then allow B cell survival and differentiation into memory B cells (B_{mem}) or long-lived plasma cells (LLPCs). These

memory subtypes are responsible for pathogen clearance and serve as protection against pathogen re-encounter.

Long-lived Plasma Cells

Germinal centers are the main source of long-lived plasma cells. This was first demonstrated by Foy et al.,[32] where CB17 (Igh b) mice were immunized with TNP-BSA and treated with anti-CD40L antibody or a control antibody. On week four, B cells from these mice were adoptively transferred into BALB/c (Igh a) recipients and challenged with TNP-KLH. The level of donor-specific IgG1 b anti-TNP antibodies was inhibited by greater than 80% in animals that received anti-CD40L antibody compared to the antibody control. Because blocking CD40L interactions abrogates germinal centers, this study correlated germinal centers as the source of LLPC generation. However, blocking CD40L can also block various steps in the B cell and T cell immune response, and thereby cannot be considered solely as germinal center specific. Nonetheless, studies with mutations in CR1/2, IL21R on B cells, and PD-1 cause defects in germinal center maturation, progression, and LLPC generation [33-35]. These cells are generated in the secondary lymphoid organs, but home to the bone marrow (BM) where they release low levels of high affinity class-switched antibody through the lifetime of an individual and serve to survey for pathogen[34, 36].

Memory B cells

Memory B cells (B_{mem}), on the other hand, are defined as antigen-specific mutated cells which remain in a resting state, are dependent on T-cell help, and have a lower threshold for antigenic stimulation[37-39]. Coico et al., [40] purified GC B cells with peanut agglutinin (PNA) and showed that secondary responses in irradiated recipient mice were derived from PNA^+ cells; linking germinal centers with memory B cells. Other studies have demonstrated that the absence of CD40 or CD40L blocked GCs and resulted in a loss of B_{mem} [41-43]. The more

correlative evidence for GC derived B_{mem} comes from studies exhibiting that these cell types tend to have a mutated BCR and class-switched isotypes[44]. Recently, the notion that B_{mem} only has class-switched isotypes has changed. Doga et al.,[45] used an AID-Cre-EYFP mouse model which enabled AID expressing cells to be permanently marked by enhanced yellow fluorescent protein (EYFP). After immunization with sheep red blood cells (SRBCs), these mice generated both IgM and switched B_{mem}. Both subtypes had mutated BCRs, although IgM B_{mem} was mutated to a lesser extent. More essential was the capacity of recall responses based on class isotype. Two studies highlighted that upon recall responses, isotype-switched B_{mem} cells differentiated into plasma cells and did not participate in GC re-entry; while IgM B_{mem} cells re-enter the GC and underwent robust isotype switching [45, 46] [Fig 2]. These findings brought forth the idea of B_{mem} heterogeneity.

As of today there is still no concordance upon the signals required for B_{mem} differentiation [47]. However, there does appear to be an association of GC dynamics with the generation of B_{mem}. Studies by Blink et al.,[48] demonstrated that germinal center derived B_{mem} appeared as early as day seven after primary immunization. This was further confirmed by Takashi et al.,[49] where B_{mem} mutations matched early GC but not late GC mutation profiles; whereas, LLPCs in the BM had a mutation profile that matched late GC, but not early GC mutation profiles. These findings show that B_{mem} arises out of the early GC and that timing may be associated with their differentiation fate. Apart from GC dynamics, some cell surface markers have been identified. Anderson et al.,[50] immunized transgenic and non-transgenic mice with nitrophenyl-chicken gamma-globulin (NP-CGG) and identified an NP-specific population of cells that expressed high levels of CD80 and low levels of CD35. Cells with this expression profile were of the IgM and IgG isotypes, and only the $CD80^{hi}$ expressers had undergone SHM. In conjunction with these findings, Tomayko et al.,[51] paired CD80

expression with expression of CD73 and PD-L2; to identify five different subsets of memory in response to NP-CGG.

Toll-Like Receptors and Germinal Center B Cell Responses

Toll –Like Receptors

Toll-like receptors (TLRs) are type I intramembrane glycoproteins that contain leucine-rich motifs on the extracellular domain which allow for ligand recognition; and a Toll/interleukin-1R (TIR) interacting intracellular domain that is responsible for signal transduction[52]. These receptors recognize pathogen associated molecular patterns (PAMPS), [53] such as lipopolysacharide (LPS) found on gram- negative bacteria cell walls, single or double-stranded viral RNA, and unmethylated CpG islands on bacterial DNA to name a few[54] [Fig 3]. TLRs are divided into two groups: those that are plasma membrane bound and recognize microbial lipids or proteins; and those that are found in the endosomal compartment and recognize microbial DNA or RNA[54]. Upon ligand activation, TLRs homodimerize or heterodimerize and recruit cytosolic TIR domain-containing adaptors in order to connect TLR receptors to downstream effector proteins. To date, five adaptor molecules have been identified in TLR signaling: MyD88, TRIF, TRAM MAL, and SARM[55]. These adaptors trigger two main pathways that are either dependent on MyD88 or TRIF to begin signal transduction. The signaling cascade leads to the nuclear translocation of transcription factors NF-$\varkappa$B, IRF3 or IRF7. These transcription factors generally promote the production of pro-inflammatory cytokines and type I interferon, respectively, which are necessary for immunologic cellular trafficking to the site of infection and activation of effector cells for pathogen clearance [56]. As of today, 13 different TLRs have been identified in mammals, 10 of which can be found in humans[54].

Toll-Like Receptors and B Cell Responses

In vitro stimulation of B cells with TLR ligands induces strong B cell proliferation and rapid differentiation into plasma cells [57-61]. While naïve and immature human B cells do not exhibit a strong response to TLR stimulation alone, co-stimulation via CD40 or BCR induces strong proliferation and differentiation of these cells. This suggests a role for TLRs in the promotion of antibody responses only in the context of B cell activation [62, 63]. In fact, purified germinal center B cells displayed enhanced expression of MyD88 mRNA compared to follicular B cells upon TLR ligation; indicating that B cells become more sensitive to TLR signal during T-dependent responses[64] .

Toll-Like Receptors and Germinal Center B cell Responses

To study the relevant importance of TLR signaling in T-dependent antibody responses *in vivo*, several models have been utilized. Kasturi et al., [65] adoptively transferred MyD88$^{-/-}$ B cells into μMT B cell deficient mice and immunized with ovalbumin (OVA) encapsulated in poly(D, L-lactic-co-glycolic acid) (PLGA) nanoparticles containing TLR adjuvants. MyD88$^{-/-}$ B cells demonstrated decreased antibody responses compared to wild type B cells. Pasare et al., [66] used the same mouse model but immunized with human serum albumin and LPS, a TLR4 ligand. MyD88$^{-/-}$ B cells were not able to form germinal centers and produced decreased levels of antigen-specific IgG. A further study by Barr et al., [67] used a mixed bone marrow chimera model where only the B cell compartment was deficient in MyD88 expression, and established that after OVA and LPS immunization IgM and IgG2c production was dependent on MyD88 signaling in B cells. If these mice were infected with *Salmonella typhimurium,* similar results were observed. These studies support the involvement of TLRs in the augmentation of T- dependent antibody responses against protein antigens.

However, protective effects of TLR ligands during the immune response encompass more than just protein antigens. Several studies have elucidated the importance of B cell TLR signaling in antiviral responses. Hou et al., [68] immunized cell-specific MyD88-deficient mice with a virus-like particle containing a TLR9 ligand and showed that germinal center dependent IgG responses were augmented and that this augmentation was dependent on MyD88 expression in B cells, but not dendritic cells. These mice were also challenged with inactivated influenza virus, containing ssRNA fragments which are ligands for TLR7. B cell MyD88$^{-/-}$ mice exhibited a 3 to 10 fold decrease in total anti-influenza IgG and IgG2 responses compared to wild type B cells. This correlated with data by Yang et al., [69]where human papillomavirus-like particles, containing major capsid protein L1, induced CSR in B cells in a MyD88 intrinsic signaling manner. Studies utilizing mouse retroviral models further supported TLR signaling in B cells as an important component for viral clearance. Browne [70] examined MyD88$^{-/-}$ B cell specific mice and their ability to clear Friend Virus (FV). These animals had significantly higher viral infectious centers and reduced anti-FV total antibody responses compared to wild type animals. Work by Walsh and colleagues [71] highlighted the involvement of TLR7 in clearance of chronic viral infection by lymphocytic choriomeningitis virus (LCMV). TLR7$^{-/-}$ mice infected with chronic LCMV exhibited decreased LCMV-specific plasma cells and IgG, as well as decreased germinal centers. All together, these findings establish a crucial role for B cell TLR intrinsic signaling in viral clearance.

Nonetheless, the mechanism involved in the regulation of the germinal center response by TLRs is an area that needs further study. While significant correlations have been demonstrated on the importance of B cell intrinsic TLR signaling for the augmentation of humoral immunity and viral clearance, very little is known about the role of TLRs in germinal center biology. Rookhuizen et al., [72] attempted to demonstrate how TLR9

enhanced germinal centers. Using dendritic cell and B cell specific MyD88$^{-/-}$ mice immunized with NP-CGG covalently linked to CpG, they demonstrated that MyD88 signaling in B cells induced ICOS expression on T_{fh}, increased NP-specific plasma cells, and enhanced high affinity NP-specific antibody during the primary and secondary responses. These findings suggest selection in the germinal center is impacted by B cell induction of ICOS on T_{fh}, thereby inducing maintenance of T_{fh} cells, which would increase the probability of GC B cell interaction with their cognate T cell. However, further studies need to be conducted to understand how GC processes like somatic hypermutation, AID expression, and B cell survival are regulated by TLRs.

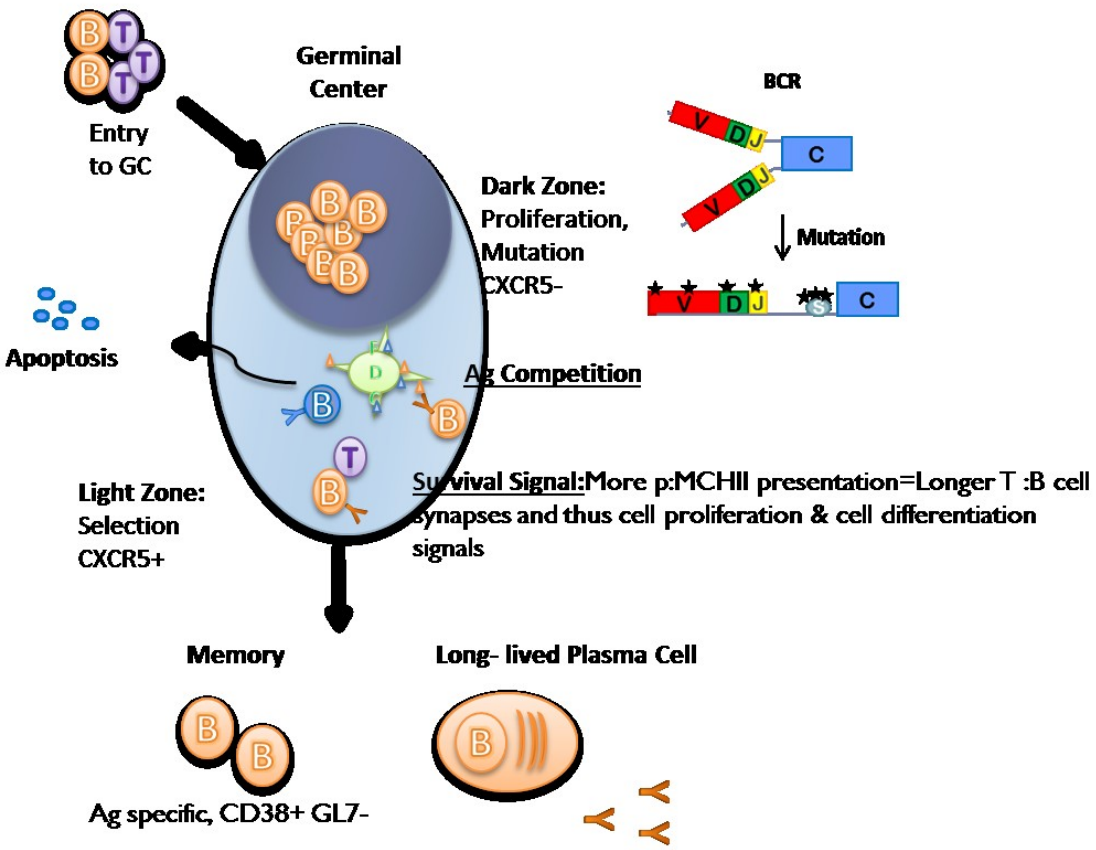


Figure 1 Germinal center reaction

Upon antigen (Ag) encounter B cells interact with cognate T cells and enter the germinal center reaction. In the dark zone of the germinal center, B cells undergo proliferation and somatic hypermutation of the variable (VDJ) and switched (S) regions in the B cell receptor (BCR). In the light zone, B cells that have acquired mutation of their BCR must undergo selection. They first compete for Ag presented by follicular dendritic cells (D). Higher affinity B cells survive and present Ag as peptide MHC class II (pMHC) to T follicular helper T cells (T). These T cells synapse with B cells of higher pMHC density and provide survival and differentiation signals. High affinity B cells are selected and exit the germinal center reaction as memory or long-lived plasma cells.

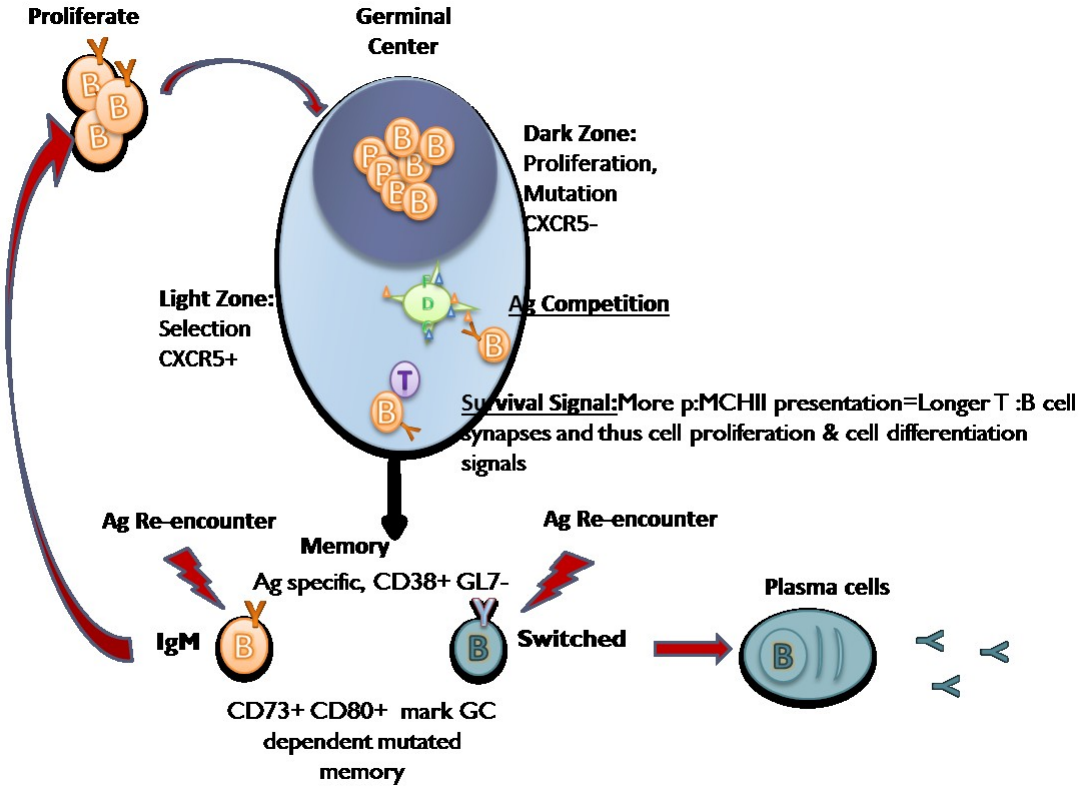
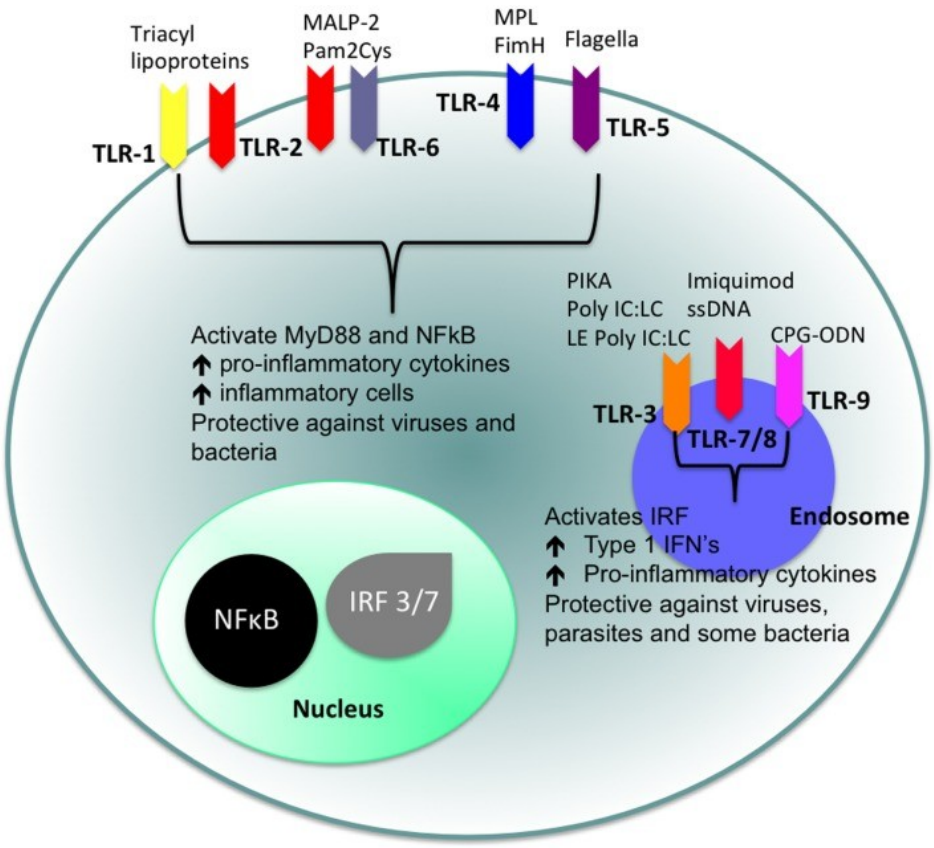


Figure 2 B memory functions upon antigen recall

Upon antigen (Ag) re-encounter, IgM memory B cells undergo a proliferative burst and enter the germinal center reaction where they can either class switch their isotype and differentiate into plasma cells or further participate in affinity maturation of their BCR to enhance specificity and generate more memory B cells. Switched memory B cells, on the other hand, rapidly differentiate into plasma cells and secrete antibody into the periphery to participate in serological responses.



Mifsud et al. Front Immunol. 2014 Mar 3;5:79

Figure 3 Toll-like receptors

Toll-like receptors (TLRs) are type I intramembrane glycoproteins that contain leucine-rich motifs on the extracellular domain which allow for ligand recognition; and a Toll/interleukin-1R (TIR) interacting intracellular domain that is responsible for signal transduction. TLRs are divided into two groups: those that are plasma membrane bound and recognize microbial lipids or proteins; and those that are found in the endosomal compartment and recognize microbial DNA or RNA. Upon ligand recognition, TLRs dimerize and induce signal transduction through the adaptor molecules MyD88 or TRIF. This leads to the nuclear localization of NFκB or IRF3/7, which results in the induction of either pro-inflammatory cytokines or type-I interferons necessary for pathogen clearance.

CHAPTER II: TLR7 Regulates Selection of Germinal Center B cells and Augments the Generation of Mutated Memory Cells

Introduction

Upon infection, the humoral response generates a germinal center (GC) reaction which produces pathogen-specific high affinity antibodies and B cell memory. While the high-affinity antibodies play a crucial role in immediate pathogen clearance, the B cell memory protects its host upon pathogen re-encounter. Recently, studies have demonstrated that innate immune receptors such as toll-like receptors (TLRs) which recognize pathogen-associated molecular patterns play an important role in shaping the B cell responses[52, 73, 74].

Purified GC B cells have enhanced MyD88 mRNA expression compared to follicular B cells upon TLR ligand stimulation[64] suggesting that GC B cells become more sensitive to TLR signaling during T-dependent immune responses. T-dependent antigen immunization of B cell MyD88-deficient mice exhibited significantly decreased antibody responses [66, 67]. However, a further study using NP-CGG in alum demonstrated that MyD88-deficient B cells responded similarly as WT B cells during the primary and secondary response; but that adding a TLR ligand greatly decreased NP-specific switched antibody in MyD88-deficient B cells compared to WT. Together, this indicates that B cell TLR signaling is not required for the generation of GC responses, but rather the augmentation of the response[64].

Moreover, vaccination with viral-like particles, virions, and nanopaticles established that B cell TLR signaling enhanced the GC reaction and augmented antigen-specific antibody responses [65, 68, 70]. Studies utilizing mouse viral models like Friend Virus and chronic lymphocytic choriomeningitis virus (LCMV) support TLR signaling in B cells as an important component for germinal center generation, antibody production, and viral clearance[70, 71, 75]. While these studies elucidate the importance of TLR B cell signaling in germinal center generation and high affinity antibody production, they are unable to demonstrate the mechanism leading to these effects.

We used a reductionist system to determine the effect of one TLR ligand on the germinal center reaction by immunizing mice intraperitoneally with a haptenated-protein, nitrophenyl-chicken gamma globulin (NP-CGG) in the presence or absence of R837, a TLR7 agonist. TLR7 engagement promotes early termination of the primary response; enhances GC B cell survival in the light zone of the germinal center; increases MHC II expression in IgM GC B cells but not switched B cells, and augments the generation of mutated B memory but not LLPCs.

Results

TLR7 promotes termination of the GC response and enhances affinity of NP-specific B cells

To understand the effect of TLR7 signaling on the germinal center reaction, we immunized 3-4 month old C57BL/6 mice intraperitoneally (i.p.) with a T-dependent antigen NP-CGG in the presence or absence of R837, a TLR7 agonist. This allowed us to compare GCs generated against antigen or antigen plus a TLR signal. Both immunization types induced a robust germinal center response at day 14 as quantified by flow cytometry as the total number of GC B cells ($CD4^-$ $B220^+$ $GL7^+$). However, on day 40 a reduction in the total number of germinal center B cells ($B220^+GL7^+$) was observed in animals immunized with antigen plus R837 [Fig. 4]

To see if the early termination of GCs during the primary response was due to improved generation of high affinity B cells, we sorted day 14 GC B cells ($B220^+GL7^+$) and amplified the Ig heavy chain locus with a Vh186.2 specific primer. We amplified Vh186.2 because it is a known V gene used during the canonical response to NP[76]. Affinity of NP-specific clones were determined by a tryptophan to leucine amino acid change of the complementary determining region 1 (CDR1) in the Vh186.2 gene sequence which leads to a 10-fold

increase in NP-binding affinity[76]. Mice immunized with antigen plus R837 exhibited a significantly higher proportion of NP-specific high affinity clones, as well as an increased number of mutations per clone [Fig 5B-C]. To further study if the enhanced affinity and mutations were due to an increase in the somatic hypermutation machinery, we examined sorted day 14 GC B cells for activation-induced deaminase (AID) expression. Message RNA expression of AID in GC B cells was similar between the immunization groups [Fig 5A]. We then inspected the Vh186.2 sequences for replacement and silent amino acid changes in the CDR regions 1-3. By calculating the replacement to silent mutations in each immunization group, we were able to examine if selection was occurring. Random mutations in a region of a protein that does not need to be conserved tends to have a replacement to silent ratio (R:S) of 2.9, if a ratio is significantly greater than 2.9, then positive selection is occurring[77]. R:S ratios for both immunization groups were greater than 2.9, indicating selection was occurring in the germinal center. Furthermore, in animals immunized with antigen and R837 the R:S ratio, while not significant, was higher compared to the NP-CGG immunized group [Fig 5D].

TLR7 stimulation promotes GC B cell survival in the light zone and MHC Class II up regulation

The germinal center is dependent on selection signals for GC B cell survival[78] and in vitro stimulation of B cells with TLR ligands has been demonstrated to induce up-regulation of anti-apoptotic factors such as c-myc and BcL-xL [79]. To investigate whether TLR7 played a role in GC B cell survival, we immunized WT mice with NP-CGG or NP-CGG plus R837 and measured B cell apoptosis 14 days later. TLR7 signaling led to a one fold reduction in the number of apoptotic GC B cell in the light zone ($CD4^-B220^+$ $CXCR5^+$ $GL7^+AnnexinV^+$

7-AAD$^-$) [Fig 6A-B]. We further examined the proportion of B cells in the light zone versus the dark zone. Absolute numbers of GC light zone B cells (CD4$^-$B220$^+$ CXCR5$^+$ GL7$^+$) and GC dark zone B cells (CD4$^-$B220$^+$ CXCR5$^-$ GL7$^+$) were similar between immunization groups [Fig 6C-D]. Increased B cell survival and mutation of GC B cells in the absence of AID upregulation, indicated that DZ B cells undergo more rounds of proliferation to enhance the mutation on their B cell receptors with TLR7 stimulation. On the other hand, absence of B cell accumulation but increased survival in the light zone, suggested cells were being selected for and perhaps exiting the germinal center reaction. Because T helper cells are more likely to form synapses with higher affinity GC B cells and polarize towards B cells with the highest amount of pMHC[29, 30], we further investigated the ability of TLR7 to enhance MHC II expression in GC B cells. TLR7 signaling significantly enhanced MHCII expression in IgM GC B cells, but not switched (Swg) GC B cells [Fig7A].

TLR7 stimulation augments the generation of mutated IgM memory

Because Tfh cells are the limiting factor for positive selection in the GC[28] and enhanced MHC presentation increases the opportunity for B cell positive selection[29-31]; we examined the memory compartment. WT mice immunized with NP-CGG plus R837 exhibited a significant increase in IgM memory (DUMP$^-$IgD$^-$B220$^+$CD19$^+$ IgM$^+$ CD38$^+$ PNA$^-$), but not Swg memory (DUMP$^-$IgD$^-$B220$^+$CD19$^+$ IgM$^-$ CD38$^+$ PNA$^-$) on day 14[Fig 7B]. To verify if TLR7 signaling in B cells was required for the augmentation of mutated memory μMT recipient animals were reconstituted with total naïve WT or TLR7$^{-/-}$ B cells and immunized with NP-CGG or NP-CGG plus R837. NP-specific mutated memory (B220$^+$NIP$^+$ CD80$^+$CD35^{lo}) was examined on day 14 after immunization. TLR7 signaling in B cells was required for the augmentation of mutated memory cells; this was validated by the absence of

memory augmentation in animals reconstituted with $TLR7^{-/-}$ B cells in the presence of TLR7 stimulation [Fig 7C-D].

TLR7 stimulation delays the generation of high-affinity switched antibodies

Positive selection leads to two fates: B memory and plasma cell differentiation[80]. To check the effect of TLR7 on plasma cell generation, WT mice immunized with NP-CGG or NP-CGG plus R837 were examined for plasmablasts, plasma cells, and NP-specific antibodies on day 14 and 40 after immunization. Total numbers of plasmablasts ($CD4^-$ $B220^+CD138^+$ or $B220^+CD138^+$) and plasma cells ($CD4^-$ $B220^-CD138^+$ or $B220^-CD138^+$) were similar between immunization groups on day 14 and 40 [Fig 8 A, Fig 9A]. NP-specific affinity matured IgG_1 and IgG_{2c} were significantly decreased with TLR7 signaling on day 14; nonetheless, by day 40 the recovery of NP- specific affinity mature switched IgG_1 and IgG_{2c} antibodies was observed in NP-CGG plus R837 immunized animals [Fig 8 B-C; Fig 9 B-C]. Thus, in conclusion TLR7 signaling in B cells enhances GC B cell selection of high affinity IgM memory on day 14 and results in a delayed secretion of NP-specific high affinity IgG_1 and $IgG2_c$.

TLR7 signaling promotes enhanced memory responses

IgM B memory cells re-enter the GC and undergo robust isotype switching [45, 46]. To study if TLR7-generated memory B cells responded better upon antigenic recall, we reconstituted μMT recipients with memory cells generated in WT mice immunized with NP-CGG or NP-CGG plus R837. Upon antigenic recall, recipients who received memory cells generated with TLR7 signaling exhibited significantly greater total cell numbers of GC B cells ($B220^+GL7^+$), plasmablasts ($B220^+CD138^+$), and plasma cells ($B220^-$ $CD138^-$) [Fig 10A-D]. The secondary IgG_1 response was significantly elevated in mice who received memory B

cells generated under NP-CGG plus R837 conditions. The caveat with this experimental set up is that both dendritic cells and B cells express TLR7 and the germinal center effect cannot solely be ascribed to TLR7 signaling in B cells. To understand if the elevated GC B cells observed upon recall are due to the memory generated by TLR7 signaling in B cells; we reconstituted μMT recipients with WT or $TLR7^{-/-}$ total naïve B cells, generated memory with NP-CGG or NP-CGG plus R837, and then re-challenged. After antigen recall, μMT recipients with WT B cells and TLR7 generated memory demonstrated a significant increase in GC B cells, but μMT recipients with $TLR7^{-/-}$ B cells and TLR7 generated memory did not. Therefore, in addition to enhancing selection of IgM mutated memory, TLR7 generated memory is functionally better in response to antigenic recall.

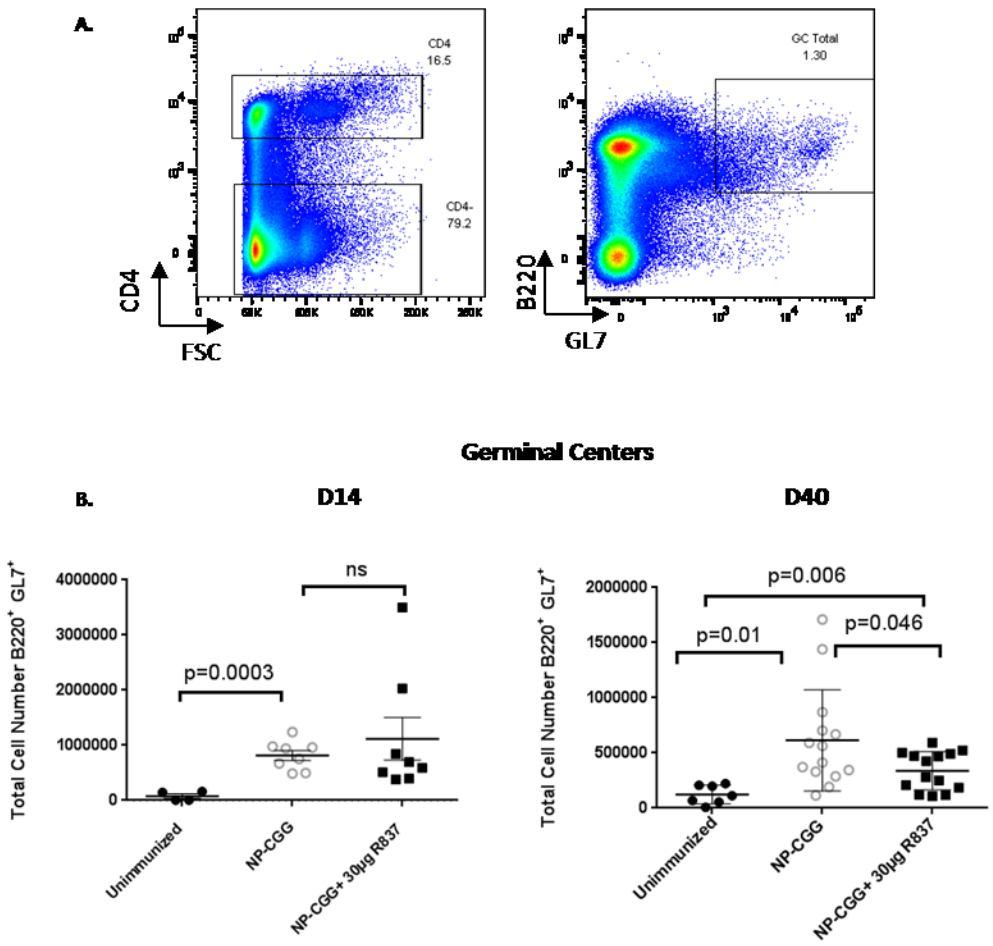


Figure 4 TLR7 promotes termination of the germinal center response by day 40

(A)Gating strategy for identification of germinal center B cells ($CD4^-$ $B220^+GL7^+$). Absolute numbers of total germinal center B cells from spleen on day 14 (B) and day 40 (C) after I.P. immunization with NP-CGG (○) or NP-CGG plus R837(■) or unimmunized controls (●). Each value symbol represents the value obtained from a single mouse; error bars represent the standard deviation.

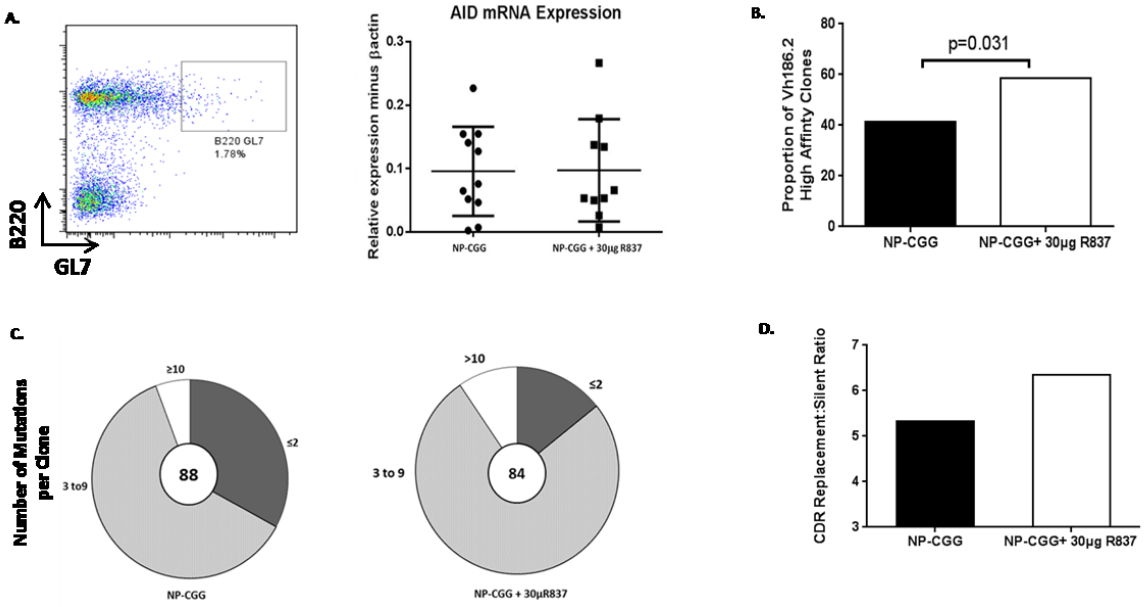


Figure 5 TLR7 enhances affinity of NP-specific B cells

(A, left) Representation of flow cytometry plot for sorting of germinal centers ($B220^+GL7^+$) 14 days after immunization; (A, right) AID mRNA expression relative to β-actin of sorted germinal center B cells on day 14 after i.p. immunization with NP-CGG (●) or NP-CGG + R837 (■). Each symbol represents an individual mouse, error bar represent standard deviation. (B) Spleens were sorted on day 14 after immunization for germinal center B cells ($B220^+GL7^+$). cDNA was utilized to amplify the Ig heavy chain locus with Vh186.2 specific primers. High affinity clones were identified by a tryptophan to leucine amino acid change on amino acid 33 of the complementary determining region 1 which leads to a 10-fold increase in NP-binding affinity. Data are from 5 independent experiments with one mouse per treatment group. (C) Distribution of mutations per clone. The number of mutated clones is shown in the center of each circle. Segments represent the proportion of clones that contain the indicated number of mutations. (D) Replacement to silent amino acid mutation ratio for complementary determining regions 1-3 (CDR1-3).

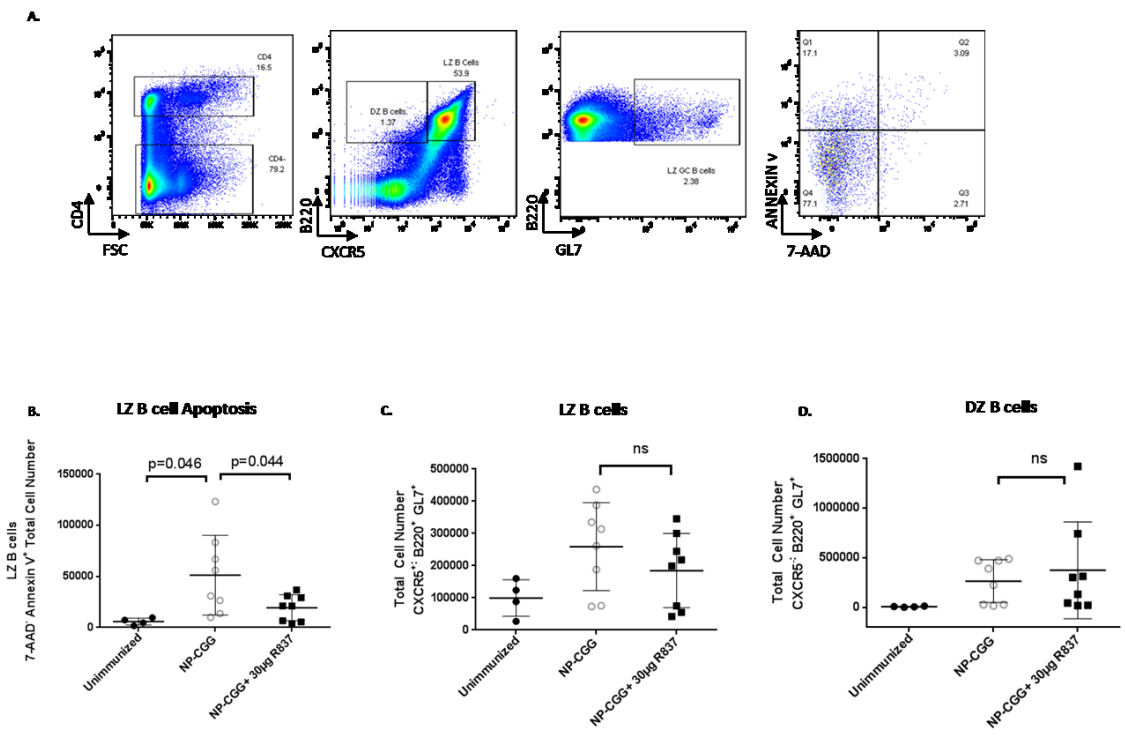


Figure 6 TLR7 signaling promotes GC B cell survival in the light zone

(A)Representative flow cytometry plots exhibiting gating scheme for enumeration of light zone (LZ) B cells ($CD4^-B220^+$ $CXCR5^+$ $GL7^+$), dark zone (DZ) B cells ($CD4^-B220^+$ $CXCR5^-$ $GL7^+$), and apoptotic B cells in the LZ ($CD4^-B220^+$ $CXCR5^+$ $GL7^+$ Annexin V^+ 7-AAD^-). Compiled data from triplicate experiments showing the absolute number of apoptotic light zone B cells (B), LZ B cells (C), and DZ B cells on day 14 after I.P. immunization with NP-CGG (○) or NP-CGG plus R837(■) or unimmunized controls (●). Each value symbol represents the value obtained from a single mouse; error bars represent the standard deviation.

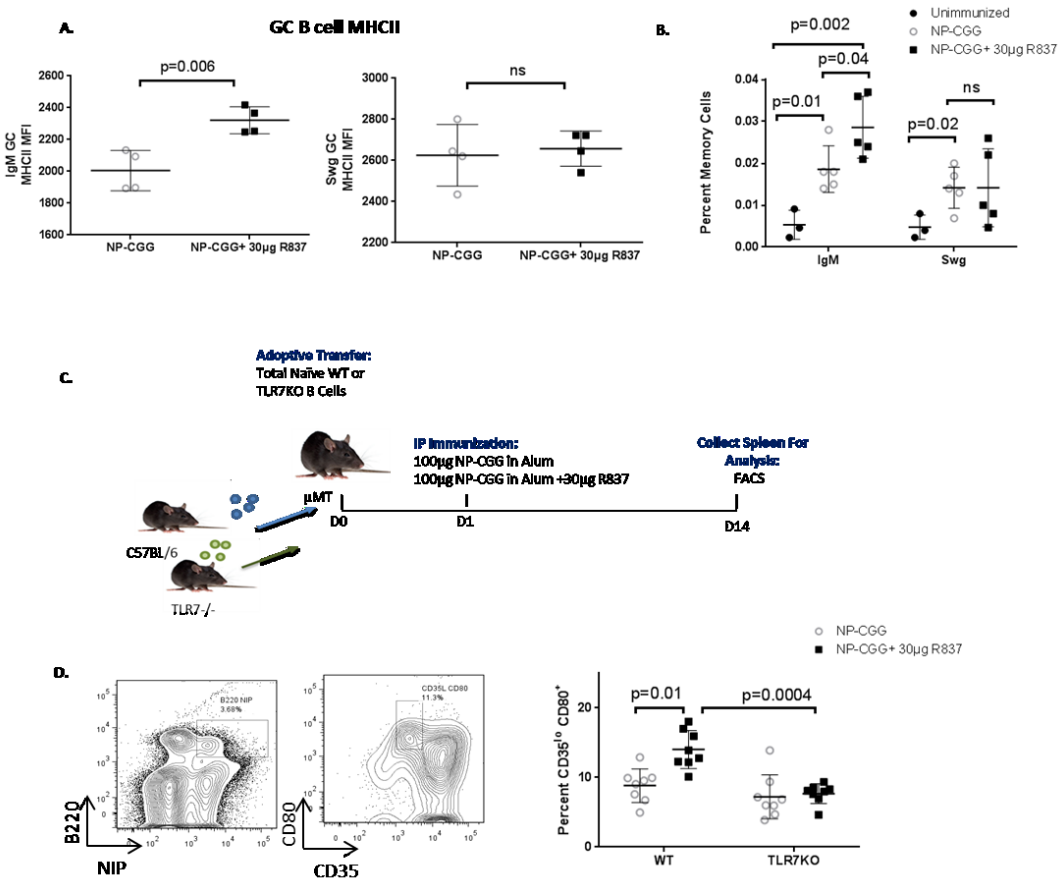


Figure 7 TLR7 stimulation enhances MHCII expression on GC B cells and augments the formation of mutated memory B cells

(A, Left)Mean fluorescence intensity of MHCII expression on germinal center IgM B cells (DUMP$^-$ CD19$^+$GL7$^+$ IgM$^+$ MHCII$^+$) or (A, Right) germinal center Swg B Cells (DUMP$^-$ CD19$^+$GL7$^+$ IgM$^-$ MHCII$^+$) on day 14 after immunization, duplicate experiments compiled. (B) Total percent IgM memory (DUMP$^-$IgD$^-$B220$^+$CD19$^+$ IgM$^+$CD38$^+$ PNA$^-$) or switched (Swg) memory (DUMP$^-$IgD$^-$B220$^+$CD19$^+$ IgM$^-$CD38$^+$ PNA$^-$) on day 14 after immunization, duplicate experiments compiled. (C) µMT recipient mice were reconstituted with C57BL/6 or TLR7$^{-/-}$ total naïve B cells to test if TLR7 expression on B cells was required for the augmentation of mutated memory. (D) Representative flow cytometry plots of NP- specific mutated memory (B220$^+$NIP$^+$ CD80$^+$CD35^{lo}) (D, left) and percent NP-specific mutated memory(D, right) of µMT recipients receiving WT or TLR7$^{-/-}$ B cells on day 14 after

immunization. Mice were immunized I.P. with NP-CGG (○) or NP-CGG plus R837(■) or unimmunized controls (●). Each value symbol represents the value obtained from a single mouse; error bars represent the standard deviation.

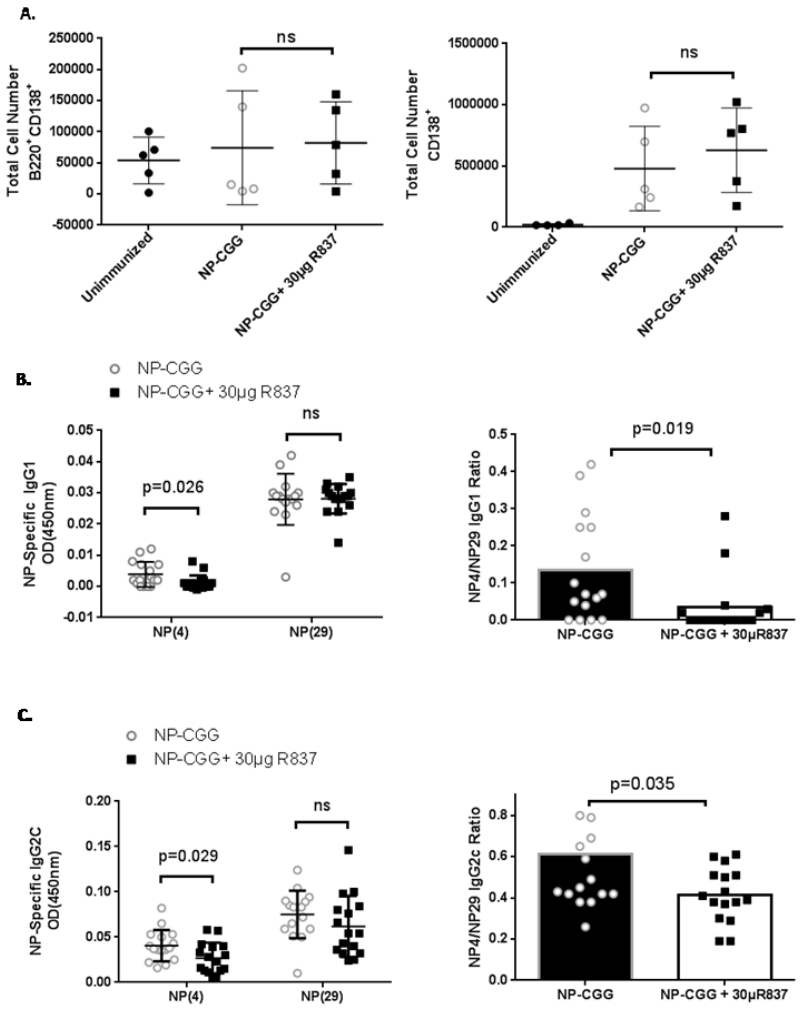


Figure 8 TLR7 stimulation delays the generation of NP-specific high affinity antibodies in early primary responses

(A)Absolute numbers of plasmablasts ($CD4^-B220^+CD138^+$) (A, left) or plasma cells ($CD4^-B220^-CD138^+$) (A, right) 14 days after immunization I.P. with NP-CGG (○) or NP-CGG plus R837(■) or unimmunized controls (●). (B) Serum NP-specific IgG_1 was measured by ELISA high affinity IgG_1 (NP_4 binding) and total IgG1 (NP_{29} binding) (B, left); affinity maturation was determined by the ratio of anti-NP_4 to anti-NP_{29} IgG_1 (B right). (C) Serum NP-specific IgG_{2c} was measured by ELISA as in (B). Each symbol represents an individual mouse; error bars represent the standard deviation.

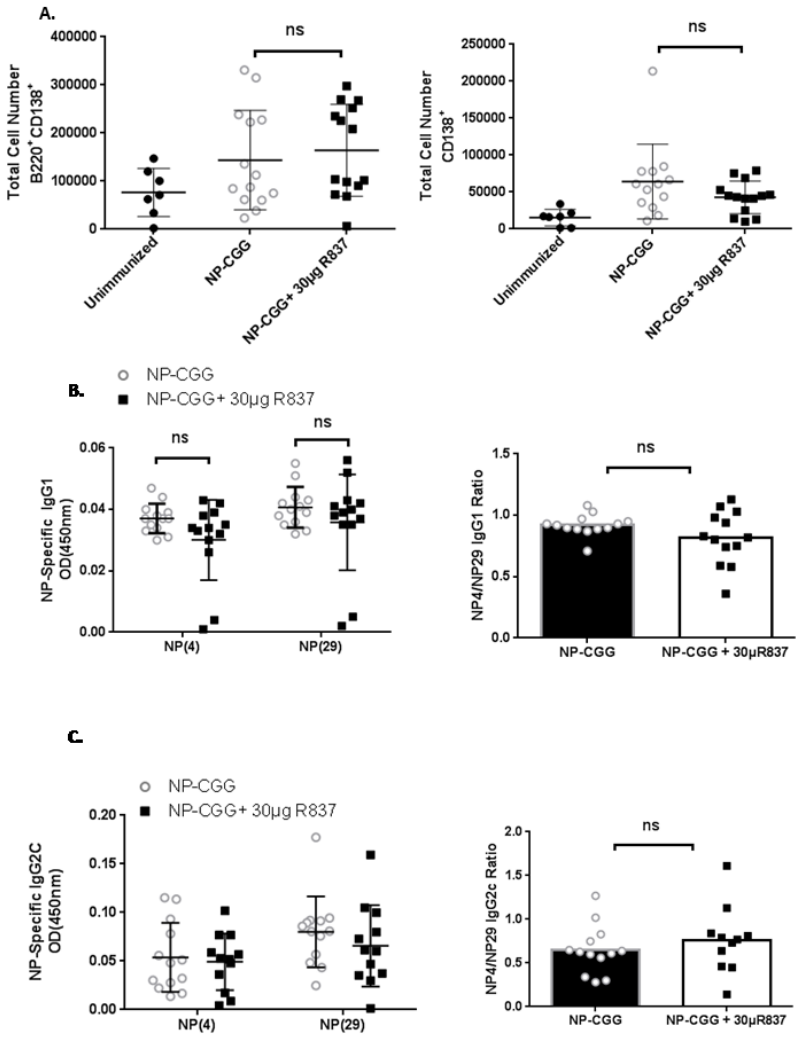


Figure 9 Generation of NP-specific high affinity antibodies recovered in late primary responses with TLR7 signaling

(A)Absolute numbers of plasmablasts ($B220^+CD138^+$) (A, left) or plasma cells ($B220^-CD138^+$) (A, right) 40 days after immunization I.P. with NP-CGG (○) or NP-CGG plus R837(■) or unimmunized controls (●). (B) Serum NP-specific IgG_1 was measured by ELISA high affinity IgG_1 (NP_4 binding) and total IgG1 (NP_{29} binding) (B, left); affinity maturation was determined by the ratio of anti-NP_4 to anti-NP_{29} IgG_1 (B, right). (C) Serum NP-specific IgG_{2c} was measured by ELISA as in (B). Each symbol represents an individual mouse; error bars represent the standard deviation.

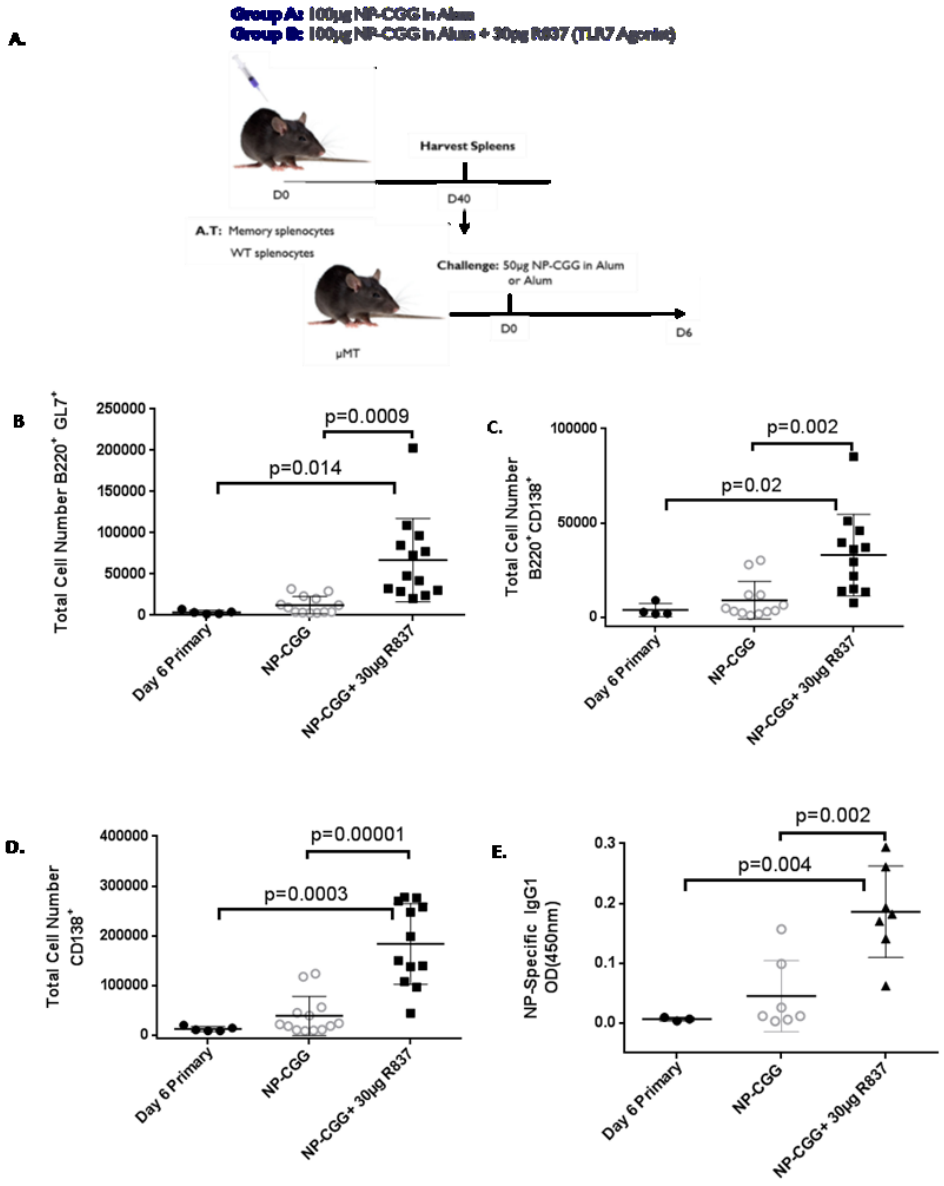


Figure 10 TLR7 signaling enhances memory function during recall responses

(A)Memory was generated in C57BL/6 mice. 40 days after immunization, µMT recipient mice were reconstituted with 14-30 x10^6 total splenocytes from mice immunized I.P. with NP-CGG(○) or NP-CGG + R837(■) or from unimmunized mice (●). Recipients were allowed to recover for 18-24hrs and were then re-challenged with NP-CGG. Six days after re-challenge, splenic GC B cells ($B220^+GL7^+$), plasmablasts ($B220^+CD138^+$), plasma cells ($B220^-$ $CD138^-$) were enumerated by flow cytometry. (B) Absolute numbers of GC B cells

($B220^+GL7^+$), (C) plasmablasts ($B220^+CD138^+$), and (D) plasma cells ($B220^-$ $CD138^-$). (E) Serum NP-specific IgG_1 was measured by ELISA. Each symbol represents an individual mouse; error bars represent the standard deviation.

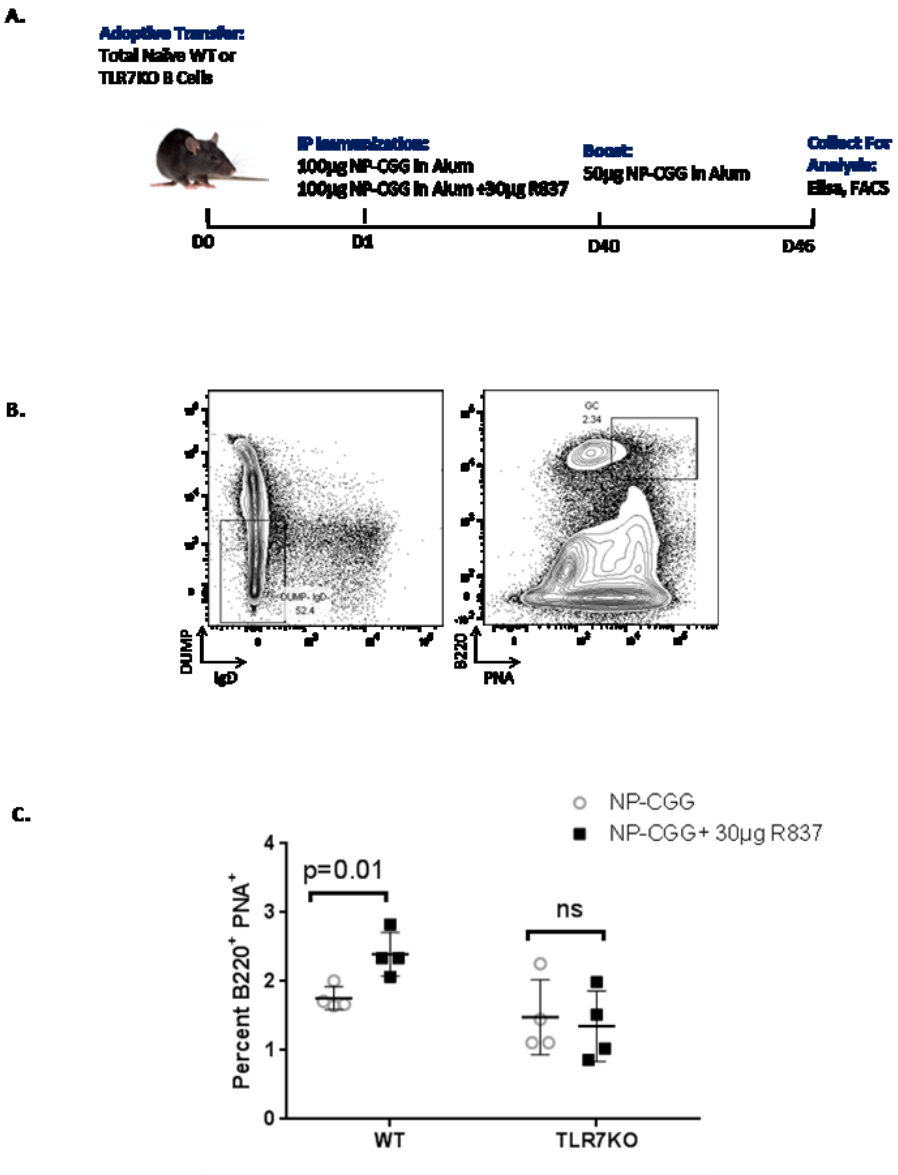


Figure 11 Enhanced germinal center responses upon recall are dependent on B cell TLR7 signaling

(A) µMT recipients were reconstituted with total naïve C57BL/6 or TLR7 $^{-/-}$ B cells to test whether B cell TLR7 signaling was the only contributor to the GC augmentation observed during re-challenge. Mice were immunized i.p. with NP-CGG (○) or NP-CGG + R837 (■). Memory was allowed to generate and 40 days after immunization, animals were re-challenged with NP-CGG. Six days post re-challenge, splenocytes were analyzed by flow cytometry. (B)Representative flow cytometry plots demonstrating gating scheme for GC B

cells ($DUMP^-IgD^-B220^+$ PNA^+) and percent GC B cell (C). Each symbol represents an individual mouse; error bars represent the standard deviation.

Discussion

TLR ligands have been demonstrated to be strong activators of B cells in vitro inducing B cell proliferation and rapid differentiation into plasma cells[57-61]. Furthermore, several studies have elucidated the involvement of TLR B cell signaling in the augmentation of T-dependent responses [65] and in protective germinal center anti-retroviral responses [68]. However, the mechanisms leading to this augmentation in responses have been poorly understood.

To study the mechanism responsible for the augmentation of GC responses upon TLR signaling, we used a reductionist system by immunizing mice intraperitoneally with a T-dependent antigen, nitrophenyl-chicken gamma globulin (NP-CGG), in the presence or absence of R837, a TLR7 agonist. In agreement with results by Palm and colleagues [81] haptenated proteins are strong immunogens and are alone capable of generating robust antibody production in absence of a TLR signal. Immunization with NP-CGG was capable of strong GC induction and IgG production on day 14; addition of a TLR7 ligand did not boost the GC response, but rather resulted in early termination 40 days after immunization. A surprising reduction of high affinity IgG on day 14 was observed, but was recovered by day 40 of the primary response. These results disagree with current data regarding primary responses where adding a TLR4, 7, or 9 ligand to protein antigen led to enhanced GC responses and augmentation of antigen-specific IgG production during early stages of the primary response[65-67, 72]. Discrepancies observed could be attributed to the route of immunization, the form in which the antigen is presented, and the mouse system utilized. While some immunizations were performed intraperitoneally, others were performed subcutaneously in the footpad; and while one looked at the systemic response, another examined the response near the draining lymph node which allowed for more restrictive

timing of studies. Antigen was delivered by various methods attached to a virus like-particle or covalently-linked to the antigen and delivered at much higher doses. The increased amount of antigen available could result in enhanced cross-linking of the BCR and thereby alter the necessity for TLR signaling in B cells.

The early termination of GCs observed during late stages of the primary response is attributed to enhanced selection of high affinity B cells into the IgM memory compartment. This is the most remarkable finding in this study. Our experiments demonstrate that TLR7 signal enhances the proportion of high affinity B cells in the germinal center; but not the somatic hypermutation machinery, as no difference was observed in the levels of mRNA for AID in GC B cells. Furthermore, the germinal centers are dependent on selection signals for GC B cell survival[78] and in vitro stimulation of B cells with TLR ligands have been demonstrated to induced up-regulation of anti-apoptotic factors such as c-myc and BcL-xL [79]. Our findings correlate with these studies. TLR7 signaling exhibited a one-fold reduction in the number of apoptotic GC B cell in the light zone, although there was no observable difference in the proportion of B cells in the light zone versus the dark zone. Absence of B cell accumulation, but increased survival in the light zone suggests cells are being selected for and exiting the germinal center reaction. Since T helper cells are more likely to form synapses with higher affinity GC B cells and polarize towards B cells with the highest amount of peptide-MHC[29, 30], we examined MHC II expression in GC B cells. TLR7 signaling significantly enhanced MHCII expression in IgM GC B cells, but not switched (Swg) GC B cells. This aligned with the augmented IgM, but not Swg memory generated with TLR signaling during the early stages of the primary response. Moreover, TLR7 deficient B cells in the presence of TLR7 ligand were not able to induce the augmented mutated memory exhibited by WT B cells in the presence of TLR7 ligand. Our findings

support several studies suggesting that TLR signal is not required for the generation of GC responses, but rather augments them [65]. Nonetheless, in this report, we provide novel findings that TLR7 directs selection of B cells into the IgM memory compartment by up-regulating the antigen presentation machinery.

Vaccinations with viral-like particles, virions, and nanopaticles have established that B cell TLR signaling enhanced the GC reaction and augmented antigen-specific antibody responses [65, 68, 70]. Our findings are in agreement with these studies regarding recall responses. Memory generated under TLR7 signal resulted in significantly greater GC reactions, plasma cell differentiation and antigen-specific IgG antibody output. The augmentation in germinal centers is attributed to B cell intrinsic TLR7 signaling, because memory generated with a TLR7 ligand in animals deficient in TLR7 lack the ability to generate augment GC responses upon recall. Our data correlate with the current hypothesis that IgM B memory cells re-enter the GC and undergo robust isotype switching [45, 46].

Lastly, our experiments are in agreement with studies demonstrating that TLR signaling upon recall enhances the GC reaction, plasma cell differentiation, and antibody responses upon recall. [64-66] Furthermore, we demonstrate TLR7 involvement in GC selection through its ability to promote GC B cell survival in the light zone and up-regulation of antigen-presentation machinery in IgM GC B cells. Moreover, our experiments suggest selection of antigen-specific GC B cells into the IgM memory pool. This memory population further supported the robust GCs generated upon antigenic recall and the GC augmentation was attributed to TLR7 signaling in B cells and not DCs. In conclusion, we demonstrate that TLR7 signal in B cell induces affinity maturation via the selection of high affinity B cells and not through processes of somatic hypermutation.

Significance

Vaccine development has been in place for decades. Nevertheless, very few adjuvants are currently approved for human use; and, the licensure of new adjuvants has taken a longer time than expected slowing vaccine formulation development [82-84]. First generation adjuvants have been restricted to insoluble aluminium salts, oil in water emulsions, and liposomes with very little understanding of their mechanism of action[82, 83]. While they provide a great safety profile, they do not expand the efficacy of vaccines. On the other hand, several second generation adjuvants- such TLR4 and TLR9 agonist- have improved immunogenicity and have demonstrated better safety profiles by limiting unwanted activation of the immune system [82, 85, 86]. In order to expedite the licensure of new adjuvants and improve the development of efficacious and safe vaccine formulations, there needs to be better understanding of the mechanisms of action by which TLR ligands induce protective adaptive responses. Here, we demonstrate how TLR7 regulates GC B cells responses in the context of protein antigens and contribute to the generation of an adjuvant platform.

CHAPTER III: Materials and Methods

Animals

C57BL/6 mice were purchased from Jackson Laboratories and bred in house (National Institute on Aging/NIH). μMT B6.129S2-Igh-6tm1Cgn/J and TLR7$^{-/-}$ B6.129S1-Tlr7tm1Flv/J mice were purchased from Jackson Laboratories. Mice were housed and all animal protocols were approved by the Animal Care and Use Committee of the National Institute on Aging.

Immunizations

C57BL/6 or μMT mice 8-12 weeks of age were immunized intraperitoneally (i.p.) with 100μg nitrophenyl-chicken gamma-globulin (NP_{31}-CGG) (Biosearch Technologies) emulsified in Alum (ThermoScientific) or with 100μg NP_{31}-CGG emulsified in Alum plus 30μg R837 is PBS (Sigma) suspended in 200μl.

Adoptive Transfer

For primary and memory recall experiments, spleens from anesthetized 9-12 week old TLR7$^{-/-}$ or C57BL/6 mice were isolated and homogenized into single cell suspensions. Cells were treated with 5mL ACK Lysis Buffer to remove red blood cells. Magnetic cell separation was performed using CD43 (Ly-48) and CD11b mouse Microbeads (Miltenyi Biotec) to obtain a pure total naïve B cell population. The purified naïve B cell population was washed 2X with cold 1X PBS and re-suspended at a final concentration of 15-30x10^6cells/100μl 1X PBS. For recall experiments C57BL/6 mice were immunized and memory was allowed to generate for 40 days. 14-30 x10^6 total splenocytes were adoptively transferred intravenously via tail-vein injection. For all other experiments, mice were adoptively transferred intravenously via tail-vain or by retro-orbital injection with 100μl of cell suspension.

Cell Sorting

Spleens were isolated and homogenized into single cell suspensions. Cells were treated with 5mL ACK Lysis Buffer to remove red blood cells. Single cell suspensions were stained with fluorescently labeled anti-B220 Fitc and anti-GL7 AlexaFlour 647(Biolegend) in FACS Buffer (1X PBS, 1%BSA, 2mM EDTA) for 30 minutes at 4°C in the dark. Labeled cells were washed 2X with 1X PBS and re-suspended in Sort Media (1X PBS, 25 mM HEPES, 1mM EDTA, 1% FBS) at $20x10^6$ cells/mL. Re-suspended cells were filtered and taken to the NIA Flow Cytometry Core Facility for sorting of the germinal center B cell population ($B220^+GL7^+$ double positive). Cells were sorted into 100% FBS.

Flow Cytometry

Spleens were isolated and homogenized into single cell suspensions. $0.5\text{-}2X10^6$ splenocytes /100µl were distributed into 96-well round bottom plates(Costar), centrifuged at 1250rpm, 5 minutes, 4 °C. Supernatant fluid was aspirated and cells were stained with antibody master mixes in FACS Buffer (1X PBS, 1%BSA, 2mM EDTA) for 30-45min in the dark at 4 °C as follows : Germinal center B cells were stained with fluorescently labeled with anti-CD4 PeCy7 ,anti-B220 Fitc and anti-GL7 AlexaFlour 660 or anti-B220 Fitc and anti-GL7(Biolegend) for day 14 and 40 primary responses, respectively . Plasmablasts and plasma cells were stained with anti-CD4 PeCy7, anti-B220 Fitc and anti-CD138 APC (Biolegend)or anti-B220 Fitc and anti-CD138 APC (Biolegend) for day 14 and 40 primary responses, respectively. Memory B cells from WT and memory recall transfer µMT animals were stained with anti-CD4, anti-CD8a, anti-F4/F80, and anti-Gr1 PeCy5 (Biolegend) as a dump gate plus anti-IgD BV421, anti-CD19 PeCy7, anti-CD38 APC (Biolegend); anti-B220 CF594 and anti-IgM PE (eBioscience) and PNA-Fluorescein (Vector Laboratories). Memory B cells on day 14 using µMT transfers were stained with NIP_{15}-Flourescein- BSA (Biosearch

Technologies), anti-B220-PerCP(Biolegend), anti-CD80-PE(BD Bioscience), and anti-CD35-BV421 (Biolegend). Apoptotic germinal center cells were stained with anti-CD4 Pecy7, anti-B220 Fitc, anti-CD185(CXCR5) BV421, anti-GL7 AF647, Annexin V PE, and 7-AAD (Biolegend). Cells were then washed 2X with cold 1X PBS; and either re-suspended in FACS Buffer (1X PBS, 1%BSA, 2mM EDTA) for analysis using the BD FACS CANTO II or fixed with 2% formaldehyde for 10 minutes at room temperature; washed with cold 1X PBS; and re-suspended in FACS Buffer (1X PBS, 1%BSA, 2mM EDTA) for analysis. Refer to Table 1 for antibody information.

ELISA

High binding 96-well flat bottom plates (FisherBrand) were coated with 100µl/well of 10µg NP_4-BSA or NP_{25}-BSA in Coating Buffer(0.186gNa_2CO_3, 0.286g $NaHCO_3$ in 1X PBS) overnight at 4°C. Plates were washed 3X with Wash Buffer (1X PBS, 0.1% Tween20) and blocked with 5% BSA for 1h at room temperature. 40-100µl of serum dilutions were plated in triplicate and incubated 2h at room temperature. Wells were washed 5X with Wash Buffer (1X PBS, 0.1% Tween20) and incubated with detection antibody, anti-IgM-HRP (Southern Biotech, 1:10,000) or anti-IgG-HRP(Southern Biotech 1:10,000) or anti-IgG2c-HRP (Thermo Scientific, 1:20,000) in 5% BSA for 1h at room temperature. Wells were washed 5X with Wash Buffer and incubated with Substrate Solution (TMB Peroxidase Substrate, Vector Labs) until a light a blue color change was noticed. Then Stop Solution (12.5mL H_3PO_4, 87.5mL ddH_20) was added and the plate read using BioRad Model 680XR Microplate Reader at 450nm. Data was presented as OD values and mice where anesthetized and orbital eye bleeds were collected.

Total RNA Isolation & cDNA Synthesis

Sorted cells were centrifuged at 1250rpm for 10minutes at 4°C. Re-suspended in 1 ml Trizol

Reagent (Invitrogen) and incubated at room temperature for 5 minutes. Then 300 µl of chloroform was added, mixture was vortexed for 30 seconds, and centrifuged at max speed for 15 minutes at 4°C. The upper aqueous layer was removed into a fresh Eppendorf tube and an equal volume of 70% EtOH was added. Mixture was applied to an RNAeasy column (Qiagen) and centrifuged at maximum speed for 30 seconds. The column was washed with 700 µl RW1 Buffer and then with 500µl RPE Buffer (Qiagen). The column was placed in a fresh Eppendorf tube with 50 µl DEPC H_2O and centrifuged at maximum speed 2X. 10 µl of 3 M sodium acetate and 300 µl of ice-cold 100% EtOH were added per sample and incubated at -20°C overnight. The mixture was centrifuged for 15 minutes, washed with 100µl 70% EtOH and centrifuged again for 10 minutes. Supernatant fluid was discarded and the pellet was air dried for 10 minutes. 20µl DEPC H_2O was added to re-suspend the RNA. Isolated RNA was stored at -80°C. cDNA was generated using dT Oligo and Superscript III First-Strand Synthesis System (Invitrogen).

IgH Repertoire Amplification

RNA was extracted from germinal center sorted B cells using Trizol reagent (Invitrogen), followed by further purification with RNeasy Mini kit(Qiagen) as stated in the total RNA isolation section. cDNA was generated using oligo dT and Superscript III (Invitrogen). Resultant cDNA was amplified using TaKaRA Taq™ (TakaRa Clontech). Each PCR reaction contained: 1µl cDNA, 0.2uM VH and IgG1 Primers, 0.2mM dNTPs, 0.1µl TaKaRA Taq, 5U polymerase. The final volume was brought to 20 µl with ddH_2O. Cycling conditions were initial melt at 94°C for 3 minutes, followed by 30 cycles of a three step program (94°C, 1 minute; 45°C, 1 minute; 72°C, 2 minute). The reaction was held at 72°C for 10 minutes and cooled at 4°C. Refer to Table 2 for primer sets.

VH186.2 Amplification

RNA was extracted from germinal center sorted B cells using TRIzol reagent (Invitrogen), followed by further purification with RNeasy Mini kit (Qiagen) as stated in the total RNA isolation section. cDNA was generated using dT oligo and Superscript III (Invitrogen). Resultant cDNA was amplified using TaKaRA Taq™ (TakaRa Clontech). Each PCR reaction contained: 2µl cDNA, 0.2µM primary reaction primers, 0.2mM dNTPs, 0.25µl TaKaRA Taq, 5U polymerase. The final volume was brought to 50 µl with ddH_2O. Cycling conditions were initial melt at 94°C for 2 minutes, followed by 30 cycles of a three step program (94°C, 1 minute; 55°C, 45 seconds; 72°C, 45 seconds). The reaction was held at 72°C for 4 minutes and cooled at 4°C. Refer to Table 2 for primer sets.

IgH Repertoire & VH186.2 Cloning

PCR reaction product plus ethidium bromide was electrophoresed on a 1% agarose gel for 35 minutes. A 400-500 base pair fragment was gel purified using QIAquick Gel Extraction Kit (Qiagen). The purified 500 base pair product was then TA cloned using StrataClone PCR Cloning Kit (Agilent Technologies). StrataClone SoloPack competent cells were used for bacterial transformation according to manufacturer's protocol (Agilent Technologies). Bacterial colonies were picked and grown overnight in a 37°C shaker with 4mL of LB broth plus ampicillin. Cultures were minipreped using QIAprep Spin Miniprep Kit according to manufacturer's protocol (Qiagen). Purified DNA was sent to be sequenced by Macrogene USA using T7 primers.

Sequence Analysis of IgH & Vh186.2 Clones

Sequencing results obtained from Macrogene USA were blasted using the NCBI/IgBlast software against their germline heavy chain immunoglobulin sequence. Mutations and amino acid substitutions were counted for each framework region and

for complementary-determining regions 1, 2, and 3 (CDRs 1, 2, &3). Mutation frequency was calculated as total mutations divided by total base pairs sequenced. High versus low affinity VH186.2 antibodies was determined by a tryptophan (W) to leucine (L) amino acid change in amino acid 33 of CDR1[76].

AID RT-qPCR

RNA was extracted from germinal center sorted B cells using TRIzol reagent (Invitrogen), followed by further purification with RNeasy Mini kit (Qiagen)as stated in the total RNA isolation section. cDNA was generated using Superscript III (Invitrogen), followed by qPCR using Power SYBR Green PCR Master Mix (Life Technologies) and the primers listed in Table 2.

Table 1 Fluorescent Antibodies

Name	Clone	Company
Germinal Centers		
B220-Fitc	RA3-6B2	Biolegend
GL7-AF647	GL7	Biolegend
CD4-PeCy7	GK1.5	Biolegend
Plasmablast and Plasma Cells		
B220-Fitc	RA3-6B2	Biolegend
CD138- APC	281-2	Biolegend
CD4-PeCy7	GK1.5	Biolegend
Apoptotic GC B cells		
CD4-PeCy7	GK1.5	Biolegend
B220-Fitc	RA3-6B2	Biolegend
GL7-AF647	GL7	Biolegend
CD185 (CXCR5)- BV421	L138D7	Biolegend
Annexin V- PE	-	Biolegend
7-AAD	-	Biolegend
Memory Cells		
DUMP		
CD4-PeCy5	H129.19	Biolegend
F4/F8-PeCy5	BM8	Biolegend
Ly6G/LyC Gr1-PeCy5	RB6-8C5	Biolegend
C8a-PeCy5	53-6.7	Biolegend
IgD-BV412	11-26c.2a	Biolegend
CD19-PeCy7	6D5	Biolegend
CD38-APC	90	Biolegend
B220-CF594	RA3-6B2	eBioscience
IgM-PE	II/41	eBioscience
PNA-Flourescein	-	Vector Laboratories
Mutated Memory Cells		
NIP(15)- Fluorescein-BSA	-	Biosearch Technologies
B220-PerCP	RA3-6B2	Biolegend
GL7-AF647	GL7	Biolegend
CD80-PE	16-10A1	DB Bioscience
CD21/35 eFlour421	7E 9	Biolegend

Table 2 Primers

Technique	Oligonucleotide (5' to 3')
AID Expression	
Aicda Forward	GGTCCAGATCGGGATCATGACCTTC
Aicda Reverse	CGGACAGAATTTTCATGTAGCCCTTCCC
β-Actin Forward	GACCTCTATGCCAACACAGTGCTG
β-Actin Reverse	CACCGATCCACACAGAGTACTTGC
Vh186.2 Amplification	
Primary Reaction	
Vh186.2 Foward_S	CAT GCT CTT CTT GGC AGC AAC AGC
IgH Cg1 Reverse_S	GTG CAC ACC GCT GGA CAG GGA TCC
Secondary Reaction	
Vh186.2 Foward_S2	CAG GTC CAA CTG CAG CAG
IgH Cg1 Reverse _S2	AGT TTG GGC AGC AGA
IgH Repertoire Amplification	
VH Forward Primers	
MH1	CTT CCG GAA TTC SAR GTN MAG CTG SAG SAG TC
MH2	CTT CCG GAA TTC SAR GTN MAG CTG SAG SAG TCW GG
MH3	CTT CCG GAA TTC CAG GTT ACT CTG AAA GWG TST G
MH4	CTTCCG GAA TTC GAG GTC CAR CTG CAA CAR TC
MH5	CTT CCG GAA TTC CAG GTC CAA CTV CAG CAR CC
MH6	CTT CCG GAA TTC GAG GTG AAS STG GTG GAA TC
MH7	CTT CCG GAA TTC GAT GTG AAC TTG GAA GTG TC
Heavy Chain Constant Region Reverse Primers	
IgG1	GGA AGA TCT ATA GAC AGA TGG GGG TGT CGT TTT GGC

References

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CURRICULUM VITAE
The Johns Hopkins University School of Medicine

Diana P. Castiblanco March 21, 2016

Educational History

Ph.D expected	2016	Program in Immunology Mentor: Dr. Patricia J. Gearhart	Johns Hopkins School of Medicine
B.S.	2010	Immunology and Infectious Disease	The Pennsylvania State University
B.S.	2010	Toxicology Mentor: Dr. Richard. Frisque	The Pennsylvania State University

Research Experience

National Institutes of Health, National Institute on Aging Baltimore, MD
Doctoral Research Scientist May 2011 – Present

Relevant Coursework: Principles of Drug Development/ Drug Discovery Case Studies/ Fundamentals of Budgeting and Financial Management/ Managing Complex Projects

- Lead, design, and execute experiments using in vivo systems to study the mechanism of Toll-like receptor 7 (TLR7) regulation of humoral responses with implications in retroviral protection
- Examine antibody repertoire and mutation upon vaccination using antigen-specific or global antibody sequencing to evaluate antibody affinity and neutralization spectrum
- Measure B cell memory generation induced by immunization with TLR7 agonist using fluorescence-activated cell sorting, Elisa and ELISPOT assays to understand memory recall and protection efficacy
- Engage in cross-functional team participation to execute 4 independent scientific projects
- Supervise, assist, and facilitate biologist on two independent projects focused on AID targeting to the immunoglobulin locus
- Present result findings at lab, institute-wide and professional meetings to discuss research efforts

The Pennsylvania State University University Park, PA
Undergraduate Research Assistant June 2007 – May 2010

- Developed miniT protein mutant BK Virus to confirm protein oncogenic activity
- Measured transformation ability of wild type versus mutant BK virus using dense focus assays
- Completed sequenced for BKV(WT9) virus variant
- Scheduled team meetings for technical strategy development to enhance transformation assays
- Established project milestone to maintain research team updated and facilitate collaboration

Leadership Experience

Immunology Training Program- The Johns Hopkins University Baltimore, MD
Chief Operating Graduate Student May 2015- Present

- Implement guidelines and systematize operations for leadership roles involving scientific communication/ collaboration, and professional development to enhance student morale
- Oversee and empower six program officers to use resources and time effectively to reach scheduled goals
- Evaluate program needs and negotiate with Board of Directors to achieve program needs, enhance student involvement, and reach resolutions to improve program evaluation

Women in Bio: Washington DC/Baltimore Chapter Baltimore, MD
Programing Events Committee: Co-Chair/Member February 2012- February 2014

- Organized monthly meetings for 12+ programing committee members; ability to multi-task and maintained poise under pressure
- Empowered project staff to meet quality standards and use resources effectively to deliver tasks on time

Eberly College of Science – The Pennsylvania State University University Park, PA
President, Bunton-Waller Fellow Student Council August 2008 –May 2010

- Coordinated bi-weekly meetings for 10+ undergraduate student representatives
- Assisted with professional development programs focused on resume writing, interviewing, and contract negotiation
- Managed educational programming for Bunton-Waller Fellows and Lenfest Scholars
- Developed templates for tracking event progression ,budgets, and event evaluations

Eberly College of Science – The Pennsylvania State University University Park, PA
Program Assistant, The Pennypacker Experience August 2007 – May 2008

- Planned, developed, and implemented 12+ academic and professional development programs
- Reviewed transcripts and individualized four-year academic plans for 45+ undergraduate students
- Created a supportive environment for residents in the First Year Science and Engineering (FISE), Mildred S. Bunton and Calvin Waller Undergraduate Fellows, and Lenfest Scholars Programs, as well as participants in the Schreyer Honors College

Scholarships and Fellowships

- **Intramural AIDS Research Fellowship,** National Institutes of Health, 2013
  - Received $45,700 renewal of funds to cover stipend and health insurance for one year
- **NIH Graduate Student Research Award (NGSRA),** National Institutes of Health, 2013
  - Place in the Top Five of a 200+ graduate student symposium
  - Received $1,000 travel award
- **Immunology Training Program Retreat Poster Winner,** Johns Hopkins University School of Medicine, 2012
  - Awarded "First Place" for poster presentation of 35+ graduate students
- **Intramural AIDS Research Fellowship,** National Institutes of Health, 2012

- Received $39,700 funds to cover stipend and health insurance for one year
- **Keystone Symposium- Mutations, Malignancy and Memory - Antibodies and Immunity,** Boston, 2012
  - Selected to serve as a Conference Assistant
  - Received $1,200 scholarship to cover registration fee, travel and lodging Intramural
- **NIAID Research Opportunities (INRO)**, National Institutes of Health, 2010The
- **Mildred S. Bunton and Calvin Waller Undergraduate Fellowship**, Penn State University, 2006 - 2010
  - Received complete tuition, room and board for 4 years
- **Smart Grant,** The Eberly College of Science, 2009
- **Summer Research Opportunities Program (SROP) Grant**, Penn State University, Summer 2009
- **Ronald E. McNair Post-Baccalaureate Research Program Grant**, Penn State University, Summer 2008
- **Summer Research Opportunities Program (SROP) Grant**, Penn State University, Summer 2007
- **Dean's List,** The Pennsylvania State University, Academic Year 2006, 2008, and Fall 2007
- **Galen Dreibelbis Endowment for Excellence in Agriculture**, The College of Agricultural Sciences**,** 2008 - 2009
- **Horace T. Woodward Scholarship**, The College of Agricultural Sciences**,** 2007
- **John N. Adam Jr. Scholarship for Excellence in Agriculture**, The College of Agricultural Sciences**,** 2007
- **Latino Academic Excellence,** PA Summit on Educational Excellence for Latino Students, 2007

Publications

Gearhart PJ, **Castiblanco DP**, and Russell LR. (2015) Exceptional Antibodies Produced by Successive Immunizations. PLOS Biology 13(12): e1002321. doi:10.1371/journal.pbio.1002321

Zanotti KJ, Maul RW, **Castiblanco DP**, Yang W, Choi YJ, Fox JT , Myung K, Saribasak H, and Gearhart PJ. (2014) ATAD5 Deficiency Decreases B Cell Division and IgH Recombination J. Immunology pii: 1401158.

Barrantes Gomez (**Castiblanco) DP**, Frisque RJ. (2010). Contribution of BK Virus miniT Protein to Viral Oncogenic Activity: The Pennsylvania State University Schreyer Honors College. Electronic Thesis. https://honors.libraries.psu.edu/paper/1381/

Presentations

Poster Presentations

- **Toll-Like Receptor 7 Involvement in Germinal Center Responses**
  - Fundamental Immunology & Its Therapeutic Potential, Cold Spring Harbor Laboratories, April 2015
- **A Role for Toll-Like Receptor 7 in Memory B cell Generation and Antibody Affinity Maturation**
  - Keystone Symposium- Prophylatic and Therapeutic Antibodies, Keystone Symposia, February 2014

- **Role for Toll-Like Receptor 7 in Class Switch Recombination and Somatic Hypermutation**
    - NIH Graduate Student Research Symposium, National Institutes of Health, January 2013
    - The 11th Annual Training Program Retreat, Johns Hopkins University School of Medicine, September 2012

Oral Presentations

- **Toll-Like Receptor 7 modulation of B cell responses**
    - Immunology Training Program Retreat, The Johns Hopkins School of Medicine, September 2014
- **Role for Toll-Like Receptor 7 in Germinal Center and Memory Responses**
    - The 12th Annual Training Program Retreat, Johns Hopkins University School of Medicine, September 2013
- **Contribution of BK Virus miniT Protein to Viral Oncogenic Activity**
    - 18th Annual National McNair Research Conference, University of Wisconsin-Milwaukee, November 2010