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Innate Signaling Pathways in the Maintenance of Serological Memory: A Dissertation

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INNATE SIGNALING PATHWAYS IN THE MAINTAINENCE OF SEROLOGICAL MEMORY

A Dissertation Presented

By

Forum M. Raval

Submitted to the
Faculty of the University of Massachusetts Graduate School of Biomedical Sciences,
Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

June 21, 2012

Immunology & Virology

INNATE SIGNALING PATHWAYS IN THE MAINTAINENCE OF SEROLOGICAL MEMORY

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ABSTRACT

Long-term antiviral antibody responses provide protection from re-infection and recurrence of persistent viruses. Using a polyomavirus (PyV) mouse model, our lab has shown that MyD88-deficient mice generate low levels of virus-specific IgG after the acute phase of infection and that these IgG responses have a skewed isotype distribution with low levels of IgG2a/c. MoreoverMyD88-deficientmice have reduced numbers of long-lived plasma cells in the bone marrow. These studies suggest an important role of MyD88-mediated signaling in long-term antiviral responses. Our lab has shown that T cell-deficient mice can also maintain long-term virus-specific IgG responses following PyV infection. The goal of this thesis is to evaluate the role of innate signaling pathways in maintaining serological memory to persistent virus infection and to elaborate on how long-term antiviral responses can be maintained in an immunocompetent or partially immune compromised, T cell-deficient host.

Regarding T cell-dependent B cell responses, I set out to investigate the upstream and downstream components of the MyD88-mediated pathways required for normal antibody isotype and long-term humoral responses.

IgG2a is a predominant immunoglobulin isotype in most virus infections. Wild type mice, in response to PyV infection, primarily induce antiviral IgG2a with some IgG1. MyD88-deficient mice in response to PyV infection display attenuated levels of virus-specific IgG2a, but normal levels of IgG1. Using Unc93B1 mutant mice (3d mice), which are defective in TLRs 3, 7 and 9

signaling, I show that 3d mice also generated low levels of virus-specific IgG2a following PyV infection. Studies in individual TLR3-/-, TLR7-/- or TLR9-/- mice displayed PyV-specific IgG2a responses similar to wild type responses. TLR7 and TLR9 double deficient mice generated similar skewed antibody isotype responses, where virus-specific IgG2a was reduced compared to wild type mice. This shows that TLR7 and TLR9-MyD88 mediated pathways are important in regulating IgG2a responses during a PyV infection.

To investigate what components downstream of MyD88 are involved in mediating IgG2a responses, I worked with IRF5-deficient mice. IRF5 is a transcription factor that is activated upon stimulation of TLR7 or TLR9-MyD88-mediated pathways. Moreover, IRF5-deficient mice cannot generate autoantibodies specifically of the IgG2a isotype in a mouse lupus model, suggesting that IRF5 plays an important function in mediating class switching to IgG2a. In vitro studies where IRF5-/- B cells were stimulated with TLR7 or TLR9 ligands also generated low levels of γ2a germ-line transcripts, suggesting a B cell-intrinsic role for IRF5 in regulating γ2a germ-line transcription. PyV infection of IRF5-deficient mice resulted in similar skewed isotypes as observed in MyD88-deficient and 3d mice. To investigate a B cell-intrinsic role for IRF5 in regulating IgG2a responses in vivo upon PyV infection, I transferred IRF5-/- B cells and WT T cells into RAG KO mice prior to infection and compared the responses of these mice with mice reconstituted with wild type B6 B and T cells. Diminished numbers of IgG2a+ B cells and reduced levels of virus-specific IgG in mice

reconstituted with IRF5-/- B cells were seen compared to mice reconstituted with wild type B cells.

Regarding the defect in long-term IgG production in MyD88-/- mice upon PyV infection, I conducted studies in IRF5-/-, 3d, single TLR3-/-, TLR7-/-, TLR9-/- and TLR7/9 double deficient mice. These studies reveal an important and redundant role for TLR7- and TLR9-MyD88 signaling in maintaining long-term anti-PyV IgG responses. To determine how MyD88 signaling affects the generation of long-lived plasma cells and memory B cells, I investigated germinal center (GC) responses in MyD88-deficient mice. A defect in GC B cell numbers is observed in MyD88-deficient mice after the acute phase of infection. The GC reaction is essential for the generation and maintenance of long-lived plasma cells and memory B cells. T follicular helper (TFH) cells are absolutely required to generate normal GC. l found reduced numbers of TFH cells in MyD88-deficient mice. Lower numbers of T FH cells suggests that poor T cell help may contribute to the diminished number of GC B cells. However, interaction with B cells is required for the formation of fully differentiated TFH cells. Along with B cell function, MyD88 signaling can affect T cell and dendritic cell function as well. Thus, it is not clear at this point whether the requirement for intact MyD88 signaling for the formation and maintenance of long-term B cell populations is completely B cell-intrinsic.

Some viruses can induce T cell-independent B cell responses, perhaps due to their complex arrays of repetitive antigenic epitopes on virions, coupled

with the induction of innate cytokines. Nevertheless, T cell help is usually necessary for generating long-term antibody responses in the form of long-lived plasma cells and memory B cells. In contrast, our lab has found that T cell-deficient mice infected with PyV develop long-lasting, protective antiviral IgG responses. I questioned whether these mice could generate TI B cell memory cells or long-lived plasma cells. I show that long-lasting anti-PyV antibody in T cell-deficient mice was not due to the presence of long-lived plasma cells or memory B cell responses.

TCRβδ deficient mice, which lack both CD4 and CD8 T cells, had ~10 a times higher virus load persisting in various organs. Therefore, I hypothesized that the high level of persistent PyV antigen, in completely T cell-deficient mice, may activate naïve B cell populations continuously, thereby maintaining the long-lasting IgG responses. Prior to PyV infection, T cell-deficient received wild type CD8 T cells, which reduced PyV loads, and this was associated with decreased levels of antiviral serum IgG over time. As in TCRβδ deficient mice, high PyV loads were detected in the bone marrow, which is the site for B cell lymphopoiesis, I questioned how B cells develop in the presence of PyV antigen and still stay responsive to PyV, generating long-term antiviral IgG responses in the periphery. Studies have shown that self-antigens that trigger both B cell receptor signaling and TLR-MyD88 signaling pathways in the bone marrow lead to the breaking of B cell tolerance and production of autoantibody in the periphery. Thus, we hypothesized that high PyV levels in the bone marrow signal

through both B cell-receptors and TLRs, allowing continuous antiviral antibody production by B cells. Using mice that are deficient in T cells and MyD88 signaling, I found that PyV-specific TI IgG levels gradually decreased, supporting this hypothesis. Thus, high PyV loads and innate signaling together can break B cell tolerance. During a persistent virus infection this can result in sustaining long-term protective T cell-independent IgG responses.

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Fang, C.-M.,Roy, S., Nielsen, E., Paul, M., Maul, R., Paun, A., Koentgen, F., Raval, F., Szomolanyi-Tsuda, E., and Pitha, P. (2012). Unique contribution of IRF5-Ikaros axis to the B-cell IgG2a response. *Genes and Immunity.*

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Raval F., Lien, E., Welsh, R., Szomonlanyi-Tsuda, E. Role of innate signaling pathways in maintaining T cell-dependent antibody responses to a persistent virus infection. (manuscript in preparation)

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AID	activation-induced cytidine deaminase
APC	antigen presenting cells
ASC	antibody secreting cell
ASC KO	apoptosis-associated specklike protein containing a caspase recruitment domain knockout
BAFF	B cell-activating factor
Bcl-2	B cell lymphoma-2
Bcl-6	B cell lymphoma-6
BCMA	B cell maturation antigen
Blimp-1	B lymphocyte-induced maturation protein 1
BM	bone marrow
Bmem	memory B cells
BCR	B cell-receptor
BTK	Bruton’s tyrosine kinase
CD4	cluster of differentiation-4
CD8	cluster of differentiation-8
CD19	cluster of differentiation-19
CD40L	cluster of differentiation-40-ligand
CD40	cluster of differentiation-40
CD45R(B220)	cluster of differentiation-45R
CD138	cluster of differentiation-138
CMV	cytomegalovirus
CpG	cytosine-phosphate-guanine

CSR	class switch recombination
CXCL9	(C-X-C motif)-chemokine-9
CXCL10	(C-X-C motif)-chemokine-10
CXCL11	(C-X-C motif)-chemokine-11
CXCL12	(C-X-C motif)-chemokine-12
CXCL13	(C-X-C motif)-chemokine-13
CXCR3	(C-X-C motif)-chemokine receptor-3
CXCR4	(C-X-C motif)-chemokine receptor-4
CXCR5	(C-X-C motif)-chemokine receptor-5
DC	dendritic cells
DKO	double deficient/knockout
DNA	deoxyribonucleic acid
Dock2	dedicator of cytokinesis 2
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
E-selectin	endothelial-cell selectin
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ER	endoplasmic reticulum
FACS	fluorescence-activated *cell sorting*
FDC	follicular dendritic cells
GC	GC
GC TFH	GC T follicular helper cell
GEF	guanine nucleotide exchange factor
GL	germ-line
GLT	germ-line transcripts
HBSS	Hank’s balanced salt solution
HSV-1	herpes simplex virus-1
i.p.	intraperitoneally

i.v.	intravenously
i.n.	intranasally
ICOS	inducible T-cell co-stimulator
ICOS-L	inducible T-cell co-stimulator-ligand
IFN	interferon
IFNα	interferon alpha
IFNγ	interferon gamma
Ig	immunoglobulin
IKKe	I-kappa-B kinase epsilon
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-21	interleukin-21
IRAK	interleukin-1 receptor-associated kinase
IRFs	interferon regulatory factors
IRF3	interferon regulatory factor-3
IRF4	interferon regulatory factor-4
IRF5	interferon regulatory factor-5
IRF7	interferon regulatory factor-7
IRF8	interferon regulatory factor-8
iTregs	inducible T regulatory cells
KO	knockout
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
LRRs	leucine-rich repeats
MACS	magnetic-activated cell sorting
MAP	mitogen-activated protein
MCMV	murine cytomegalovirus
MCV	Merkel cell polyomavirus

MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
MHV68	murine gamma herpesvirus 68
MITF	microphthalmia-associated transcription factor
mRNA	messenger ribonucleic acid
MTA3	metastasis-associated 1 family member 3
MyD88	myeloid differentiation primary response gene 88
MZ	marginal zone
NF-kB	nuclear factor-kappa B
NIK	nuclear factor kappaB-inducing kinase
NK	natural killer
NKT	natural killer T
NP	4-hydroxy-3-nitrophenylacetyl
NP-CGG	4-hydroxy-(3-nitrophenyl)-acetyl-conjugated to chicken gamma globulin
OVA	ovalbumin
P-selectin	platelet selectin
PAMPs	pathogen-associated molecular patterns
PAX5	paired box protein-5
PBS	phosphate buffered saline
PC	plasma cells
PD-1	programmed death 1
PFU	plaque forming unit
PGK	phosphoglycerate kinase-1
PI3-K	phosphatidylinositol 3-kinase
PNA	peanut agglutinin
PP2A	protein phosphatase 2
Prdm-1	PR domain zinc finger protein 1

PSGL-1	P-selectin glycoprotein ligand 1
PyV	polyomavirus (mouse)
qPCR	quantitative polymerase chain reaction
RAG	recombinase activating gene
RSV	respiratory syncytial virus
RNA	ribonucleic acid
SAP	signaling lymphocytic activation molecule (SLAM)-associated protein
SCID	severe combined immunodeficiency
SDF-1	stromal cell-derived factor-1
SH2D1A	SH2 domain-containing protein 1A
SHM	somatic hypermutation
SLAM	signaling lymphocytic activation molecule
SLE	systemic lupus erythematosus
SV40	simian virus 40
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TBK1	TANK binding kinase-1
TCR	T cell-receptor
TCRαβ	T cell-receptor-alpha/beta
TCRβδ/ TCRαβγδ	T cell-receptor beta/delta
TCRγδ	T cell-receptor-gamma/delta
TD	T cell-dependent
TFH	T follicular helper cells
TGF-β	transforming growth factor beta

TFR	regulatory T follicular helper cell
Th	helper T cells
TI	T cell-independent
TI-1	T cell-independent type I
TI-2	T cell-independent type II
TIRAP	TIR domain containing adaptor protein
TLR1	Toll-like receptor-1
TLR2	Toll-like receptor-2
TLR3	Toll-like receptor-3
TLR4	Toll-like receptor-4
TLR5	Toll-like receptor-5
TLR7	Toll-like receptor-7
TLR9	Toll-like receptor-9
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	tumor necrosis factor receptor (TNFR)-associated factor
TRAF3	tumor necrosis factor receptor (TNFR)-associated factor-3
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing- adaptor-inducing interferon
TSPyV	trichodysplasia spinulosa-associated polyomavirus
VLP	viral-like particles
VP1	viral protein-1
VP2	viral protein-2
VP3	viral protein-3
VSV	vesicular stomatitis virus

WT	wild type
Xid	X-linked immunodeficiency
XBP-1	X-box-binding protein

CHAPTER I: Introduction

A. Rationale and outline for thesis

This thesis will examine the role(s) of innate signaling pathways in the maintenance of long-term antibody responses during a persistent virus infection in the presence and in the absence of T cell help. Antibody responses play multiple roles in virus infection. During the acute phase of infection, efficient antibody production can aid in viral clearance. Additionally, the long-term antibody responses generated during an acute infection by the formation of long-lived plasma cells (PC) and memory B (Bmem) cells help to prevent re-infection with the same virus as well as the recrudescence or reactivation of persistent infections, and the development of disease. I wanted to investigate how long-lasting antibody responses are generated and maintained during a persistent viral infection to achieve low-level viral persistence resulting from the delicate balance between antiviral host mechanisms and virus replication.

To accomplish this goal, I utilized a mouse model of polyomavirus (PyV) infection, in which both T cell-dependent (TD) and T cell-independent (TI) B cell responses to viral antigens are generated (1). PyV infection of wild type (WT) mice results in low-level, life-long viral persistence with no associated disease. In immune compromised mice, such as mice lacking T cells, PyV persists at higher viral loads. These high viral loads in the absence of appropriate anti-tumor responses can result in tumor formation (2). Thus, normal immune responses are

required for a disease-free, life-long persistence of PyV infection in mice. My studies expanded on a previous finding of our laboratory that showed mice deficient in myeloid differentiation primary response gene 88 (MyD88) have defects in long-term humoral immunity in response to PyV infection (3). MyD88 is a vital adaptor linking certain cytokine receptors (e.g. IL-1R/ IL-18R) and toll-like receptors (TLRs) to intracellular signaling pathways. These results strongly suggest that innate receptors are required for sustaining antibody responses. My studies will demonstrate that MyD88-deficient (MyD88-/-) mice have defective memory B cell responses and, as previously established, lack long-lived bone marrow (BM) plasma cells and have skewed immunoglobulin G (IgG) isotypes. I want to determine the molecules upstream and downstream of MyD88 required in preserving long-term antiviral TD B cell responses. Investigating the role of MyD88-mediated pathways, I show that TLR7/TLR9- MyD88-IRF5 pathways are important for antiviral IgG2a/c isotype production and that these pathways are likely to play an essential role in the generation of long-lived IgG responses to virus infection. By addressing the MyD88-mediated mechanisms involved in long-term humoral immunity, I show that a defect in MyD88 is associated with poor maintenance of germinal center (GC) responses. Thus, both innate receptors (TLRs) and helper CD4 T cells play an important role in maintaining long-lasting antiviral antibody responses to a persistent viral infection.

T cell-deficient (TCR (T cell receptor) βδ KO) mice can generate long-lasting virus-specific IgG (Immunoglobulin G) responses following PyV infection,

suggesting that PyV can induce TI B cell memory-like response (1, 4). Generally, TI B cell responses are thought to generate short-term antibody responses. However, other models have suggested that certain TI antigens such as 4-hydroxy-(3-nitrophenyl)-acetyl-Ficoll (NP-Ficoll) can elicit a memory B cell response defined as quiescent, nonsecretory, and isotype switched cells (5). B cell proliferation to NP-Ficoll-secondary challenge was tested, but not recall antibody responses (5).

Using a novel memory B cell assay, I will demonstrate that long-term PyV-specific IgG responses in TCRβδ KO mice are not due to TI recall memory IgG responses but are instead due to continuous activation of nascent B cells emerging from the bone marrow into an environment of persistent virus infection. I will also show that innate MyD88-mediated signaling and persistent high viral loads are essential for maintaining long-lasting TI IgG responses in the PyV model.

This thesis will describe how innate receptors directly and indirectly influence the preservation of long-lasting IgG responses to PyV infection in mice. Furthermore, this thesis will provide an insight into the generation and maintenance of long-term B cell responses that may also be applied to other persistent virus infections.

B. Humoral Immunity: TD versus TI B cell responses

Depending on the type of antigen, B cells can generate TD or TI B cell responses. TD B cell responses generated by protein antigens result in GC formation and the development of memory and long-lived B cell responses. During a viral infection, a B cell requires two signals to be fully activated. The viral antigen cross-linking the BCR (B cell receptor) provides the first signal, while the second signal is from helper T cells. With the help from specialized CD4 T cells known as T follicular helper cells (TFH), GC are formed, where B cells go through massive rounds of proliferation, somatic hypermutation (SHM), class switch recombination (CSR) and a selection process that results in affinity-matured, antigen-specific long-lived B cell populations. (Figure 1.1)

TI antibody responses are divided into two basic categories: TI-type I (TI-1) or TI-type II (TI-2) antibody responses. TI antibody responses are generated outside the follicle in response to various TI antigens. TI-1 antibody responses are generated by B cell mitogens (e.g. lipopolysaccharide (LPS)) that cause polyclonal B cell proliferation. Antigens with repetitive structures such as bacterial polysaccharides generate TI-2 antibody responses (6). TI-2 antigens strongly activate the B cell receptor (BCR) and T cell help is not required. TI-2 antigens are identified by their inability to induce an antibody response from BTK deficient (Xid mice) B cells (7). BTK is important in B cell maturation and for signaling

Figure 1.1- General schematic of TD and TI B cell responses

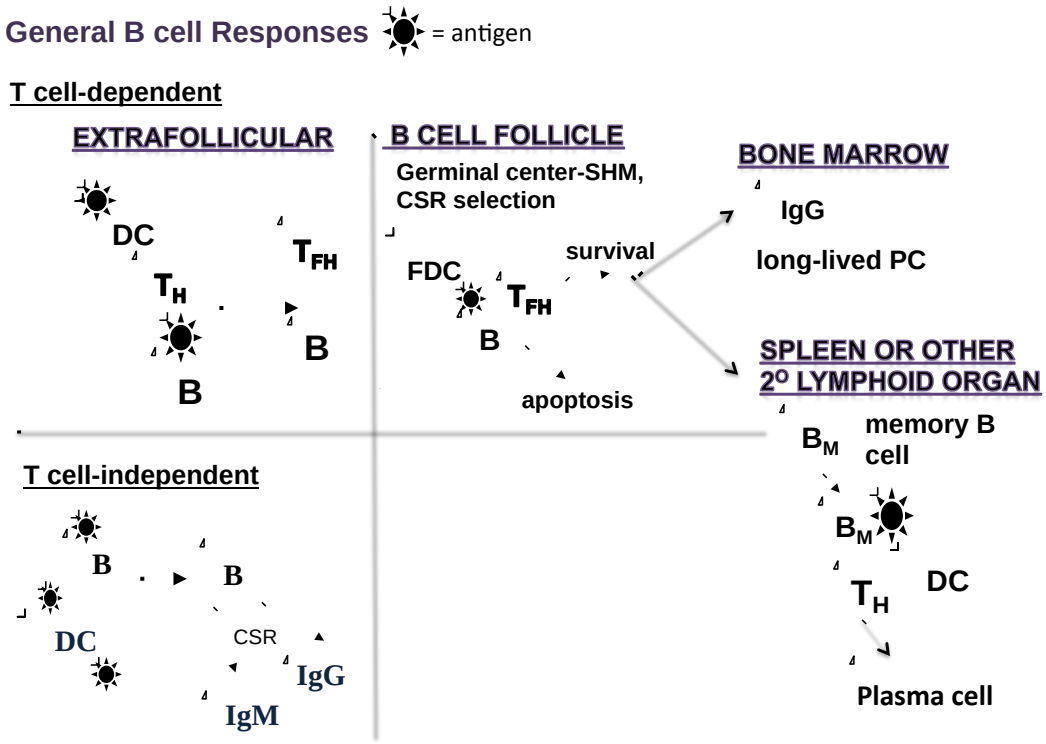


Figure 1.1: B cells generate antibody either in the presence (upper part) or absence (bottom part) of T cell-help. Long-term T cell-dependent antibody responses require the formation of GC responses, while T cell-independent antibody responses are generally thought to be short-lived.

downstream of the B cell receptor. In a TI B cell response, activation of B cells requires interaction of BCR and/or TLRs with antigen, along with help from cytokines. TI B cell responses are generally thought to be extrafollicular, short-lived and devoid of GC. (Figure 1.1) Under certain conditions such as immunization with high doses of TI antigen (NP-Ficoll), GC can form (8). However, these TI GC are rapidly induced, have a short duration, and do not support SHM in the absence of T cell help (9).

C. B cell subsets

B1 and B2 B cell subsets are important in generating TI and TD B cell responses. B1 cells are divided into two types of B cells: B1a and B1b cells. B1b cells generate a TI B cell response and develop in the fetal liver. B1a and B1b cells are self-renewing and are generally found in the peritoneal cavity (6, 10). B1b and B1a cells represent an innate response by B cells and these cells produce natural antibody (11). The surface expression of CD5 distinguishes B1b (CD5-) and B1a (CD5+) cells (6, 10).

B2 B cells include marginal zone (MZ) B cells and the follicular B cells. MZ B cells are found surrounding the lymphoid follicle in the spleen and lymph nodes. MZ B cells, in the spleen, sense blood-borne antigens and provide a first line of B cell defense against antigens. MZ B cells generate a rapid, early, TI B cell response. MZ B cells are different from B2 B cells by expressing lower levels of CD23, a low affinity Fc epsilon receptor, and high levels of CD21, a

complement receptor. (11-13) B1a, B1b and MZ B cells have an important role in TI B cell responses. (6)

Follicular B cells are found within the B cell follicles in secondary lymphoid organs. The activation of follicular B cells is usually dependent on T cell help. Follicular B cells are phenotypically described as cells with high levels of CD21 and CD23. Activation of follicular B cells leads to GC responses, which are crucial for the formation of long-lived B cell populations. (11)

D. Antibody function and antiviral B cell responses

B cells that function in a TD or TI manner have multiple effector functions. They secrete antibodies, secrete soluble immunomodulatory factors (cytokines) and affect the activity of other cells via surface molecules. B cells are APCs that can activate T cells by their major histocompatibility complex (MHC) and processed peptide complexes. Antibodies are the secreted form of the BCR. Immunoglobulin (antibody) is, for the most part, a homodimeric protein that contains 2 heavy chains and 2 lights chains that are connected by disulfide bonds. Antibody can also be described by the antigen-binding region (Fab or variable region) and by their Fc (or constant) region.

Secreted antibodies can circulate throughout the body and provide protection against pathogens by multiple mechanisms. For example, antibodies can be neutralizing,(14) which means that antibodies can abolish the ability of pathogens to be infectious. Specifically, during a virus infection, antibodies can attach to a

viral attachment protein and prevent viral entry into host cells. If a virus were to attach, neutralizing antibodies can interfere with virus penetration and un-coating of the virus. Second, antibodies can bind to pathogens and attract phagocytic cells (macrophages) and mast cells, which can help control the pathogen. Thirdly, antibodies can induce antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC occurs when virus-infected cells are coated with IgG, which allows NK cells to bind the Fc fragment of the antibody via Fcγ receptors. Crosslinking the Fc receptors signals the NK cell to kill the target cell. (11)Finally, antibodies coated on pathogens can activate complement-mediated lysis of infected cells (11). In response to viral infections, along with the mechanisms mentioned above, antibodies can prevent viral pathogenesis. If virus enters cells, antibodies can interrupt the virus life cycle within cells, such as preventing viral protein and ribonucleic acid (RNA) synthesis. Antibodies specific for a surface glycoprotein of Sindbis virus resulted in decreased viral protein synthesis and a cessation to infectious virion production (15). Antibodies can also make virus infections more severe, altering tropism by enabling the internalization of virus particles into various cell types. For instance, a virus that normally does not target macrophages would not initiate virus-specific innate responses by macrophages. However, specific antibody-virus complexes can be internalized by Fc-receptor-expressing macrophages, which can trigger a strong innate response against the virus. An example of this process is an antibody-dependent enhancement in secondary dengue infection (16).

E. Germ-line gene rearrangements: B cell development, CSR and SHM

In order for B cells to recognize specific pathogens and elicit effector functions, BCRs must go through several germ-line gene rearrangements during development in primary and peripheral lymphoid organs. B2 B cells develop in the bone marrow (primary lymphoid organ) and then migrate to secondary lymphoid organs. Similar to TCR gene rearrangements, the BCR rearranges its heavy and light chains via VDJ recombination. BCR gene rearrangements take place within the bone marrow. The immunoglobulin heavy chain is rearranged first in the early pre-B cell stage in the bone marrow. If this rearrangement is successful, surrogate light chain proteins form the pre-BCR which signals to stop heavy chain rearrangement and to begin light chain rearrangement. There are two light chain genes present in mouse and humans. The kappa light chain rearranges first, and if unsuccessful, meaning that the rearrangement does not result in a light chain protein, then the lambda chain is rearranged. It is during these VDJ gene arrangements that the variable region of the antibody is generated. Once heavy and light chain gene rearrangement has successfully occurred, then the immature B cell, which expresses IgM goes through central tolerance (11, 17). Central tolerance is a process that takes place during B cell development in the bone marrow. Immature B cells that recognize self-antigens with high affinity are deleted or changes occur to BCR specificities. Immature B cells that strongly react to self-antigens have four end-fates: clonal deletion, receptor editing, anergy or immunological ignorance. The B cells that survive

central tolerance can migrate out of the bone marrow. In the periphery, B cells further mature through two transitional stages (T1 and T2), which results in surface expression of IgD on mature naïve B cells. (11, 18)

B cells coming out of the bone marrow express IgM, and express IgD as they mature. Once activated through the BCR, B cells lose IgD expression and can express IgM or undergo class switching and secrete different antibody isotypes. The cytokine environment and T cell help dictates which isotype(s) the B cells will generate. Class switch recombination (CSR) is a change in the constant region of the antibody heavy chain. Activation-induced cytidine deaminase (AID) is required for CSR (19). Changes in the constant region of the heavy chain results in a switch in the class of antibody, generating various isotypes such as IgG1, IgG2a, IgG2b, IgG3, IgA or IgE (18, 20). CSR requires germ-line (GL) transcription at the targeted constant region of the antibody to activate specific isotype promoters in response to cytokines, antigen and other co-stimulatory signals. In vitro models have shown that cytokines, along with LPS, can direct the generation of specific isotypes by regulating the expression of GL transcripts (GLTs) (20). IL-4, transforming growth factor-beta (TGF-β), and IFNγ can regulate specific isotype production. IL-4 induces the production of IgG1, IgG4 and IgE. Adding IFNγ, in addition with LPS, produces high levels of IgG2a. B cells stimulated with TGF-β can generate IgA (20). The variable region of antibodies is further mutated through somatic hypermutation (SHM), which can

help generate antibody with higher affinity for antigen. CSR and SHM occur during a GC response in a TD B cell response.

F. TD B cell response: GC reaction

GC B cell responses are generated with the help from CD4 T cells. GC responses are necessary for the generation of high affinity, class switched, long-lived plasma cells and memory B cells.

GC are formed in secondary lymphoid organs such as the spleen and lymph node. GC are developed when antigen-activated B cells, along with help from CD4 cells, seed within the center of the follicle. Once the GC matures, two major compartments are formed: the light and dark zones. The light zones are found within secondary lymphoid organs, towards the outer portion of the GC follicle, near the source of antigen, while the dark zones are found within the inner zones of GC follicle. The dark zone consists of centroblasts (proliferating GC B cells) and the light zone is made up of centrocytes, which are GC B cells that can further differentiate into long-lived PCs, and Bmem cells. The light zone contains follicular dendritic cells (FDCs) and GC T follicular helper cells (GC TFH cells) (21, 22). FDCs can bind large quantities of antigen in immune complexes, through which GC B cells test BCR binding affinity to antigen. Generally, proliferation and SHM occurs in the dark zone, while the GC B cell selection based on affinity maturation and further differentiation happens in the light zone. GC B cells can migrate between the dark and light zones of the GC (22). GC B

cell selection is important for the selection of high affinity B cells that will form the long-lived PC and memory B cell populations.

Plasma cells can be generated at multiple stages following a B cell's encounter with antigen. B cells can differentiate into extrafollicular plasma cells upon initial encounter with antigen, generating short-lived plasma cells. Alternatively, activated B cells can migrate within the B cell follicle and generate GC responses. GC B cells are seen in mice starting around 5-6 days post exposure to antigen. Once the GC has formed, proliferating B cells (early G1 phase) first enter the dark zone in response to the chemokine stromal cell-derived factor-1 (SDF-1) that attracts (C-X-C motif)-chemokine receptor-4 (CXCR4) positive B cells. After hours in the G1 phase in the dark zone, B cells (centroblasts) begin to downregulate CXCR4. Then, (C-X-C motif)-chemokine receptor-5 (CXCR5) upregulation allows GC B cells to migrate into (C-X-C motif)-chemokine-13 (CXCL13) positive, FDCs-rich light zones. It is thought that centroblasts have low levels of surface Ig (BCR), while centrocytes increase their surface Ig expression. Also within the light zones, the BCR is further mutated in the Ig variable region through SHM creating B cells with varying affinities for antigen. GC B cells scan FDCs, binding antigen, and then go through a GC B cell selection process by presenting antigen to GC TFH cells (23). Centrocytes with a low-affinity BCR that triggers inadequate BCR signaling after antigen exposure or harbor a self-reactive BCR are induced to undergo apoptosis. GC B cell apoptosis occurs in a Fas-FasL dependent manner (24). Thus, some important

markers that are used to identify GC B cells are Fas(25), GL-7 (a T and B cell activation marker) (26) and peanut agglutinin (PNA). B cells that have high affinity or strong BCR signaling for antigen will survive and further differentiate into long-lived plasma cells or memory B cells. (22) Memory B cells can also differentiate into plasma cells upon re-encountering the same antigen.

It is not clear what differentiates short-lived plasma cells from long-lived plasma cells. However, there is a transcriptional program that dictates the formation of all forms of plasma cells (27). (Figure 1.2) Transcription factors paired box protein-5 (PAX5), microphthalmia-associated transcription factor (MITF), B cell lymphoma 6 (Bcl-6) and metastasis-associated 1 family, member 3 (MTA-3) can play a role in inhibiting plasma cell development. PAX5 is required for B cell development and for GC responses, but it inhibits X-box-binding protein 1 (XBP-1), which is highly expressed in plasma cells. Bcl-6 is required for GC B cell formation, but it is a negative regulator of B lymphocyte-induced maturation protein 1 (Blimp-1), a transcription factor crucial for plasma cell development. Bcl-6 and MTA3 can work together to mediate the repressive activity of Bcl-6 (27).

The generation and preservation of GC responses require different molecules and cell populations. Bcl-6 is a transcription factor that is

Figure 1.2- Gene expression program in GC B cells and plasma cells

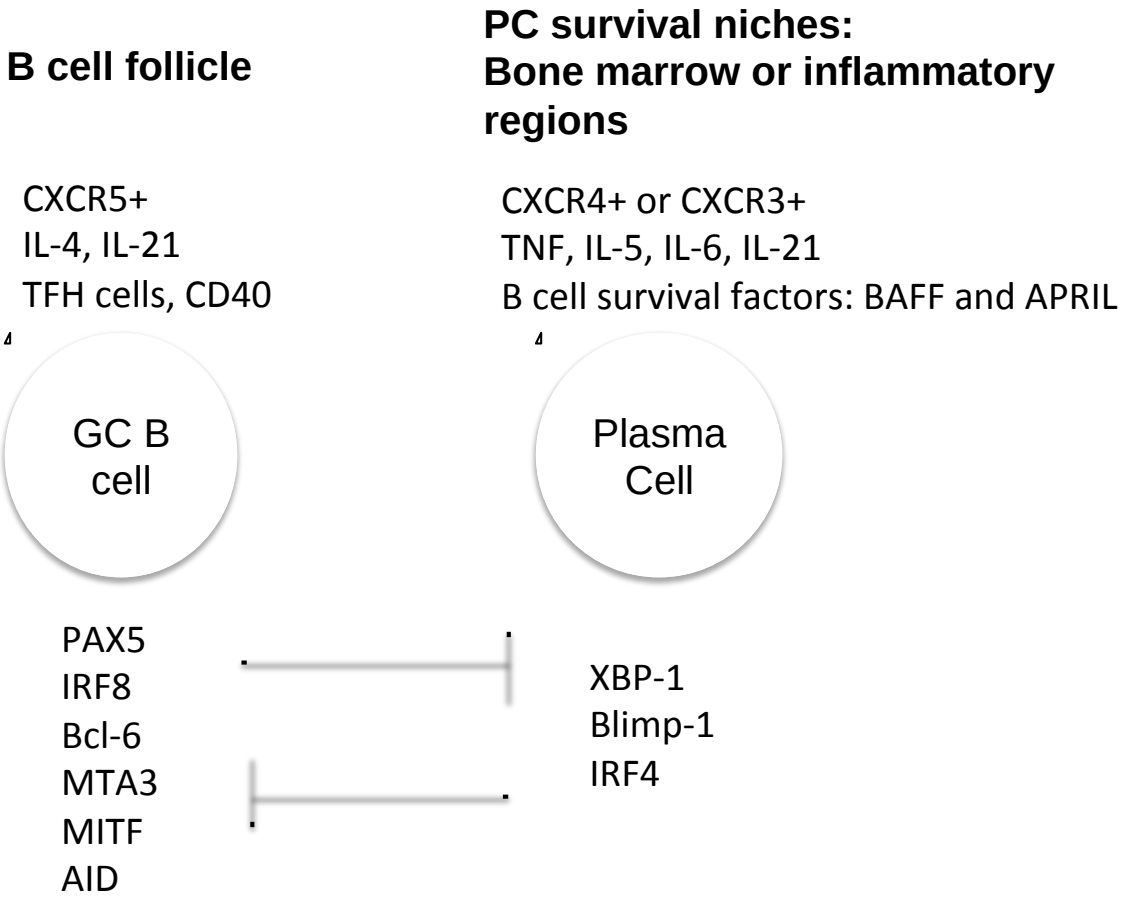


Figure 1.2: Differentiation of GC B cells into PC requires a unique gene expression profile. Transcription factors that are important for GC B cells need to suppress factors required for PC differentiation. Certain chemokine receptors and cytokines also attract B cells into either the B cell follicle or PC survival niches.

essential for the generation of GC B cells (28). The formation and maintenance of GC depend on signals from helper T cells and CD40. Specialized CD4+ T cells known as TFH cells are required for the maintenance of GC responses. TFH cells will be discussed in more detail in the T cell section. TFH cells provide costimulatory signals such as CD28 and inducible T-cell co-stimulator (ICOS). Cytokines secreted by helper T cells such as IL-4 and IL-21 are required for preserving the GC response. CD40-CD40 ligand interactions are crucial because CD40 signaling induces AID expression, as does IL-4 signaling. CD40 also helps GC B cells survive by increasing expression of anti-apoptotic molecule, B cell lymphoma-extra large (Bcl-xL). GC TFH cells, fully differentiated and a specialized form of TFH cells present in the GC, help in the survival and selection of high affinity centrocytes (GC B cells located in the light zone of GCs), preserve the GC response by recycling some centrocytes into centroblasts by providing signals that can lead to the differentiation of long-lived plasma cells and Bmem cells. (18, 20, 22)

G. Two forms of B cell memory: Long-lived Plasma Cells and Memory B cells

Long-lived plasma cells and memory B cells maintain serological memory. Long-lived plasma cells and memory B cells are both derived from GC B cell responses (18, 22, 29). Long-lived plasma cells reside in the bone marrow and continuously secrete antibody. Long-lived plasma cells can provide protective

antibody responses for a long duration, in many cases for a lifetime. Upon lymphocytic choriomeningitis virus (LCMV) Armstrong infection, mice can generate protective antibody responses that last one-year post infection. (30) Long-lived PCs express the transcription factor, Blimp-1, and CD138, also known as syndecan-1. (18, 31) Memory B cells can be found in secondary lymphoid organs such as the spleen, lymph node or Peyer's patches. When memory B cells re-encounter antigen, these cells can differentiate into plasma cells and secrete antibodies at a faster rate. Human memory B cells are defined by high CD27 expression, but there is no known marker that defines murine memory B cells (32). For this reason, I developed an adoptive transfer assay that can detect memory B cells based on their recall function. This assay will be discussed further in the materials and methods section.

It is not clear what signals are required for the differentiation of GC B cells into either long-lived plasma cells or memory B cells. Many groups have generated different models to understand this differentiation process. Some believe that the generation of long-lived plasma cells and memory B cells is a stochastic, random mechanism (24). Evidence against the stochastic model shows that the over-expression of Bcl-2, an anti-apoptotic factor, in B1 B cells leads to memory B cell differentiation in a GC-independent manner (33). This suggests that over-expression of an anti-apoptotic factor can change the output of plasma cells and memory B cells (33). Studies have suggested that the development of long-lived plasma cells and memory B cells is dependent on the

affinity of their receptors. Long-lived plasma cells have been shown to have high affinity receptors, while memory B cells can have low affinity receptors. Memory B cells can be of low affinity, un-switched and appear earlier than long-lived plasma cells (24). It was also reported that the quality and quantity of signals from GC TFH cells to GC B cells could determine GC B cell fates (34). For instance, T cells were shown to polarize to sites where MHC:peptide complexes were abundant. Thus, those GC B cells that have high affinity for antigen can more frequently present to GC TFH and receive signals for their differentiation than GC B cells that have low affinity for antigen (34). Other studies have shown that CD40L signaling is important for memory B cell formation, but not for long-lived bone marrow plasma cells, suggesting that different signals from helper T cells can influence the fate of a GC B cell (35).

Even though the exact mechanism of how GC B cells choose their fate is unclear, several different molecules have been found to be important for the maintenance of long-lived B cell populations. Transcription factors Bcl-6 and Pax5 need to be suppressed in order for GC B cells to begin to differentiate into long-lived plasma cells. (18) In addition to Blimp-1 and XBP-1, plasma cell survival requires other signals including cytokines (tumor necrosis factor(TNF), IL-5, IL-6, IL-21), chemokines, and B cell survival factors, B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL). BAFF and APRIL bind, with different affinities, to the B cell maturation antigen (BCMA) receptor, BAFF-receptor and transmembrane activator and calcium-modulator and cyclophilin

ligand interactor (TACI). (Figure 1.2) These B cell survival factors have different roles in promoting B cell activation and function and also in prolonging the persistence of plasma cells in the bone marrow. (36) It has also been shown, in an influenza virus (flu) model, that long-term antibody responses in mice require TACI. (37)

IL-6 may help in activating PR domain zinc finger protein 1(*Prdm1),* the gene that encodes Blimp-1. The migration of plasma cells to the bone marrow requires signals from chemokines such as CXCL12. Reduced expression of CXCR5 and the up-regulation of CXCR4 and CXCR3 are essential for plasma cells to migrate to their survival niches. CXCL9, CXCL10 and CXCL11 are ligands for CXCR3, while CXCL12 attracts CXCR4 expressing cells. CXCR3 is up-regulated on plasmablasts in the presence of inflammatory cytokines such as IFNγ (18). CXCR3 plays a role in recruiting B cells to inflamed tissues. CXCL12 is secreted by the stroma in the bone marrow and also by the red pulp within spleens, which attract post-GC B cells to the bone marrow or other lymphoid organs such as the spleen. In the absence of CXCR4, plasma cells are not detected in the bone marrow. (18, 29) Endothelial-cell selectin (E-selectin) ligand, platelet selectin (P-selectin) ligand, and CD44 have been shown to be important adhesion molecules on plasma cells. (18) These adhesion molecules help traffic the plasma cells to their survival niches: either inflammatory regions (for example inflamed synovial tissue) or the bone marrow. Once in the bone marrow, long

lived plasma cells are provided with survival signals from the bone marrow environment.

All of the above mentioned interactions and steps are necessary for the formation and maintenance of naturally functioning B cell memory (23). TD B cell responses not only require helper CD4 T cells but also signals from innate receptors such as TLRs. TLR signaling can be important for long-lasting TD and TI B cell responses. In the next two sections, the role of T cell help and TLR signaling in mediating B cell responses will be further discussed.

H. Role of T cell help for TD B cell responses-TFH cells

CD4 T cells are a heterogeneous population of T cells which recognize antigen in the context of MHC class II (major histocompatibility complex class II) and respond by secreting a number of different cytokines. Unlike CD8 T cells, CD4 T cells are rarely associated with cytolytic function. However, they do play a role in supporting CD8 T responses and B cell differentiation into GC B cells and subsequently memory B or plasma cells (38).

Naïve CD4 T cells can differentiate into various T helper (Th) cell subsets depending on the antigen stimulation and cytokine environment (39). Each of the CD4 helper subsets are defined by a unique master transcription factor that dictates the helper T cell lineage (40). IFNγ and IL-12 trigger naïve CD4 T cells to differentiate into Th1 cells, which secrete IFNγ, lymphotoxin α, and IL-2 (38). Th1 cells up-regulate a transcription factor, T-bet. Th2 cells differentiate in the

presence of IL-4 and IL-2 and up-regulate the transcription factor GATA-3. The signature cytokine secreted by Th2 cells is IL-4, but IL-5, IL-13, IL-25 and IL-10 can also be generated (39). Th1 cells provide defense against intracellular pathogens, while Th2 cells against extracellular parasites. Other T helper cell populations include Th17 cells and inducible T regulatory cells (iTregs). Th17 cells are generated by the presence of TGFβ, IL-6, IL-21 and IL-23. Th17 cells up-regulate the transcription factor, RORγt and produce IL-21, IL-17α and IL-22 (41). iTregs are developed by TGF-β and IL-2, up-regulate FoxP3 and secrete TGF-β, IL-35 and IL-10. iTRegs play an important role in immune tolerance (38, 42).

A distinct subset of CD4 T cells with a unique phenotype and cytokine production was identified in the GC of human tonsils (43). TFH cells are specialized to help generate and maintain the GC B cell response. GCs will develop and begin forming the dark and light zones but, without the help from TFH cells, the GC falls apart (22). TFH cells also provide important co-stimulatory and differentiation signals to GC B cells as previously described.

TFH cells have been studied in both humans and mice. Human TFH cells express certain markers that are not expressed by mouse TFH cells, such as CD57 (21, 22). In humans and in mice, there are multiple different types of TFH cells and these are described by their unique location in the interfollicular zone, within the follicle or within the light zone of the GC (44). Different TFH cells are also defined by various cytokine production (21, 23). Expression of CXCR5, Bcl-6

(45) and the ability to help GC B cells during TD B cell responses are common features of human and mice TFH cells (28).

Many of the molecules expressed on TFH cells that are important for B cell help are expressed by all T helper cells (e.g., CD40L), but TFH cells also express transcription factors and chemokine receptors which distinguish them from other helper T cells. CXCR5+ CD4 T cells express a gene profile that is distinct from Th1 and Th2 cells (46). Th1 and Th2 cells can also provide some help to B cells, but TFH cells have a unique role in maintaining TD GC B cell responses (21, 44).

TFH cells express important co-stimulatory molecules necessary for GC B cell survival and differentiation such as CD40L and ICOS. In the absence of CD40-CD40L or ICOS or ICOSL, GC are not developed (47). Upon activation, TFH cells also secrete cytokines including IL-10, IL-21 and IL-6. IL-10 is an important growth and differentiation factor for B cells in humans and is secreted upon activation of ICOS signaling (48). In the absence of both IL-6 and IL-21, virus-specific antibody responses are abolished. IL-6 plays an important role in the differentiation of plasmablasts, mainly of extrafollicular B cells, and also helps up-regulate Blimp-1. IL-21 is important for plasma cell differentiation from GC B cells (49).

Bcl-6, a transcriptional repressor, is one factor that defines TFH cells (45). The expression of Bcl-6 is tightly regulated and mainly expressed in GC B cells and TFH cells. Bcl-6, within B cells, is the transcription factor that determines a

GC B cell lineage commitment. Bcl-6 also negatively regulates Blimp-1. The dynamics between these two opposing transcription factors determine a GC B cell fate or a long-lived plasma cell-lineage commitment. (28, 31, 50)

TFH cells also express high levels of signaling lymphocytic activation molecule (SLAM)-associated protein (SAP). SH2 domain-containing protein 1A (SH2D1A) encodes SAP, a 14kDa adaptor protein (51). The association of SAP with SLAM family receptors is central for its function as an adaptor molecule (51). The SLAM family includes Ly9, Ly108, CD84, 2B4, CRACC and SLAM (CD150), each of which is expressed on T cells, B cells, macrophages and dendritic cells (51). SAP-deficient (SAP $^{-/-}$) mice lack natural killer T (NKT) cells, have impaired NK cell and cytotoxic T lymphocyte (CTL) cytolytic activity, are reduced in Th2 type cytokine production, have major defects in the formation of GCs and have reduced long-term humoral immunity (3, 51). SAP$^{-/-}$ mice acutely infected with LCMV show comparable levels of IgG+CD138+ plasma cells compared to wild type mice (52). However, at day 30 post LCMV infection, SAP$^{-/-}$ mice have approximately 150 fold fewer LCMV-specific memory B cells per spleen than a WT mouse under the same conditions (52). This indicates that SAP $^{-/-}$ mice are incapable of developing or maintaining a long-term humoral response against LCMV. Thus, the role of SAP within TFH cells is crucial for normal GC B cell responses in a TD-manner. TFH cells can express other molecules as well such as PD-1. Staining with antibodies against P-selection glycoprotein ligand 1 (PSGL-1) and Ly6C, a hematopoietic cell differentiation antigen, can also be

used to detect TFH cells. PSGL-1lo and Ly6Clo splenocytes expressed TFH cell markers such as CXCR5, ICOS and PD-1 (53).

The exact mechanism required for TFH cell differentiation is still unclear. As mentioned before, the cytokine environment and co-stimulatory signals provided by antigen presenting cells can help differentiate naive CD4 T cells into various T helper cells. It is not clear whether the TFH cells are a unique lineage or if they differentiate from Th1 or Th2 polarized cells (21). IL-6 and IL-21 are necessary but not sufficient for the generation of TFH cells indicating there are other cytokines that are required for TFH cell development (49).

It is believed that interactions with B cells are also necessary for the generation and full differentiation of TFH cells. CD4 T cells transferred into B cell KO mice (μMT KO) were not able to differentiate into TFH cells, suggesting a major role of B cells in TFH cell development. (31) Furthermore, the absence of ICOS-L (ligand) hinders TFH cell development. The expression of ICOS-L on B cells is regulated by the noncanonical NF-κB pathway that is induced upon the activation of the BAFF receptor on B cells (54). In the absence of an important signaling component within the noncanonical nuclear factor-kappa B (NF-κB) pathway (Nuclear factor kappaB-inducing kinase (NIK) knockout mice), there are defects in the development of TFH cells. TFH cell defects were absent upon the delivery of recombinant ICOS-L to NIK KO animals (54). Thus, B cells play an important role in the generation of TFH cells.

I. Role of TLRs in B cell responses

TLRs play an important role in innate and adaptive immune responses. The precise role of these innate receptors in mediating long-term antiviral protection was further investigated in this thesis.

TLRs are innate receptors that recognize pathogens by detecting pathogen-associated molecular patterns (PAMPs) (55, 56) Twelve mammalian TLRs have been identified which recognize specific ligands and activate downstream adaptor proteins. TLRs recognize various types of ligands and can be found in different cellular locations. Some TLRs are found on the surface of cells (TLRs 1/2, 4,5,6,8,10,11,12) and others in endosomes (TLRs 3,7 and 9). The sensing domain of extracellular or endosomal TLRs contains leucine-rich repeats (LRRs), a transmembrane region and a cytoplasmic domain. LRRs fold into a sickle-shape sensing particular ligands. (57, 58) Some surface TLR ligands identified thus far include triacylated lipoproteins (TLR1/2), diacylated lipoproteins (TLR2/6), LPS (TLR4) and flagellin (TLR5). Some surface TLRs are also able to sense viral proteins. For example, TLR2 and TLR4 can recognize viral protein from CMV, HSV-1, and measles virus (16). Endosomal TLRs are nucleic acid sensors that recognize single-stranded ribonucleic acid (ssRNA) (TLR7), double-stranded ribonucleic acid (dsRNA)(TLR3), and cytosine-phosphate-guanine (CpG)–rich deoxyribonucleic acid (DNA) (TLR9) (57). (Figure 1.3)

Figure 1.3- Schematic of TLR signaling pathway

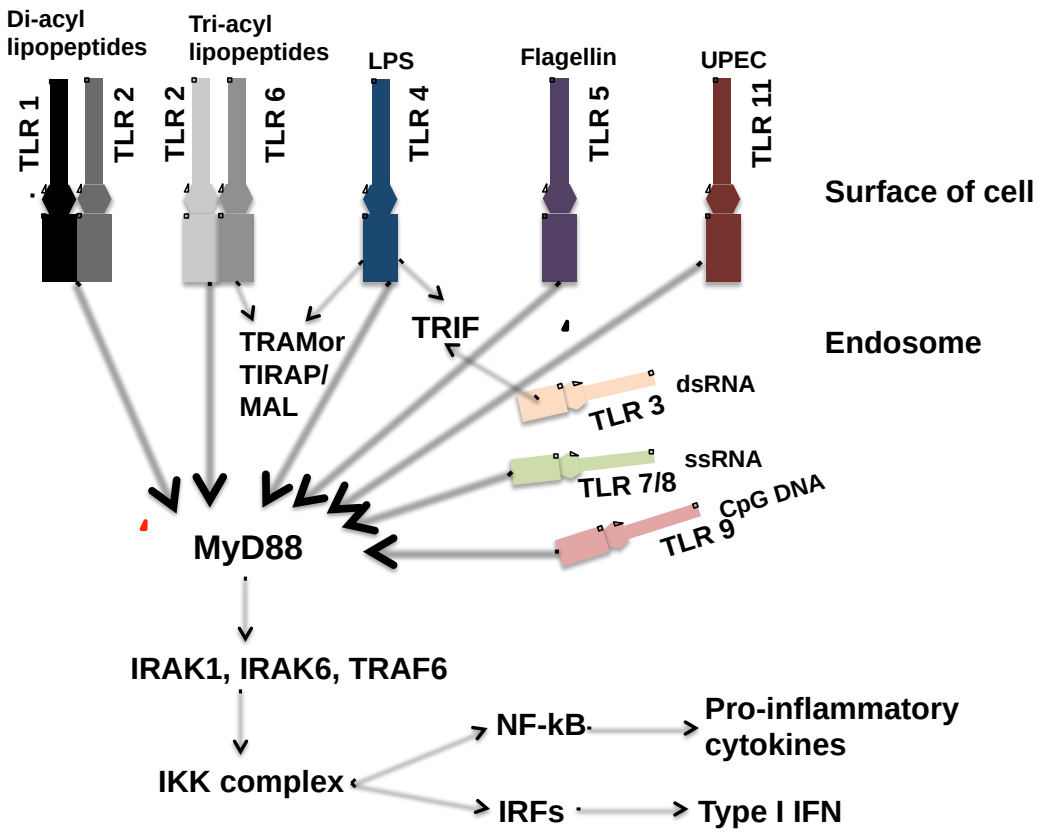


Figure 1.3: Most of the TLRs signal through MyD88, IRAK, TRAF proteins, and the IKK complex to activate NF-kB and IRFs.

Upon ligand engagement, TLRs activate downstream adaptor proteins, such as MyD88, that mediate signaling downstream resulting in activation of transcription factors such as NF-kB, mitogen-activated protein (MAP) kinases and interferon regulatory factors (IRFs). The activation of these transcription factors results in the production of inflammatory cytokines (e.g. IL-1, IL-12, IL-6, and TNF) or type I interferon (IFN). (55, 59) (Figure 1.3)

IRFs are a family of transcription factors. Various IRF molecules can form homodimers or heterodimers upon activation that can regulate type I interferon responses and activate other IFN-induced genes or inflammatory responses. There are 9 IRF genes, two of which, IRF3 and IRF7, have been found to have an important role in type I interferon production. IRF7 primarily is involved in interferon alpha (IFNα) production (60). In addition to innate responses, IRF4 and IRF8 are essential for B cell activation and differentiation. IRF8 is expressed in GC B cells, whereas IRF4 is essential for the generation of plasma cells. (Figure 1.2) However, the role of IRF5 in B cells is not well understood. IRF5 is activated by its interaction with a complex that consists of MyD88 tetramers, interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK4, and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) following TLR activation (61). Besides MyD88, IRF5 can also be activated by TANK binding kinase-1 (TBK-1). Once activated, IRF5 can form homodimers, translocate into the nucleus and activate transcription of type I interferon and inflammatory cytokines (62). Unlike IRF3 and

IRF7, IRF5 is primarily involved downstream of TLR-MyD88 pathways such as TLR7 and TLR9 pathways (62, 63) (64).

Within DCs, activation of specific TLRs and transcription factors leads to the production of inflammatory cytokines, dendritic cell migration to lymph nodes, increased antigen presentation, and up regulation of co-stimulatory molecules resulting in normal T cell priming and activation (56). It has been shown that in the absence of MyD88, in response to TLR4 ligands, DCs can still induce NF-kB and certain IRFs, but inflammatory cytokines are not generated (55). Thus TLR4 can signal both in a MyD88-dependent and independent fashion.

Some TLRs can activate NF-kB and induce IFNα and IFNβ in a MyD88-independent manner. TLR4 can signal through other adaptor proteins known as TIR domain containing adaptor protein (TIRAP, also known as MAL), TIR domain containing adaptor-inducing IFN (TRIF), and TRIF-related adaptor molecule (TRAM). (65) TLR3 is the only TLR that does not signal through MyD88, but signals only through TRIF. TRIF can form a complex with certain kinases such as TBK-1 and IkappaB kinase-epsilon (IKKε) and other proteins such as TRAF3 that can help induce IRF3, IRF7 or NF-kB (55, 65).

TLR signaling can affect B cell activation indirectly and directly. The absence of MyD88 leads to poor GC responses in response to HSA-LPS and low antibody production in response to various antigens such as flagellin. (66) This suggests that TD B cell responses not only require help from CD4 T cells, but may also require intact TLR signaling within B cells. It has been shown that TLR

signaling in B cells affects antibody class switching and also enhances antigen presentation by B cells (66). The importance of TLR-MyD88-mediated signaling for antigen-specific B cell responses to autoantigens that respond to TLR ligands and to other inert antigens such as ovalbumin (OVA) conjugated to LPS, and 4-hydroxy-(3-nitrophenyl)-acetyl-conjugated to chicken gamma globulin (NP-CGG) in alum has been shown (67). An immune response to a virus infection is different from an immune response generated by a protein antigen. In a flu model, it was shown that MyD88 signaling was important for generating flu-specific IgG2a responses (68). Furthermore, in other studies, B cell deficient mice reconstituted with MyD88-/- B cells failed to produce virus-specific IgG in response to respiratory syncytial virus (RSV) infection (69).

These studies show that innate signaling contributes to adaptive B cell responses to various antigens. Several studies have described the immune responses that are required to control acute and chronic viral infections with high virus load and pathogenicity. In contrast, how immunocompetent hosts successfully control a low-level, persistent viral infection without causing disease is not well understood. To elucidate the role of innate and adaptive immune responses in controlling a persistent virus infection, I utilized a model of mouse polyomavirus (PyV) infection.

J. Mouse polyomavirus (PyV) infection model

PyV, a DNA tumor virus, is a useful model to study immune responses to a low-level, persistent infection in its natural host. PyV is a member of the *Polyomaviridae* family of viruses. Polyomaviruses share many common features in terms of their structure and they have strict host specificity. Polyomaviruses can be divided into three genera: *Orthopolyomavirus, Wukipolyomavirus and Avipolyomavirus*. *Orthopolyomavirus* and *Wukipolyomavirus* contain the mammalian viruses while the genus *Avipolyomavirus* contain the avian-viruses. Simian virus 40 (SV40), 4 human viruses: BK, JC, Merkel cell polyomavirus (MCV), trichodysplasia and spinulosa-associated polyomavirus (TSPyV), rodent polyomaviruses, and mouse polyomavirus are some viruses that make up *Orthopolyomavirus*. Four human polyomaviruses:, WU, KI, HPyV6 and HPyV7 are included in the genus *Wukipolyomavirus* (70).

Polyomaviruses are common among humans. Most humans are infected by a number of different polyomaviruses such as the JC, BK, KI, WU and Merkel cell carcinoma (MCV) viruses. Studies have shown similar seropositivity to BK, WU, KI and MCV viruses in healthy adult populations and in children under 21, suggesting that primary PyV exposure may occur in childhood (71). These infections are mostly sub-clinical and asymptomatic, but in people with severe immunodeficiency some human polyomaviruses (BK, JC and MCV) can cause severe pathology. For example, BK virus has been shown to be associated with the failure and rejection of kidney transplants (72) and with BK virus-associated

nephropathy in patients receiving immunosuppressive drug treatment (72). JC virus has been associated with progressive multifocal leukoencephalopathy (PML) under immune suppressive conditions (73-75). Of note, MCV is different from other human polyomaviruses as it is close to mouse PyV in phylogeny. Thus far, MCV is the only PyV directly linked to tumor formation in humans (76).

Mouse polyomavirus (PyV) was the first polyomavirus discovered as the agent responsible for inducing multiple ('poly') tumors ('oma') in mice (77). This thesis will refer to mouse polyomavirus as 'PyV'. PyV is a natural pathogen of mice, and causes a persistent infection in immunocompetent mice characterized by low-level virus replication. PyV was first discovered when neonatal mice were infected with cell extracts from leukemic tissue taken from murine leukemia virus-infected mice (78). The surprising finding that the neonate mice developed salivary gland tumors suggested a tumor-inducing agent within this extract which was later identified as PyV (78).

Mouse PyV, similar to other polyomaviruses, is a small, icosahedral shaped (40-50 nanometer in diameter), nonenveloped, double-stranded DNA (dsDNA) virus. The PyV genome is circular, 5.3kb in length, encodes 3 regulatory proteins: large T, middle T, small T, and 3 structural late proteins: viral protein 1 (VP1), viral protein 2 (VP2), and viral protein 3 (VP3) (79). VP1 is the major protein that makes up the PyV capsid. Small, middle and large T antigens are essential for the viral life cycle, and they also interact with growth signaling pathways that can help promote tumorigenesis. The large T antigen is important

in initiating DNA replication. Large T antigen can also interact with the product of the retinoblastoma susceptibility gene, pRB, and drive a resting cell into the S phase of division. The small T antigen can help maximize viral replication by interacting with protein phosphatase 2 (PP2A). Small T antigen can inhibit the enzymatic activity of PP2A, resulting in overactive AKT or phosphatidylinositol 3-kinase (PI3-K) signaling pathways resulting in cell proliferation. The middle T antigen of mouse PyV, known as the transforming protein, interacts with cellular proteins (pp60 of the c-src family) and affects cell growth signaling pathways (80).

PyV infection can cause tumors in different organs due to the ability of PyV to infect various cell types. PyV can infect and replicate within the nucleus of monocytes, macrophages, dendritic cells, fibroblasts and epithelial cells (81). Unlike certain human polyomaviruses, mouse PyV has not been shown to be able to replicate within lymphocytes such as B and T cells. Infectious virus and genome copies of PyV DNA can be found in tumor tissues. Within cells, PyV genome can be maintained episomally or can integrate within host DNA.

PyV interacts with receptors containing sialic acid for the linkage of PyV to host cells. Specifically, mouse PyV can use α2,3-linked sialic acid-containing structures as receptor for attachment to cells (82). Also, other receptors such as sialic acid-containing gangliosides and α4β1 integrin receptor can be used as co-receptors for the attachment of mouse PyV to cells. (82-84) Since

sialoglycoproteins are ubiquitously expressed, PyV can infect a broad range of cell types.

K. Immune control of persistent virus infections in mice

A collaboration of innate, T cell, and B cell responses are necessary for effective long-term control of persistent viruses. Innate responses delay virus replication and spread and they also help shape the adaptive immune responses. Innate signals from antigen presenting cells, such as dendritic cells (DC), provide co-stimulatory signals and generate a cytokine environment that influences the appropriate antiviral adaptive response. The adaptive response provides viral antigen specificity and memory. Different infections involve various mechanisms that are important in viral control or clearance. Some viral infections such as MCMV greatly depend on innate cells such as NK cells, but other components of the immune system are still required for complete control. Other viral infections such as LCMV strongly rely on the cytolytic activity of CD8 T cells (85). Humoral immunity can also be important in the clearance of some acute viral infections such as Sindbis virus, and plays an important role in maintaining life-long protection in many virus infections (15).

L. Immunological control of PyV infection

An important consequence of impaired control of PyV infection is tumor formation. Immunocompetent adult mice are resistant to PyV-induced tumor formation after PyV infection regardless of their genetic background. Certain

strains of mice such as C3H or Balb/c are susceptible to tumor formation if infected as neonates. B6 mice have been known to be resistant to PyV-induced tumors (86). Neonates of certain susceptible strains infected with PyV develop a variety of tumors of mesenchymal and epithelial origin later in life (2, 86). Originally the resistance of adult mice to PyV-induced tumors was thought to be due solely to the ability of PyV to grow well in neonatal, but not in adult tissues. Although PyV grows somewhat better in neonatal tissues than in adult mouse tissues(87), experiments done with SCID mice infected as adults demonstrated that adult mice can also support high levels of virus replication (88). Therefore, tumor resistance in adult mice is mainly due to both antiviral and antitumor immune responses. It was shown that high tumor susceptibility in some H-2k mouse strains that were neonatally infected is due to the presence of a superantigen that deletes Vβ6 CD8 T cells (89). Vβ6 CD8 T cells in H-2k mice have specificities for middle T antigen-derived class I epitopes on PyV-infected cells and PyV tumor cells (89). Thus, the absence of Vβ6 CD8 T cells makes mice susceptible to PyV-induced tumor formation.

The role of CD8 T cell responses to PyV infection has been extensively studied. CD8 T cells have been shown to significantly control PyV-induced tumors (2, 90). Studies with neonatally thymectomized mice and congenically athymic mice have shown that T cells can prevent tumors induced by PyV. (91) Mice defective in MHC class I-restricted antigen presentation have an increased susceptibility to PyV-induced tumors (90). Studies have shown that CD8 T cell

protection from PyV-induced tumors is perforin-, Fas-, and TNFα- independent but dependent on IFNγ (92, 93).

CD4 T cells provide help to other cells including CD8 T cells and B cells. The role of CD4 T cells in the maintenance of CD8 T cell responses can vary in different infections. During a PyV infection, CD4 T cells have been shown to be important for recruiting newly primed CD8 T cells during the persistent phase of PyV infection (94). CD4 T cell responses also seem to be important in maintaining antiviral antibody responses at late time points post PyV infection (94).

In contrast to immunocompetent mice that can maintain a low-level of PyV persistence after infection and survive without disease, mice without an adaptive immune system succumb to PyV infection within weeks. SCID mice that lack T and B cells showed anemia, splenomegaly and massive myeloproliferation upon PyV infection (88). The acute myeloproliferative disease in these mice was due to uncontrolled PyV infection with very high viral loads resulting in death within 2 weeks following infection(88). NK cell depletion did not change the outcome of infection and NK cells were shown not to directly regulate PyV load (88).

Confirming the importance of T cell responses in preventing PyV-induced tumors, studies have shown that PyV infection of adult TCRβδ KO mice results in tumor formation. Surprisingly, mice that lack αβ T cells (TCRαβ KO mice) but still have γδ T cells are resistant to tumor formation after infection. (95) Furthermore, virus-induced tumors appear early in mice that lack NK cells and all T cells (E26

transgenic mice), compared to TCRβδ KO, which have NK cells and are only T cell-deficient. Thus, γδT cells and NK cells also contribute to the resistance to PyV-induced tumors and they can protect mice from tumor formation in the absence of T cells. (95)

Humoral immunity is not sufficient for directly controlling PyV-induced tumors (2), but antibody responses can lower PyV loads (96). TCRβδ KO mice infected with PyV survive 6-8 months post infection, till the time when tumors develop (95), while SCID mice die within 2 weeks of PyV infection (88). This suggests that a TI antibody response can control PyV loads and help mice survive in the absence of T cells (88, 96, 97). Mice deficient in B cells (μMT mice) still maintain tumor resistance (98). Passive immunization with PyV-immune serum prevented tumor formation in neonatal mice prior to PyV infection. However, administration of PyV-immune serum into neonatal mice after the start of infection did not protect mice from tumors (99).

The majority of the antibody response to PyV is directed at the major capsid protein, VP1. Upon PyV infection of WT mice, IgM (immunoglobulin M) appears at 4 days post infection and virus-specific IgG starts to be detectable around 7 days post infection (3, 3, 97). The peak of the PyV load in various organs of WT mice such as spleen, liver, kidney and lung is 5-6 days post infection (2, 3). The peak of antiviral IgG antibody responses is detected between 21-28 days post infection in WT mice (3).

The role of TI antibody in PyV control and protection was further investigated in TCRβδ KO animals. Upon PyV infection, T cell-deficient mice efficiently generate a TI antibody response consisting of virus-specific IgM and IgG, and they can survive several months post infection. (1, 96, 100) The TI IgG responses in T cell-deficient mice were reduced to ~ 10% from WT titers that were measured in mice 14 days post PyV infection (1). Of note, TI IgG responses primarily consisted of virus-specific IgG2a and IgG2b (4). PyV-specific TI IgG3 was present at very low and variable levels and IgG1 was undetectable (4). T cell-help is known to be important for the induction of IgG1. The finding that TI IgG responses can confer protection from PyV infection (96) suggests that IgG2a and IgG2b responses play an important anti-PyV role in T cell-deficient mice.

PyV was defined as a T cell-independent type II (TI-2) antigen when BTK (bruton's tyrosine kinase) deficient B cells transferred into PyV-infected SCID mice (with no T cells) could not generate an antibody response (100). BTK deficient cells were obtained from Xid (X-linked immunodeficiency) mice that display impairments in B cell receptor signaling and B cell survival (7). The PyV capsid, which consists of 72 pentameric capsomeres of the VP1 protein, represents the repetitive ligand that is capable of stimulating a TI-2 antibody response. Comparative studies with either VLPs (virus-like particles) given at a dose of 70 hemagglutination units (HAU) or live PyV infection have shown that live virus is needed to generate a TI anti-PyV IgM and IgG. VLPs can only induce IgM in TCRβδ KO mice (4). Furthermore, studies that block CD40-CD40L

signaling by anti-CD40L antibodies in T cell-deficient mice or studies using CD40-/- mice further showed that the lack of CD40-CD40L signaling greatly reduces the TI anti-PyV IgG response. This suggested that in the absence of T cells, other cells could provide the CD40L, which plays an important role in TI antibody responses to PyV infection. (101) These data suggest that, in an immune compromised host, antibody responses, generated in the absence of T cells, may help control the PyV load.

Our lab has investigated the role of the innate adaptor molecule MyD88 in mediating long-term TD B cell responses to PyV infection. MyD88-deficient mice had low levels of total virus-specific IgG starting at 2-3 weeks and lasting untill 1.5 years (latest time point tested) post PyV infection when compared to WT mice (3). The isotype usage among PyV-specific antibodies in WT mice was primarily IgG2a with some IgG1. PyV-specific antibodies in MyD88-/- mice, at 3 weeks post infection, were skewed towards IgG1 with low levels of IgG2a (3). In addition to skewed isotypes, MyD88-/- mice also had fewer (25-fold less) long-lived plasma cells in the bone marrow than WT mice (3). To further investigate long-term antibody responses, GC responses were tested. It was shown that MyD88-/- mice can generate normal frequencies of GC B cells as WT mice at 7 and 14 days post infection, indicating that MyD88-/- mice can initiate normal T cell-dependent IgG production and GC B cell responses to an acute PyV infection (3). Thus, MyD88-mediated pathways are not needed to initiate, but are required to maintain protective antibody responses to a persistent virus infection.

Overall, varying degrees of immunodeficiencies can lead to different pathologies, morbidity and mortality in response to PyV infection. The aforementioned information suggests that there is a complex immune response required for the control of this low-level persisting virus. A defect in any arm of the immune system can change the delicate balance between the disease-free host and virus replication, where increasing PyV loads result in pathology such as tumor formation. A strong and long-lasting humoral response is necessary to keep a persistent virus such as PyV controlled within hosts. Given the importance of MyD88 in the generation of a long-lasting humoral response, I sought to examine the role of innate signaling pathways in the formation and maintenance of long-lasting B cell responses to PyV infection.

M. Thesis Objectives

The longevity of antibody responses generated by B cells after infection, immunization or in the course of autoimmune diseases is of high importance. Life-long humoral immunity is desirable after natural infections or vaccinations to prevent the same kind of infection and disease for the lifetime of the host.
At the same time, autoreactive B cell responses need to be short-lived in order to avoid autoimmune pathology. The goal of the work presented in this thesis is to understand what is required to sustain a long-term T cell-dependent (TD) and T-cell independent (TI) antibody response to a persistent virus infection, specifically how innate receptors affect long-term B cell responses. To undertake this task, I

used mice that were deficient in one or several different toll like receptors or adaptors found downstream of TLRs. These studies helped identify which TLR(s) pathways affected long-term TD antibody responses. I also performed adoptive transfer experiments to examine memory B cell responses in MyD88-/- mice. Using mice that were deficient in IRF5, a transcriptional regulator downstream of the TLR-MyD88 pathways, allowed me to further unravel the mechanism behind the isotype antibody defects observed in MyD88-/- mice.

TCRβδ KO mice infected with PyV are capable of generating long-lasting VP1 specific IgG. I wanted to examine how T cell-deficient mice were able to maintain long-term antibody responses to PyV without T cell help. My findings suggest that high antigen levels and the involvement of innate pathways lead to the maintenance of long-lasting antiviral IgG responses in PyV-infected TCRβδ KO mice even in the absence of a “TI memory” response.

CHAPTER II: Materials and Methods

A. Mice and Infection

All the mice used in the studies were on the C57BL/6 (B6) background. TCRαβ KO, TCRβδ/TCRαβγδ KO, TCRγδ KO and SCID mice were originally obtained from the Jackson Laboratory (Bar Harbor, Maine), and colonies of these mice were maintained in the Department of Animal Medicine of the University of Massachusetts Medical School under specific pathogen free conditions. TCRβδ KO/ MyD88-/- mice were bred by crossing TCRβδ KO mice and MyD88-/- mice and maintained at the animal facilities at the University of Massachusetts Medical School. MyD88-/- mice were originally provided by Dr.K. Alugupalli (Thomas Jefferson University, Philadelphia, PA). TLR7-deficient (TLR7-/-), TLR3-deficient (TLR3-/-), TLR9-deficient (TLR9-/-), TLR2-deficient (TLR2-/-), TLR4-deficient (TLR4-/-), IRF5-deficient (IRF5-/-), MyD88/TRIF double knockout (DKO), UNC93B1 deficient (3d) and TLR7/9 double deficient mice were generously provided by the labs of Dr. Egil Lien and Dr. Kate Fitzgerald at University of Massachusetts Medical School. MyD88/TRIF DKO and TLR3-/- mice were also provided by the labs of Dr. Susan Swain and Dr. Dick Dutton. IRF5-/- mice were backcrossed to C57BL/6 for 10 generations and CD11c-cre-IRF5-floxed mice were obtained from Dr. Egil Lien. IRF5-floxed mice were originally generated by Dr. Paula Pitha at Johns Hopkins. All knockout mice were maintained in the

Department of Animal Medicine of the University of Massachusetts under specific pathogen free conditions.

Mice were used between 8 and 12 weeks of age and virus infections were done intranasally (i.n.) or intraperitoneally (i.p.) with ~10^6 PFU of PyV strain A2. All the procedures using animals were done according to protocols approved by the University of Massachusetts Medical School Animal Care and Use Committee.

B. VP1-specific Enzyme-linked immunosorbent assay (ELISA)

VP1-specific ELISAs were conducted as previously described (101, 102). Briefly, purified recombinant VP1 protein (0.1 µg/ml in carbonate buffer) was used to coat 96 well plates (using 50ng/well). VP1 was generously provided by Dr. Robert Garcea. Bound Ab was detected using biotin-conjugated goat Abs specific for mouse IgG; and streptavidin-conjugated HRP (Vector Laboratories). ELISA plates were developed using BD OptEIA TMB Substrate Set (BD Pharmingen), and reactions were stopped with 2 N sulfuric acid. Optical densities were read at 450 nm by $THERMO_{MAX}$ microplate reader and SoftMax software. Data are represented as optical density (o.d.) versus serum dilution (raw data), or data converted into IgG titers or shown as IgG concentration expressed in arbitrary units. The IgG titers represent end point titers, which are defined as the highest serum dilutions showing optical density in the ELISA above the values of negative control serum. The arbitrary units are derived from a standard curve

generated with various dilutions of a positive control serum in the same experiment.

C. VP1-specific Enzyme-linked immunosorbent spot (ELISPOT)

To determine the number of VP1-specific Ab-secreting cells (ASC), multiscreen HTS filter plates (Millipore) were coated with purified VP1 protein (0.1 g/ml in carbonate buffer, 50µl/well). Unlabeled goat anti-mouse Ig (H+L) (Southern Biotech) coated wells were used for determining total IgG ASC, and some wells were just coated with carbonate buffer (no coat controls). The plates were incubated overnight at 4°C and blocked the next day for 30 min at 37°C with RPMI plus 10% FBS. Cells were plated in duplicates in 0.2 ml at a concentration of 10^7 cells/ml and then followed by 2-fold dilutions. Cells were then incubated for 4 h at 37°C. Following incubation the cells were discarded and the plates were washed and blocked with 1% BSA in PBS for 30 minutes at 37C. Bound Ab was detected using biotin-conjugated goat Abs specific for mouse IgG and streptavidin-conjugated HRP. Plates were developed with Elispot AEC Substrate Set (BD Pharmingen) according to the manufacturer's protocol. Spots were counted using the CTL ImmunoSpot (Cellular Technology Ltd.). The data are shown as antibody secreting cell (ASC) frequencies.

D. Quantitative PCR (qPCR) to measure viral DNA genome copy number

DNA was prepared from organ homogenates by digestion with proteinase K (Sigma) at 55°C overnight, followed by phenol extraction and RNase-A treatment

(10u/µl, Promega). The PCR amplification was performed using SYBR Green Master Mix Kit from (Applied Biosciences). Briefly, 50 µl reaction mix containing 25µl of 2X master mix, 20 pmol each of forward and reverse primer (Invitrogen), and 250ng of the DNA sample were used. The following primers were used: β actin forward CGA GGC CCA GAG CAA GAG AG; β actin reverse CGG TTG GCC TTA GGG TTC AG; PyV VP1 forward CCC CCG GTA CAG GTT CAG TCC CAT CAT; VP1 reverse GGC ACA ACA GCT CCA CCC GTC CTG CAG. The amplification conditions for VP1 were 95°C for 10 min, followed by 37 cycles of 95°C for 30 sec, 65°C for 20 sec, 72°C for 45 sec. PCR amplification with the β actin primers used the following conditions: 95°C for 10min, then 40 cycles of 95°C for 30 sec, 62°C for 25 sec, and 72°C for 25 sec. Negative controls included a sample with no DNA substrate, and DNA from uninfected mouse organs. Three-fold serial dilutions of DNA prepared from uninfected mouse organs (starting from 750ng) were used to generate a standard curve for β actin PCR. For PyV standards we used a recombinant plasmid containing the VP1 coding sequences and made dilution series from $2x10^8$ copies to 20 copies. All the reactions were run in duplicates. The obtained PyV copy numbers were normalized to β actin, which reflected the amount of mouse genomic DNA present, and the results were expressed as PyV genome copies/ µg organ DNA.

E. Quantitative PCR (qPCR) to measure IRF5 transcript levels

MACS-purified CD11c-positive and CD11c-negative cells from splenocytes were stimulated with IFNβ overnight. The following day, total RNA was isolated from these cells using the RNEasy Mini Kit; cat: 74104; Qiagen, following the manufacturer's protocol. RNA concentration was determined by nanodrop. cDNA synthesis was performed using QuantiTect Reverse Transcription kit (cat: 205311; Qiagen) by following instructions provided by the manufacturer. qPCR was performed using the QuantiFast SYBR Green PCR kit (cat: 204054; Qiagen). Briefly 25 µl reaction mix containing 12.5µl of 2X master mix, 10 pmol each of forward and reverse primer (Invitrogen), and <100ng of the cDNA sample was used.

The following primers were used: β actin forward CGA GGC CCA GAG CAA GAG AG; β actin reverse CGG TTG GCC TTA GGG TTC AG; IRF5 forward CTT CAG TGG GTC AAC GGG; IRF5 reverse TGT ACG GCT GAG GTG GCA T. PCR amplification with the β actin and IRF5 primers used the following conditions: 95°C for 5min, then 40 cycles of 95°C for 10 sec, and 60°C for 30 sec. All the reactions were run in duplicates. β-actin was used for normalization and fold change was calculated using delta delta CT method ($2^{(-\Delta\Delta CT)}$)

F. Flow cytometry

To examine the expression of cell surface markers by flow cytometry, splenocytes were treated with 0.84% NH4Cl to lyse RBC and then washed with

FACS buffer (PBS plus 1% FCS, 2 mM EDTA, and 0.02% NaN3). Cells were treated with purified anti-CD16/32 (Fc block; clone 2.4G2; BD Pharmingen) and then stained for 30 min at 4°C with Abs diluted in FACS buffer at 1:200, unless stated otherwise. Abs used in these studies included FITC-rat anti-mouse T and B cell- activating Ag GL7 (clone GL7; BD Pharmingen), PECy7-rat anti-mouse CD19 (clone 1D3; BD Pharmingen), PE-hamster anti-mouse CD95 (BD Pharmingen), Pacific Blue-rat anti-mouse B220 (CD45R) (clone RA3-6B2; BD Pharmingen), Alexa Fluor 647 anti-Bcl-6 (clone K112-91; BD Pharmingen), PE-anti-mouse Bcl-6 (clone mGI191E; ebioscience, used at 1:100), APC Rat anti-mouse CD184/CXCR4 (clone 2B11/CXCR4; BD Pharmingen), PE-Cy7-Rat anti-mouse CXCR5 (clone 2G8; BD Pharmingen, used at 1:50 in room temperature), PecrcpCy5.5- anti-mouse CD279 (PD-1) (clone 29F.1A12; biolegend), Alexa 700 anti-mouse CD4 (clone RM4-5; BD Pharmingen), Pacific blue- anti-mouse CD3ε (clone 145-2C11; BD Pharmingen), PE-Cy5-anti-mouse CD278 (ICOS) (clone 7E.17G9; ebioscience), APC-anti-mouse/rat FoxP3 (clone FJK.16s; ebioscience), PE-Rat anti-mouse CD162/PSGL-1 (clone 2PH1; BD Pharmingen), and FITC-Rat anti-mouse Ly-6C (clone AL-21; BD Pharmingen) Flow Cytometry Analysis: at least 100,000 events were counted using a LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star).

G. Intracellular staining (ICS)

Cells were prepared as described in part I. Surface staining of cells was performed first, following the surface stain, cells were permeabilized using the FoxP3 Ebioscience kit. For 2-21/2 hrs at 4C. Cells were washed and blocked again with Fc block for 5-10 min at room temperature. Following blocking and no intermediate wash step, antibodies against nuclear proteins such as Bcl-6 and FoxP3 were added in Perm Buffer for 20-30 min at 4C. Following intracellular staining, cells were washed twice with perm buffer and twice with FACS buffer. Cells were resuspended in 250ul of FACS buffer and ran on the LSR II.

H. B cell memory assay

Spleens were harvested from uninfected and PyV-infected TCRαβ KO, TCRγδ KO, TCRβδ KO, MyD88-/-, and wild type B6 mice. SCID recipients were given10^7 splenocytes intravenously. Following 1-day post cell transfer, some of the SCID mice in each group were infected with PyV (~10^6 pfu intraperitoneally). Five to 7 days post infection, VP1 specific Elisa was performed to detect recall IgG responses from the sera of SCID mice.

I. Preparation of cells

Single cell suspensions were made from spleens, peripheral blood or bone marrow. For all cases, using .84% NH_4Cl solution lysed red blood cells. Spleens were homogenized in RPMI1640 (Invitrogen) and then filtered through nylon

mesh. Blood for cells was taken from tail vein in heparinized tubes, however blood for serum was taken in non-heparinized tubes and spun at 5500rpm for 5 min to collect serum. Bone marrow was taken by removal of the femur and tibia bones from both legs and flushing of the bone marrow from the bones using RPMI1640 and a 10 mL syringe. RPMI1650 was supplemented with 10% FCS, LG and PS.

For adoptive transfer experiments, cells were washed re-suspended in HBSS (Hank's Balanced Salt Solution) and transferred into recipient mice intravenously at 10^7 cells/ml.

J. MACS enrichment and adoptive transfer of cells

Spleens cells harvested from Ly5.1 B6 mice were pooled and treated with 0.84% NH_4Cl to lyse RBC and then re-suspended in MACS buffer as instructed by the various manufacturer protocols (Miltenyi biotec MACS kit). CD8 T cell isolation kit (Miltenyi biotec,) for negative selection of CD8 T cells was used to purify CD8 T cells from the splenocytes. Pan T cell isolation kit II (Miltenyi biotec; 130-095-130) was used to purify all mouse T cells by depletion of non-T cell targets. Pan B cell isolation kit (Miltenyi biotec; 130-095-813) was used for the negative selection of all mouse B cells. CD11c mouse microbeads (Miltenyi biotec; 130-052-001) were used to enrich CD11c+ cells from splenocytes. FACS analysis was performed before and after MACS selection on the splenocytes.

Adoptive transfer of cells: Cells were re-suspended in HBSS and intravenously transferred into recipient mice. For CD8 T cell transfer experiments, $7x10^6$ cells were intravenously (i.v.) transferred into each of 5 TCRβδ KO mice. Purified10^7 IRF5-/- or WT B cells along with 1.2 $x10^6$ WT T cells were transferred into RAG1/2 KO recipients i.v.

CHAPTER III: Role of innate signaling pathways in maintaining a long-term antiviral TD B cell response

Innate immunity plays an important role in the defense against viruses, but for many viruses it is not sufficient to clear the infection, and adaptive immunity is essential for survival (for example, PyV is lethal in SCID mice) (88). Adaptive immunity is important to prevent reinfection, since immunological memory to infections is a unique attribute of the adaptive immune system. A strong and long-lasting humoral response is necessary to keep the virus controlled within hosts. Two distinct long-lived B cell populations usually manifest B cell memory: long-lived PC and memory B cells. Both are the products of B cell responses generated with CD4 T cell help. Viral infections, unlike protein immunizations such as NP-CGG, can induce inflammatory signals and activate innate receptors. Innate signals have been shown to play an important role in promoting B cell responses (66). Previously, our lab has shown that MyD88-/- mice infected with PyV generate normal acute B cell responses but have defects in long-term humoral immunity (3). In addition to having defects in long-term antibody responses, MyD88-/- mice were shown to have skewed isotype antibody responses to PyV. MyD88-/- mice generate normal levels of virus-specific (VP1) IgG1 and very low levels of VP1-specific IgG2a and IgG2b (3). Mice deficient for MyD88 also lack long-lived plasma cells in the bone marrow when compared to wild type mice (3). In addition to TLRs, MyD88 is also found downstream of the IL-1R (IL-1 receptor) and the IL-18R (IL-18 receptor). Previous experiments from

our lab showed that IL-1R KO and IL-18R KO mice had no defects in long-term antibody production upon PyV infection,(3) indicating that these cytokine receptors are not involved in the antibody defects observed in MyD88-/- mice. Thus, this chapter will further investigate the long-term humoral defect in MyD88-/- mice and describe which innate signaling pathways play a role in sustaining long-term humoral antiviral responses.

A. Diminished B cell memory responses to PyV in MyD88-/- mice

Since our lab had previously identified a lack of long-lived plasma cells in MyD88-/- mice upon PyV infection, I questioned whether recall memory B cell responses were also impaired in MyD88-/- mice. To test B cell recall responses in MyD88-/- mice, I developed a functional recall memory assay. This assay was based on demonstrating early (day 5-7) recall IgG responses by memory B cells from SCID mice that were adoptively transferred with PyV-infected splenocytes. In these assays, SCID mice that received splenocytes from PyV-immune mice and were subsequently infected with PyV would give early, strong VP1-specific IgG recall responses. On the other hand, control groups, such as mice that received non-immune spleen cells, or mice that received PyV-immune splenocytes but were not challenged with PyV, would generate very low or undetectable VP1-specific IgG recall responses in their sera at this early time point. In this assay, SCID mice were reconstituted with either of the following groups of splenocytes: naïve splenocytes from WT mice, naïve splenocytes from

MyD88 -/- mice, PyV-immune splenocytes from WT mice, or PyV-immune MyD88-/- mice. On the day of cell transfer, some of the reconstituted SCID mice from each experimental group were infected with PyV, whereas others were left uninfected. Seven days post cell transfer and PyV infection, VP1-specific IgG responses were measured in the sera to determine the recall response. (data not shown) SCID mice given PyV-immune MyD88-/- splenocytes, along with a re-infection, were not able to generate a strong memory B cell response, as did SCID mice given PyV-immune WT cells. Therefore, in addition to the reduced long-lived PC population in the bone marrow, MyD88-/- mice had also diminished B cell recall memory in response to PyV infection.

Next, I investigated which pathways are involved in MyD88-mediated regulation of long-term humoral immunity to PyV. It was further examined what molecular mechanisms underlie the isotype skewing of the antiviral IgG responses of MyD88-/- mice.

B. Role of TLR-MyD88 pathways in mediating anti-PyV IgG2a responses

The major isotype that is generated in response to most virus infections in mice, including PyV, is IgG2a (103). Viral infection generates antiviral interferon gamma (IFNγ) responses, which studies have shown helps induce IgG2a (102). IgG2a mediates various antiviral effector functions dependent on the antigen and the immune environment. As mentioned before, MyD88-/- mice generate low levels of VP1-specific IgG2a and normal levels of IgG1 (3). WT mice, in response

to PyV infection, generate high levels of IgG2a and lower levels of IgG1 (3). IgG1 and IgG2a have neutralizing properties, but these antibodies have different effectiveness in mediating a variety of other effector functions because of their Fc portions. IgG2a, via its Fc portion, can activate the complement cascade, bind to activating Fc receptors with high affinity (104)(106) and induce ADCC (105). However, the Fc portion of IgG1 interacts with a lower affinity with Fc receptors (106). These differences in affinities of FcγR binding of IgG2a, IgG2b and IgG1 Fc leads to, generally, a more effective protection by antiviral IgG2a and IgG2b compared to IgG1 in virus-infected hosts (106)(107).

It is important to note that C57BL/6 mice express the Igh-1b allotype, which is referred to as IgG2c. Other mouse strains express the Igh-1a allotype known as IgG2a (108). In this thesis, when describing the isotype responses, IgG2a will be used, although the background of the mice used is B6. Since MyD88-/- mice generate low levels of PyV-specific IgG2a, I investigated which receptors upstream of MyD88 (TLRs) are involved in mediating antiviral IgG2a responses.

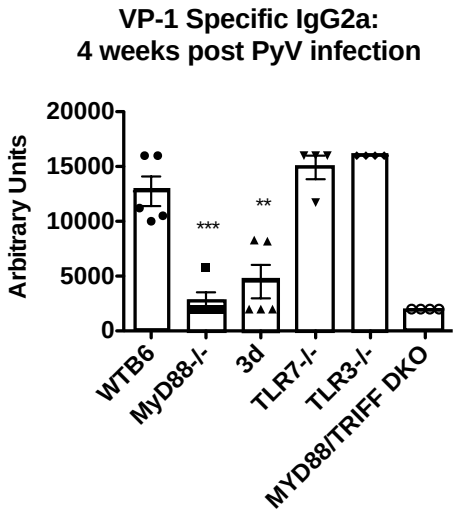
Endosomal TLRs (TLR 3,7/8, and 9) are known as ‘viral sensors’ and recognize nucleic acids. In order to investigate the role of nucleic-acid-sensing TLRs in the regulation of IgG2a production, we used Unc93B1 mutant mice (also referred to as ‘triple D’ (3d) mice). Unc93B1 is an endoplasmic reticulum (ER) transmembrane chaperone protein that plays an important role in the translocation of TLRs 3,7 and 9 from the ER into endosomes where these TLRs

interact with their ligands and become activated (109). Thus, a point mutation within Unc93B1 (H412R) abrogates its ability to traffic the endosomal TLRs,(109) essentially creating mice that are deficient in TLRs3, 7 and 9 signaling (110).

3d mice generated similar levels of VP1-specific IgG1 as WT and MyD88-/- mice at 5 weeks post PyV infection. (Figure 3.1b) However, unlike WT mice that generated normal VP1-specific IgG2a responses, 3d mice produced reduced levels of virus specific IgG2a, similar to that of MyD88-/- mice. (Figure 3.1a) Since 3d mice cannot signal through TLRs 3,7 and 9, we investigated these TLRs individually to assess if one or more endosomal TLRs are involved in controlling isotype responses. TLR9 recognizes CpG DNA (PyV is a small DNA virus) and TLR7 is known to recognize ssRNA. (55) The ligand for TLR3 is dsRNA and does not signal through MyD88 but signals via another adaptor protein known as TRIF (55). Experiments with TLR9-deficient (TLR9-/-) mice, TLR7-deficient (TLR7-/-) mice, and TLR3-deficient (TLR3-/-) mice did not display defects in VP1-specific IgG2a responses as seen in 3d mice and MyD88-/- mice. (Figure 3.1a-c) However, as expected, VP1-specific IgG2a responses were low in MyD88/TRIF double KO mice, similar to what has been observed in MyD88-/-. (Figure 3.1a)

Figure 3.1- VP1-specific isotype antibody responses are not skewed in individual TLR3, TLR7 and TLR9 deficient mice, only in 3d or MyD88-/- mice

a.



b.

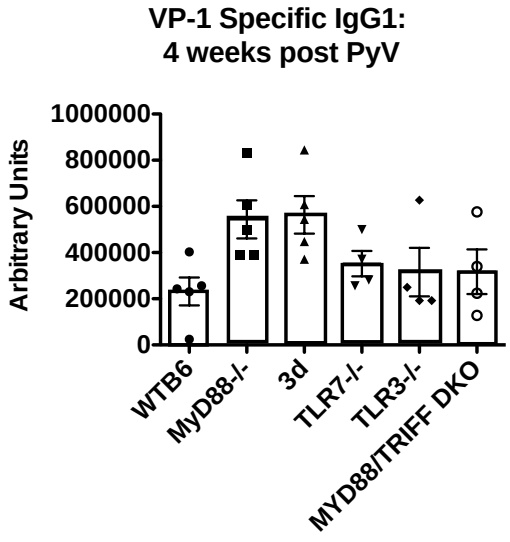


Figure 3.1 (cont.)- VP1-specific isotype antibody responses are not skewed in individual TLR3, TLR7 and TLR9 deficient mice, only in 3d or MyD88-/- mice

c.

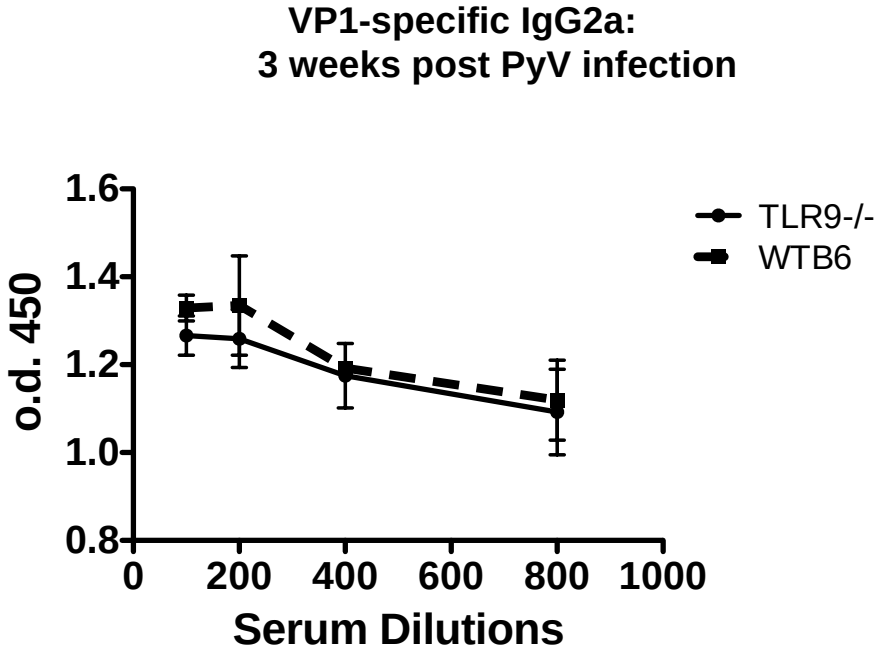


Figure 3.1- VP1-specific isotype antibody responses are not skewed in individual TLR3, TLR7 and TLR9 deficient mice, only in 3d or MyD88-/- mice

(a-b)VP1- specific IgG2a and IgG1 responses in TLR7-/- and TLR3-/- mice along with 3d, MyD88-/- and WTB6 mice 4 weeks post PyV infection. (c) PyV-specific IgG2a responses in TLR9-/- (n=4) and WT (n=2) mice 3 weeks post PyV infection. Mean +/- SD is shown ***p=.002, **p=.0037

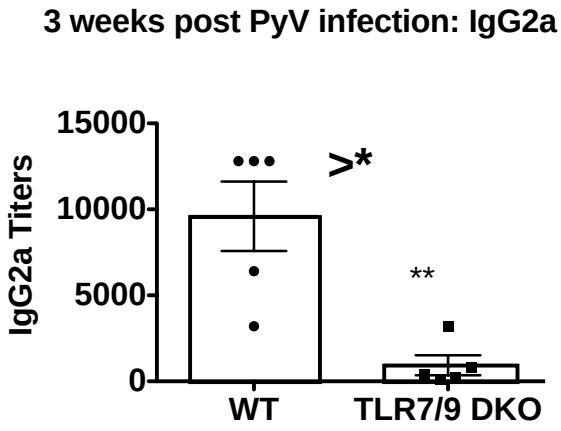
TLR7-/- or TLR9-/- mice did not have defect in VP1-specific IgG2a production. However, attenuated levels of VP1-specific IgG2a were observed in 3d and MyD88-/- mice, suggesting an importance of TLR7 and TLR9 in isotype responses to PyV infection. To evaluate the role of both TLR7 and TLR9 in mediating IgG2a responses, TLR7/TLR9-double deficient (TLR7/9 DKO) mice were infected with PyV. At 3 weeks post PyV infection, TLR7/9 DKO mice generated low-levels of VP1-specific IgG2a and normal levels of VP1-specific IgG1 when compared to WTB6 mice. (Figure 3.2a-b) All of these studies show that TLR7 and TLR9 are play an important role in the generation of IgG2a in response to PyV infection.

C. Role of IRF5 in regulating B cell-intrinsic IgG2a responses

Interferon regulatory factors are transcriptional mediators that play an important role in type I interferon induction, apoptosis, and antiviral immune responses (60). IRF4, IRF5, and IRF8 have been shown to be important in B cell responses. IRF4 is essential for CSR and plasma cell differentiation by regulating Blimp-1 expression in B cells (111).Conditional deletion of IRF4 in GC B cells inhibited plasma cell differentiation post the GC response (111). Furthermore, IRF4 deficient mice did not express the enzyme AID (111). IRF8 plays a role in B cell-lineage commitment as IRF8 deficient mice have low numbers of pre-pro-B cells and an increase in myeloid cells (112). In B cells, IRF8 induces Bcl-6 expression which is important for GC B cell responses (24).

Figure 3.2- TLR7/9 DKO mice have a defect in the generation of VP1-specific IgG2a, but not VP1-specific IgG1

a.



b.

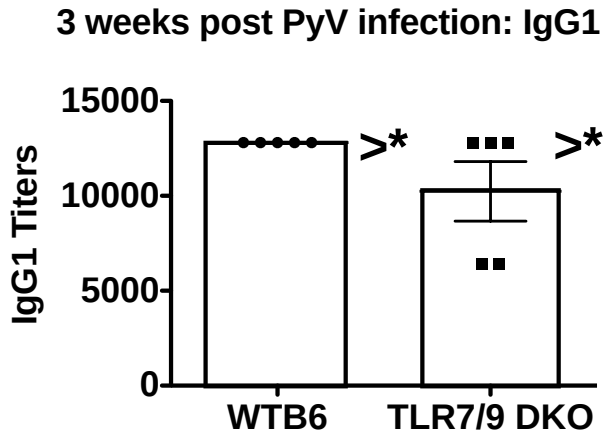


Figure 3.2- TLR7/9 DKO mice have a defect in the generation of VP1-specific IgG2a, but not VP1-specific IgG1

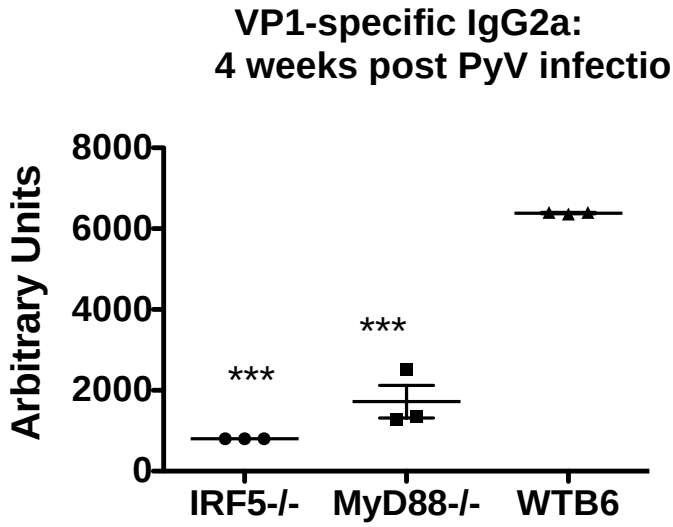
VP1-specific IgG2a and IgG1 responses detected in the sera of WTB6 and TLR7/9 double deficient mice 3 weeks post PyV infection. Mean +/- SD is shown >* The IgG2a or IgG1 titers are equal or greater than 128000. **p value <.01

IRFs, including IRF5, are transcription factors closely linked to the TLR pathway and therefore to MyD88. It was important to further investigate IRF5 because it is known that IRF5 interacts with MyD88 upon TLR7 and TLR9 activation (62). IRF5 is important in type I interferon responses, but can also have an antiviral role as shown by the susceptibility of IRF5-deficient mice to virus infections (62). IRF5 expression is induced upon viral infection or by TLR7 or TLR9-MyD88 pathways (62, 113). Furthermore, polymorphisms in IRF5 have been shown to increase susceptibility to systemic lupus erythematosus (SLE) (114). In mouse models of pristine-induced lupus, IRF5-deficient mice were not able to generate anti-nuclear autoantibodies of the IgG2a isotype, suggesting that IRF5 may regulate IgG2a responses (115). Stimulating IRF5-deficient (IRF5-/-) B cells in culture with TLR7 and TLR9 ligands resulted in low levels of germ-line transcripts (GLTs) of γ2a (116). The reconstitution of IRF5-/- B cells by retrovirus-delivered IRF5 increased γ2a GLTs, supporting a IRF5-specific and B cell-intrinsic role of IRF5 in mediating IgG2a expression (116).

Therefore, we tested antiviral IgG2a responses in PyV-infected IRF5-/- mice. At 4 weeks post PyV infection, IRF5-/- and MyD88-/- mice had low levels of VP1 specific IgG2a when compared to WT mice. (Figure 3.3a) However, IRF5-/- and MyD88-/- mice generated VP1-specific IgG1 similar to WT mice. (Figure 3.3b) To further test whether B cell-intrinsic IRF5 is required for mediating IgG2a responses, an in vivo adoptive transfer experiment was performed. RAG1/2 KO mice, which lack B and T cells were reconstituted with MACS-purified B cells

Figure 3.3- Low levels of VP1-specific IgG2a in IRF5-/- mice

a.



b.

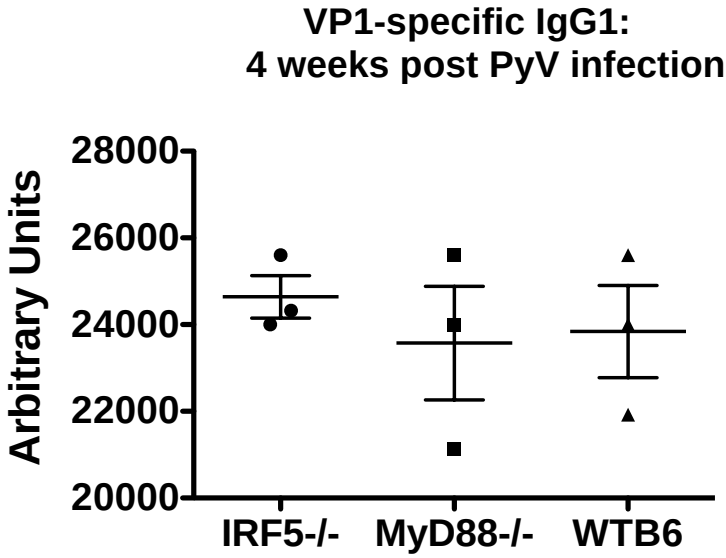


Figure 3.3- Low levels of VP1-specific IgG2a in IRF5-/- mice

IRF5-/-, MyD88-/-, and WTB6 infected with PyV were bled 4 weeks post infection to determine virus-specific isotype-switched antibody responses. VP1- specific (a) IgG2a and (b) IgG1 levels determined from the sera of these mice. Mean +/- SD is shown. ***p value < .001

from either WTB6 or IRF5-/- mice and with purified WT T cells. After the transfer of cells, the two groups of RAG1/2 KO mice, which differed in the expression of IRF5 only in their B cell populations, were infected with PyV. VP1-specific total IgG levels were lower in RAG1/2 KO mice reconstituted with IRF5-/- B cells than with WT B cells 3 weeks post infection. (Figure 3.4a) Moreover, staining for IgG2a+ B220+ B cells 6 weeks post infection revealed that RAG1/2 KO mice with IRF5-/- B cells generated lower frequencies of IgG2a+ B cells in the spleen compared to RAG KO mice with WT B cells. (Figure 3.4b)

To test a role for IRF5 in DCs for CSR to IgG2a in response to PyV infection, we tested antiviral antibody responses in IRF5 floxed CD11c-Cre mice that are deficient for IRF5 only in CD11c+ cells (referred to as CD11c-IRF5-/- mice). Exon 2 and exon 3 of IRF5 is floxed out in CD11c-IRF5-/- mice (116). CD11c-IRF5-/- mice showed no significant defects in VP1-specific IgG2a responses at 3 weeks post infection. (Figure 3.5a) To confirm the cell type-specific IRF5 defect in the mice used in these studies, CD11c+ cells were MACS-enriched from spleens of CD11c-IRF5-/- and WTB6 mice to determine IRF5 transcript levels. Following enrichment of cells, CD11c-positive and CD11c-negative cell populations, along with whole splenocytes from IRF5-deficient mice, were stimulated with IFNβ overnight. Quantitative-PCR determined levels of IRF5 transcripts in the various cell populations. (Figure 3.5b) Thus, this study confirmed that IRF5 was conditionally absent from CD11c+ cells such as DCs. CD11c-IRF5-/- mice were able to generate PyV-specific IgG2a at similar levels

Figure 3.4- B cell-intrinsic role of IRF5 in mediating IgG2a responses to PyV infection

a.

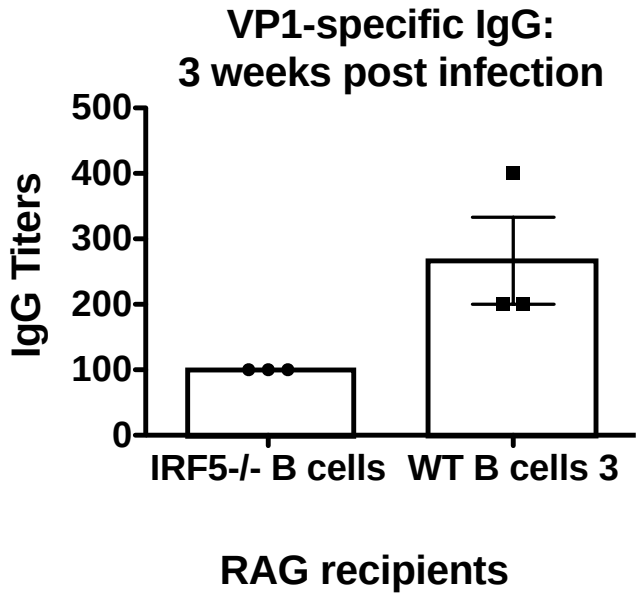


Figure 3.4 (cont.)- B cell-intrinsic role of IRF5 in mediating IgG2a responses to PyV infection

b. IgG2a+ B cells in RAG KO mice at 6 weeks post PyV infection

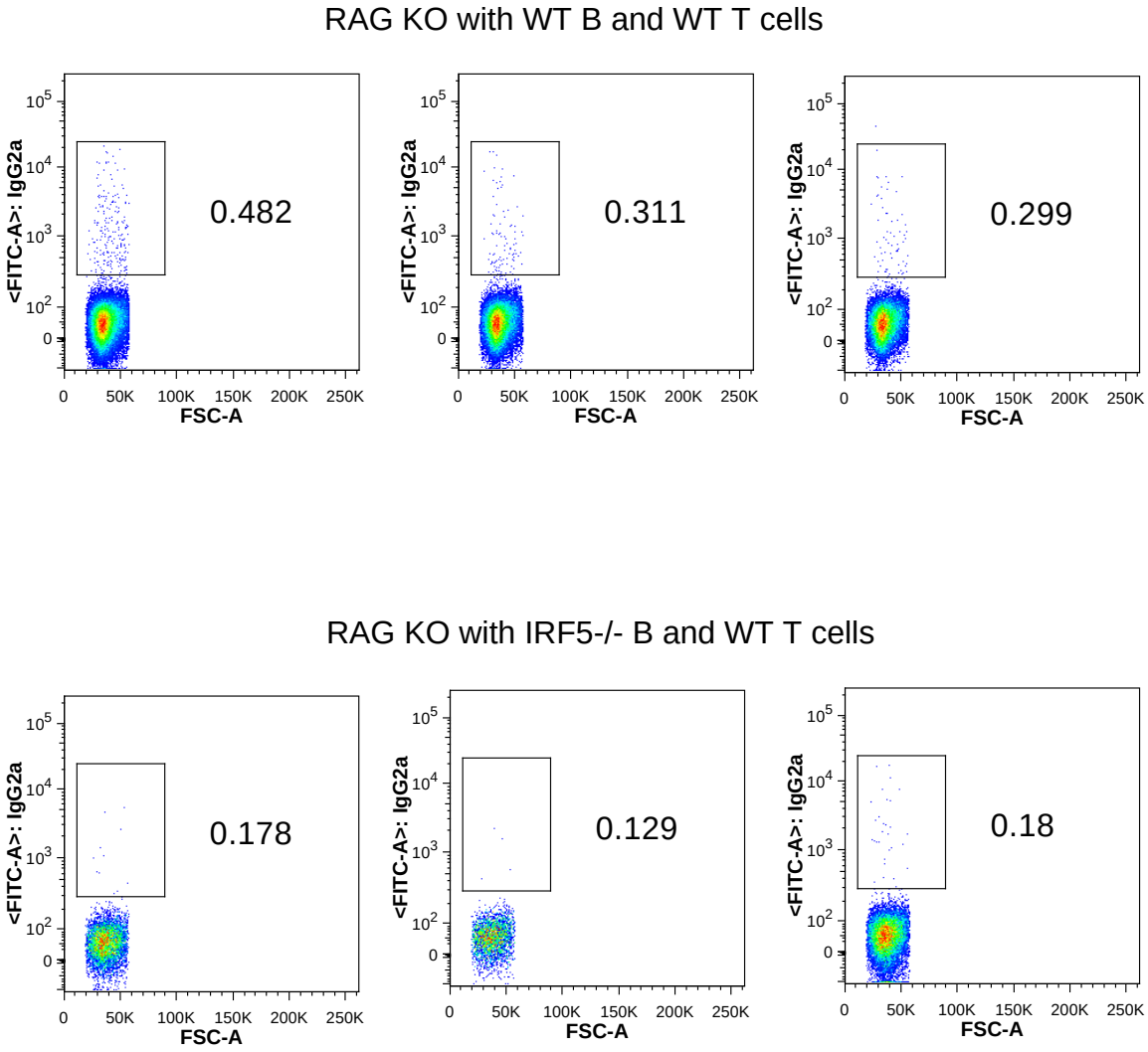


Figure 3.4- B cell-intrinsic role of IRF5 in mediating IgG2a responses to PyV infection

RAG1/2 KO mice were reconstituted with MACS-purified B cells from either WTB6 or IRF5-/- mice and WT T cells. After the reconstitution of cells, RAG1/2 KO mice were infected with PyV. (a) 3 weeks post infection, VP1- specific ELISA was performed to detect total IgG levels in the sera of RAG1/2 KO mice. (b) 6 weeks post infection, splenocytes from reconstituted RAG1/2 KO mice were examined for IgG2a+ B cells. IgG2a+ cells were gated on B220+ B cells. Mean +/- SD is shown for part a.

Figure 3.5-VP1-specific IgG2a responses are not affected in CD11c-IRF5 conditional knockout mice

a.

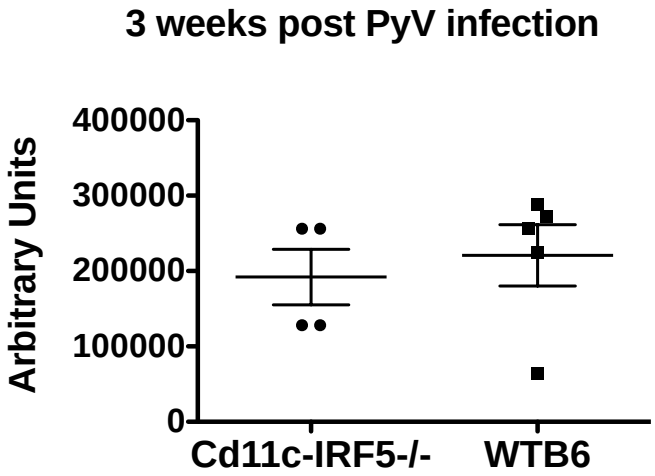


Figure 3.5-VP1-specific IgG2a responses are not affected in CD11c-IRF5 conditional knockout mice

b.

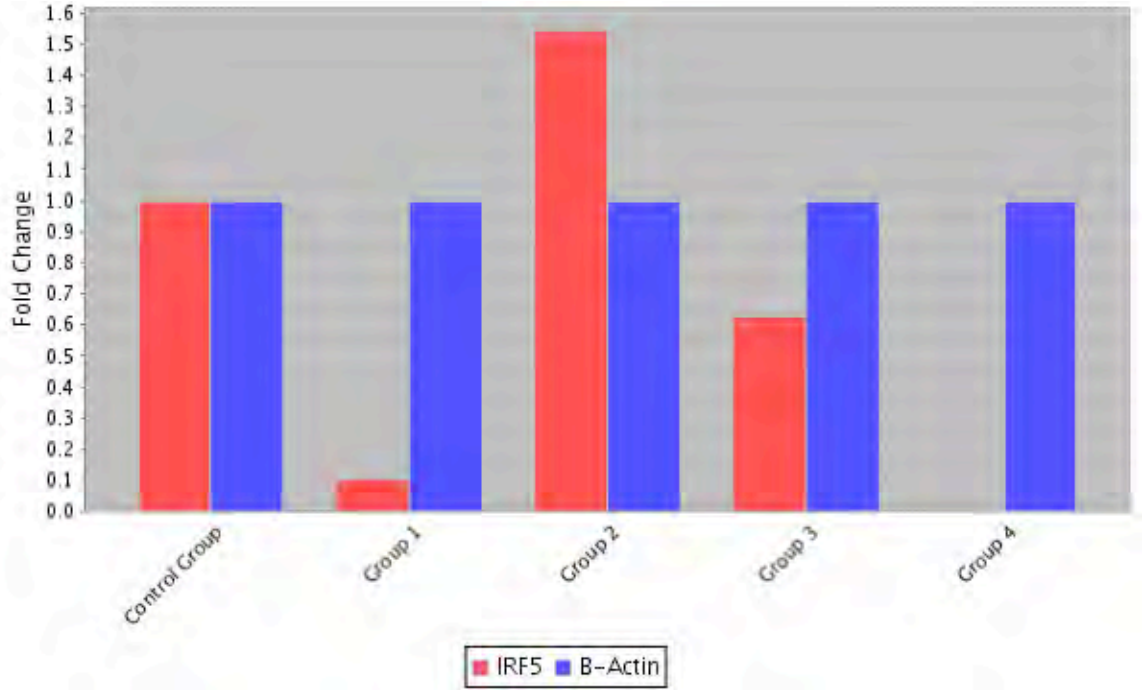


Figure 3.5- VP1-specific IgG2a responses are not affected in CD11c-IRF5 conditional knockout mice

Cd11c-IRF5-/- and WTB6 mice infected with PyV were bled at various time points post infection. (a) VP1- specific IgG2a responses at 3 weeks post infection. (b) Spleen cells were MACS-enriched for CD11c positive and negative cells from CD11c-IRF5-/- and WT mice. Following enrichment, cells were stimulated overnight with IFNβ and analyzed by q-PCR for IRF5 transcripts. Samples were normalized to b-actin and WTB6 CD11c-positive cells were set at the control group. IRF5-/- mice were used as a negative control. β-actin was used as the housekeeping control gene in this assay. The data are expressed as fold change relative to WTB6 levels of IRF5.

Control group: CD11c+ cells from WTB6 mice, WT IRF5 expression; Group 1: CD11c+ cells from CD11c-IRF5-/- mice; Group 2: CD11c negative cells from CD11c-IRF5-/- mice; Group 3: CD11c negative cells from WTB6 mice; Group 4: Whole splenocytes from complete IRF5-/- mice.

as WT mice which is consistent with a B cell-intrinsic role of IRF5 in mediating IgG2a responses to PyV infection.

In collaboration with Paula Pitha, we have shown that IRF5 mediates the induction of IgG2a by regulating the expression of the transcription factor Ikaros. The Ikaros family of transcription factors is important in the development and maturation of both lymphocytes and non-lymphoid cells. Ikaros has been shown to play a role in B cell selection and differentiation (117). Ikaros is also involved with allelic exclusion at the Igk locus (118, 119). Ikaros can function as both a transcriptional activator and repressor (117). Ikaros knockout mice are not viable(120). Studies by Fang et al utilized mice that contain a hypomorphic mutation (disrupts gene at a lesser degree) in Ikaros ($Ikaros^{L/L}$), which reduces Ikaros expression to ~10% of normal levels (117). $Ikaros^{L/L}$ mice display high serum levels of IgG2a and IgG2b and lower IgG1 responses (119).

IRF5-/- B cells stimulated with TLR ligands or cytokines induced higher levels of *Ikzf1* (the gene that encodes for Ikaros protein) transcripts and protein levels than WT stimulated B cells (116). Retrovirus delivered IRF5 downregulated *ikzf1* transcripts and restored γ2a GLTs, suggesting that IRF5 can negatively regulate *Ikzf1* expression and that Ikaros expression down-regulates IgG2a production (116). IRF5 was also shown to bind upstream of the transcriptional start site to an IRF binding site located in the *Ikzf1* promoter region (116). Through transient transfection assays, IRF5 was observed to stimulate *Ikzf1* promoter transcriptional activity (116). All of these studies provide evidence

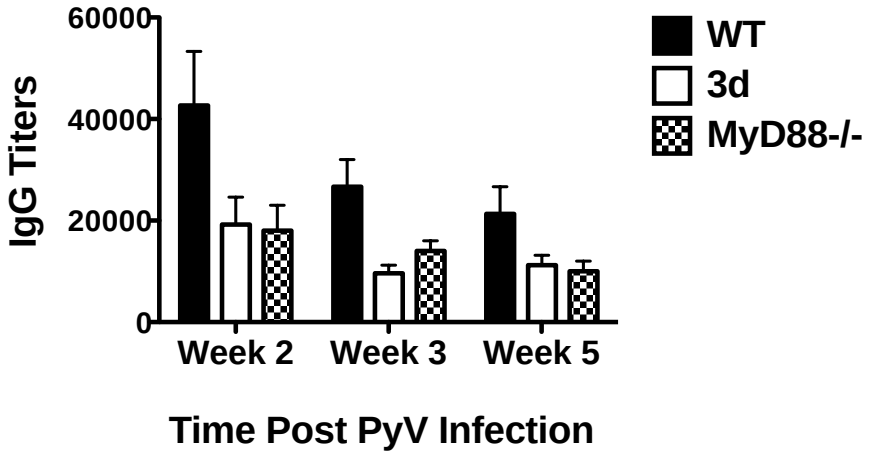
that IRF5 negatively regulates Ikaros expression and also enhances IgG2a production. Together these studies describe a B cell-intrinsic mechanism for IRF5 in regulating IgG2a responses.

D. The role of TLRs in sustaining long-term antibody responses to PyV infection

I utilized 3d mice to study the role of endosomal TLRs in long-term TD antibody responses to PyV infection. I infected 3d mice along with WT and MyD88-/- mice with PyV. 3d mice behave much like MyD88-/- mice in regards to long-term virus-specific IgG responses and the number of long-lived plasma cells in the bone marrow in response to PyV infection. Upon PyV infection, 3d mice generated low-levels of VP1- specific IgG like MyD88-/- mice starting 2 weeks post infection, and the defect was maintained until the latest time point tested, 7 weeks post infection. (Figure 3.6a-b) At 8 weeks post infection, approximately a 10-fold decrease in the number of VP1-specific ASCs was observed in the bone marrow of 3d mice compared to ASCs in the bone marrow of WT mice. (Figure 3.6c) There were no significant differences in splenic ASCs between 3d and WT mice. The defect in long-lived PC in the bone marrow in 3d mice is similar to the defect in long-lived PCs observed in MyD88-/- mice. (Figure 3.6c) Studies with 3d mice suggest a role for TLR7-and TLR9-MyD88 mediated pathways in

Figure 3.6- Defective long-term humoral responses to PyV infection in 3d mice

a.



b.

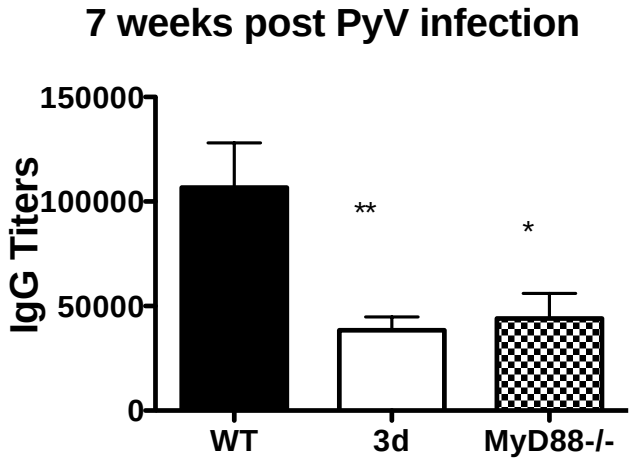


Figure 3.6 (cont.)- Defective long-term humoral responses to PyV infection in 3d mice

c.

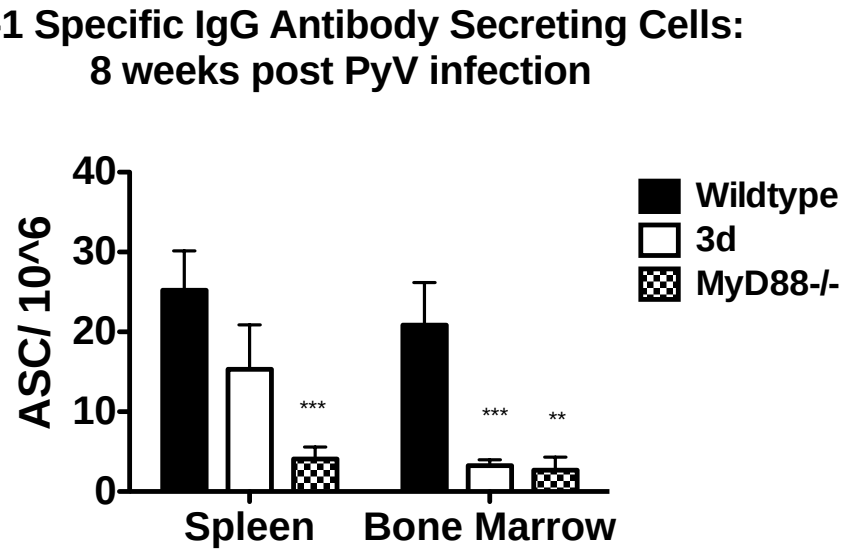


Figure 3.6- Defective long-term humoral responses to PyV infection in 3d mice

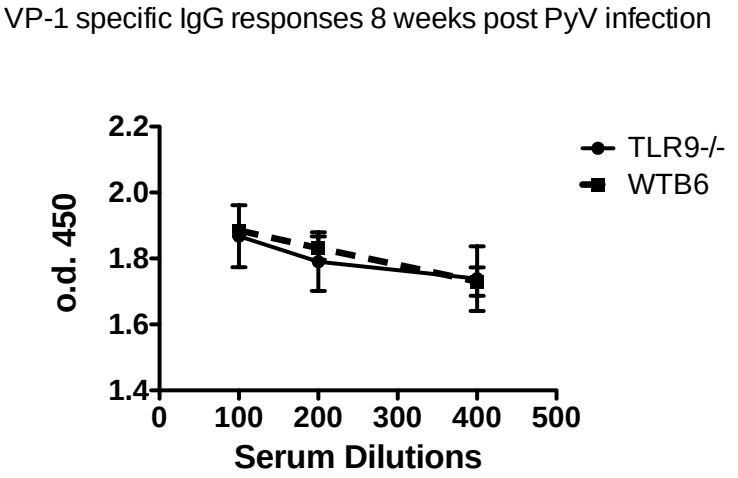
(a-b) Long-term VP1-specific IgG in 3d (n=5) mice, MyD88-/- (n=4), and WT (n=3) mice from 2 to 7 weeks post infection detected by ELISA. Mean +/- SD is shown. (c) ELISPOT assay to detect VP1-specific ASC in the spleen and bone marrow of 3d, MyD88-/- and WT mice at 8 weeks post infection (same mice from part a). *p value <.05, **p value <.01, ***p value < .001

sustaining long-term B cell responses to PyV infection. The TLR3-TRIF signaling pathway may also play a MyD88-independent role in mediating long-term humoral immunity to PyV infection. Therefore, it was important to investigate the role of individual TLR3, 7, and 9 in this model. Initial studies were conducted with TLR9 deficient mice because it recognizes CpG DNA and PyV is a DNA virus. Upon infection with PyV, TLR9-/- mice had comparable long-term antibody production to WT mice as seen in Figure 3.7a, which shows total PyV-specific IgG levels at 8 weeks post infection, the latest time point tested. Furthermore, similar numbers of PyV-specific ASCs in the spleen and bone marrow of TLR9-/- mice and WT mice were observed at 1.2 years post PyV infection. (Figure 3.7b) These data suggest that there were no defects in long-lived plasma cells as previously observed in MyD88-/- and 3d mice. It is probable that other TLRs are compensating for the loss of TLR9 in TLR9-/- mice. However, these studies suggest that TLR9 alone is not required for maintaining long-term antibody responses.

TLR7 was the next individual TLR to examine. TLR7 is known to recognize ssRNA and can signal through MyD88 and activate IRF5. When TLR7-/- mice were infected with PyV, we observed mild defects in B cell responses. One experiment showed that TLR7-/- mice had significantly lower levels of VP1-specific IgG compared to WT mice at 3 and 5 weeks post infection. (Figure 3.8a) However, the difference in virus-specific IgG was not seen at 7 weeks post infection. (Figure 3.8a) In the same experiment where defects in antibody levels

Figure 3.7- TLR9 deficient mice generate similar long-term VP1-specific humoral responses to WT mice

a.



b.

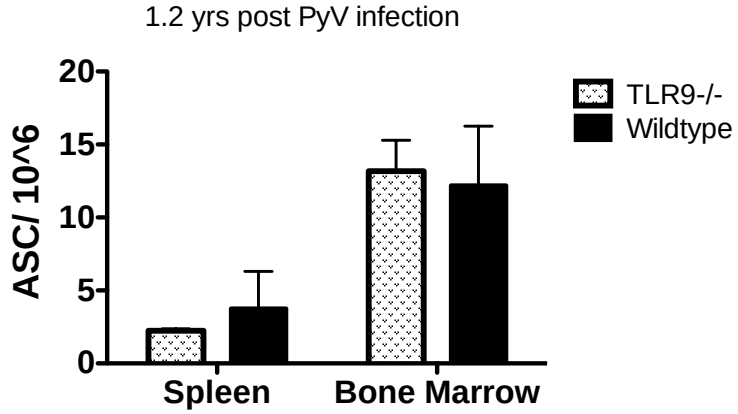
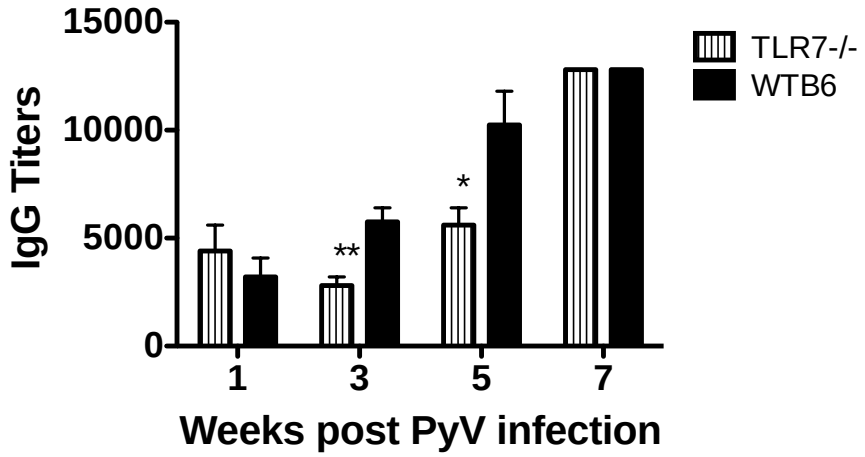


Figure 3.7- TLR9 deficient mice generate similar long-term VP1-specific humoral responses to WT mice

(a) Long-term PyV-specific IgG in TLR9-/- (n=4) and WT (n=2) mice 8 weeks post infection. (b) Splenic and bone marrow ASCs in TLR9-/- mice 1.2 years post infection. Mean +/- SD is shown

Figure 3.8- Mild defects in long-term humoral responses to PyV infection in TLR7-/- mice

a.



b.

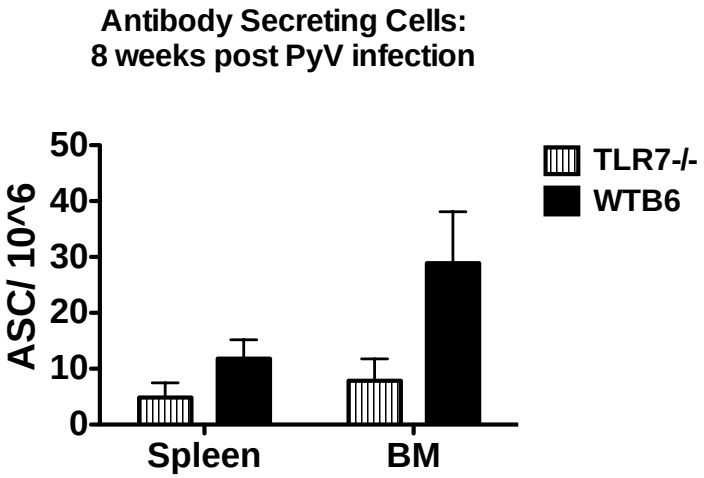


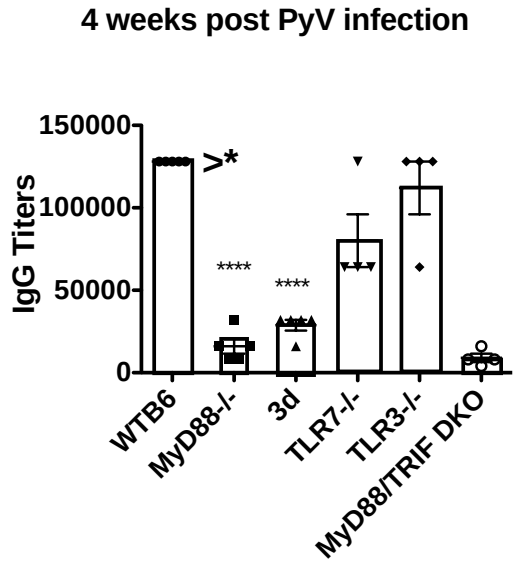
Figure 3.8- Mild defects in long-term humoral responses to PyV infection in TLR7-/- mice

(a) VP1-specific IgG levels in TLR7-/- (n=4) and WT (n=5) 1 to 7 weeks post infection. (b) VP1-specific ASC in the bone marrow 8 weeks post infection. Mean +/- SD is shown. *p value <.05, **p value <.01

were observed early after infection, TLR7-/- mice showed lower numbers (not significant) of virus-specific long-lived plasma cells in the bone marrow when compared to WT mice. (Figure 3.8b) Long-term B cell responses in TLR7-/- were inconsistent since TLR7-/- mice showed significant defects only in one experiment. The next three experiments with TLR7-/- mice displayed no significant differences between TLR7-/- and WT mice in B cell responses upon PyV infection. TLR7-/- mice had slightly diminished long-term PyV-specific IgG (figure 3.9a) at 4 weeks post infection, but this difference was not significant. Similarly, a small and not significant decrease was seen in the generation of long-lived plasma cells in the bone marrow at 8 weeks post infection in TLR7-/- mice. (Figure 3.9 b-c) TLR3-/- mice had normal levels of long-term VP1-specific IgG (shown at 4 weeks post infection). (Figure 3.9a) Individual TLR3-/- and TLR7-/- mice generated similar numbers of long-lived PCs to WT mice, unlike in MyD88-/- and 3d mice. (Figure 3.9 b-c) These studies with TLR3-/- mice suggest that TLR3-TRIFF signaling pathway is dispensable for long-term B cell maintenance against PyV infection. These data also suggest that TLR7 and TLR9, by themselves, may have a redundant role in anti-PyV B cell responses. It is also possible that TLR7 and TLR9 are compensating for each other in single TLR7-/- and TLR9-/- mice, since that has been shown to occur in other models (121). Since single TLR7-/- and TLR9-/- mice appear to have redundant roles and do not have defects in long-term anti-PyV B cell responses, it was important to determine the role of both TLR7 and TLR9 in long-term B cell responses to

Figure 3.9- Individual TLR3 and TLR7 deficient mice do not display low levels of long-term VP1-specific IgG or lower numbers of long-lived PC as 3d or MyD88-/- mice

a.



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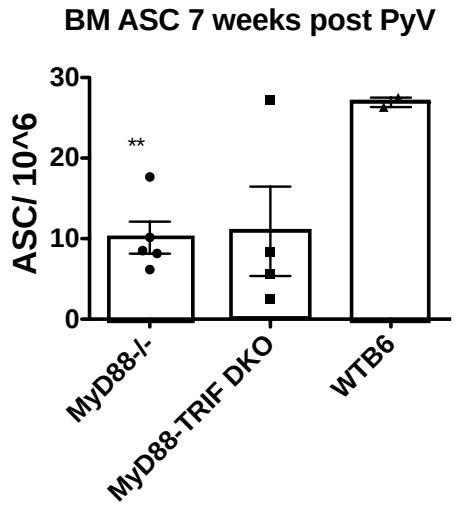


Figure 3.9 (cont.)- Individual TLR3 and TLR7 deficient mice do not display low levels of long-term VP1-specific IgG or lower numbers of long-lived PC as 3d or MyD88-/- mice

c.

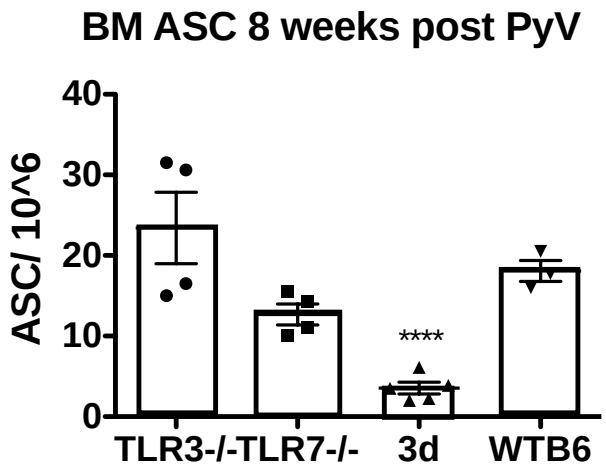


Figure 3.9- Individual TLR3 and TLR7 deficient mice do not display low levels of long-term VP1-specific IgG or lower numbers of long-lived PC as 3d or MyD88-/- mice

(a) VP1-specific IgG in 3d, MyD88-/-, individual TLR3-/-, TLR7-/-, and WTB6 mice at 4 weeks post infection. (b) ASCs in the bone marrow of MyD88-/-, MyD88/TRIF DKO and WTB6 mice at 7 weeks post PyV infection. (same mice from part a) (c) ASCs in the bone marrow of TLR7-/- , TLR3-/-, 3d and WTB6 mice 7 weeks post infection. (same mice from part a) Mean +/- SD is shown. >* The IgG2a or IgG1 titers are equal or greater than 128000. **p value <.01, ****p value < .0001

PyV infection. TLR7/9 DKO mice infected with PyV generated low-levels of VP1-specific total IgG 3 weeks post infection (Figure 3.10), as seen in MyD88-/- and 3d mice. These data support a redundant role of TLR7 and TLR9 in generating long-term IgG production in response to PyV infection.

Viral proteins can activate some surface, non-endosomal TLRs, such as TLR2 and TLR4 (16).There has been a report that TLR2 and TLR4 on APCs mediate cytokine production upon PyV infection (122). The different Th1 or Th2 cytokine responses to PyV infection result in PyV-induced tumor resistance or susceptibility, respectively (122). This suggests that surface TLR2 and TLR4 can be important in the context of PyV-induced tumor formation and in PyV recognition. Thus, it was important to investigate the role of TLR2 and TLR4 in mediating antibody responses to PyV infection. Studies using single TLR2-deficient (TLR2-/-) and TLR4-deficient (TLR4-/-) mice did not show defects in long-term VP1-specific IgG production (Figure 3.11a) or in long-lived PC in the bone marrow. (Figure 3.11b) These data suggest that neither TLR2 nor TLR4 signaling pathways influence long-lasting antibody responses to PyV infection.

It is important to remember that these innate signaling pathways are complex and redundant. No single TLR knockout mice showed the severely defective antibody responses that were observed in MyD88-/- mice and the 3d mice. All the data taken together suggest that TLR7 and TLR9 play an important, redundant role in maintaining long-term anti-PyV B cell responses.

Figure 3.10- Defective long-term PyV-specific IgG levels in TLR7/9 DKO mice

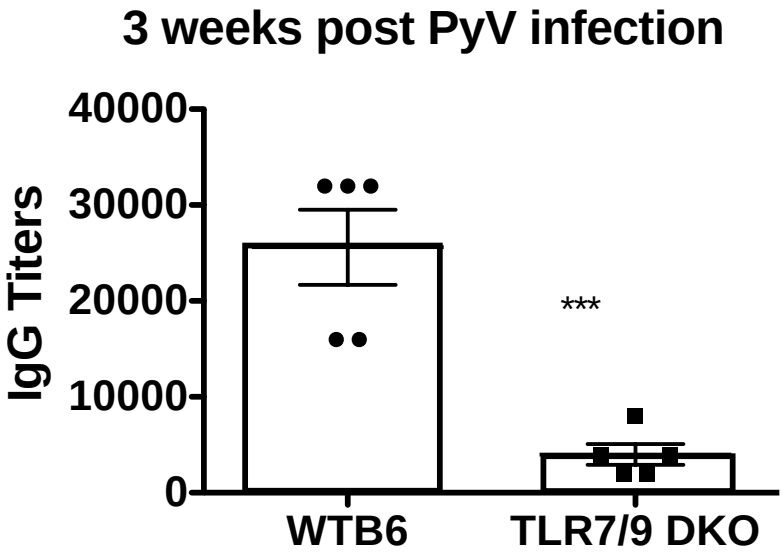
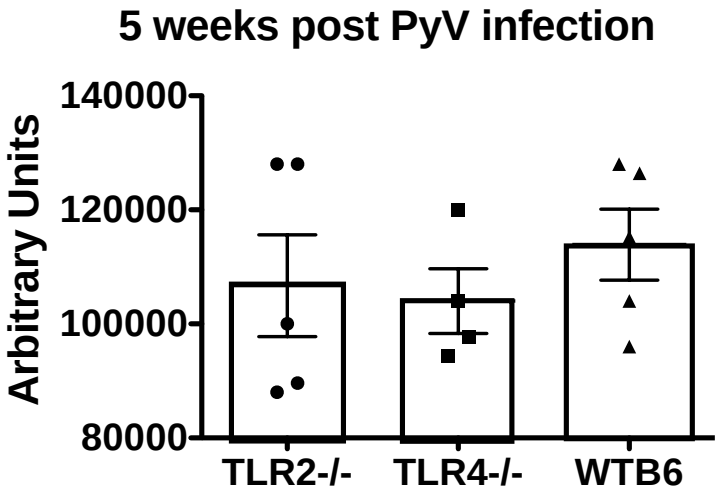


Figure 3.10- Defective long-term PyV-specific IgG levels in TLR7/9 DKO mice

VP1-specific IgG levels detected in the sera of WTB6 (n=5) and TLR7/9 DKO (n=5) mice 3 weeks post infection. Mean +/- SD is shown. ***p value < .001

Figure 3.11- TLR2-/- and TLR4-/- mice generate long-term B cell responses to PyV infection similar to WT mice

a.



b.

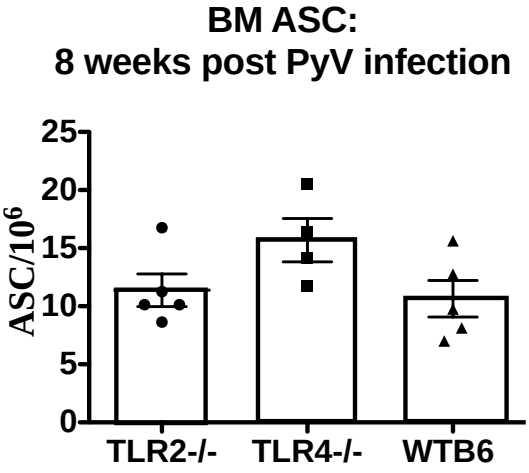


Figure 3.11- TLR2-/- and TLR4-/- mice generate long-term B cell responses to PyV infection similar to WT mice

VP1- specific ELISA in TLR2-/- (n=5) and TLR4-/- (n=4) and WT (n=5) mice. (a) VP1-specific IgG at 5 weeks post infection. (b) Bone marrow ASC in TLR2-/-, TLR4-/- and WTB6 mice 8 weeks post infection. Mean +/- SD is shown

E. The role of IRF5 in maintaining long-term antibody responses to PyV infection

Since I have shown that TLR7 and TLR9 are important in long-term antibody responses to PyV infection and that TLR7 and TLR9 pathways activate IRF5, I investigated IRF5 in long-term antibody responses to PyV infection. IRF5-/- mice generate lower levels of virus specific total IgG than WT mice in response to PyV infection. (Figure 3.12a) The defect in PyV-specific IgG in IRF5-/- mice is similar to the defective long-term PyV-specific IgG responses observed in MyD88-/- mice. (Figure 3.12a) Testing the generation of long-lived plasma cells, we found that the IRF5-/- mice developed very low numbers, 1-3/10^6 ASCs, in the bone marrow, compared to WT mice (~20/10^6 ASCs) 8 weeks post infection. (Figure 3.12b) MyD88-/- mice also showed similar defects in BM ASCs as IRF5-/- mice at 9 weeks post infection. (Figure 3.12c) These studies suggest that the TLR-MyD88-IRF5-mediated pathway is important in maintaining long-term antibody responses to PyV infection. However, it is not clear if the TLR-MyD88-IRF5 pathway plays a direct role in B cells to mediate long-term anti-PyV B cell responses.

To study the role of IRF5 within CD11c populations (DCs) in maintaining long-term PyV-specific humoral responses, I infected CD11c-IRF5-/- mice with PyV. Studies in CD11c-IRF5-deficient mice show no significant differences in VP1-specific IgG or in the generation of long-lived PC in the bone marrow. (Figure 3.13a-b) These data suggest that the absence of IRF5 in CD11c+ cells

Figure 3.12- Defective VP1-specifc long-term IgG and fewer long-lived plasma cells in IRF5-/- mice

a.

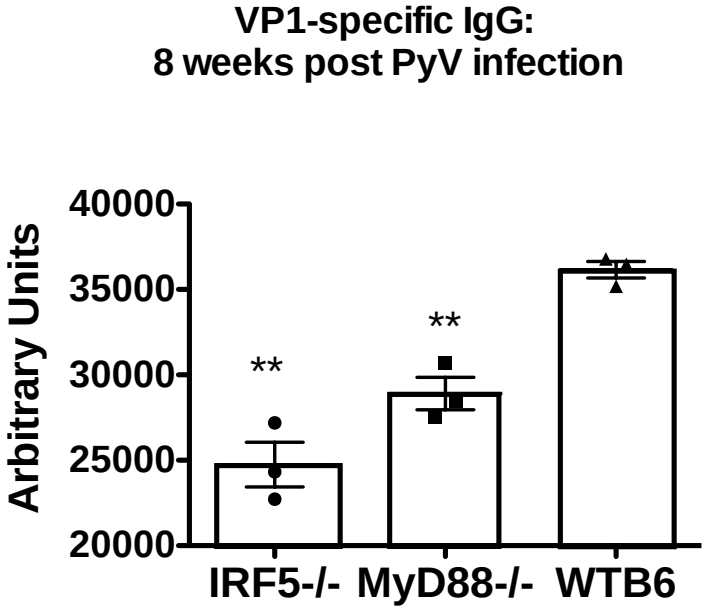
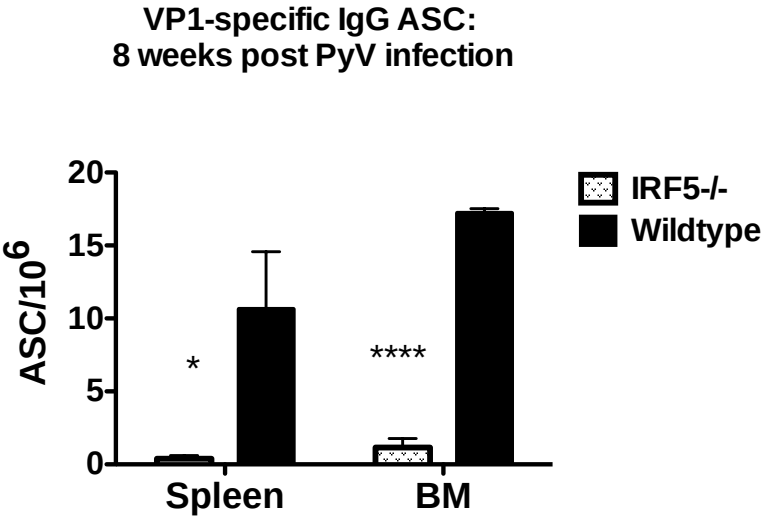


Figure 3.12(cont.)- Defective VP1-specifc long-term IgG and fewer long-lived plasma cells in IRF5-/- mice

b.



c.

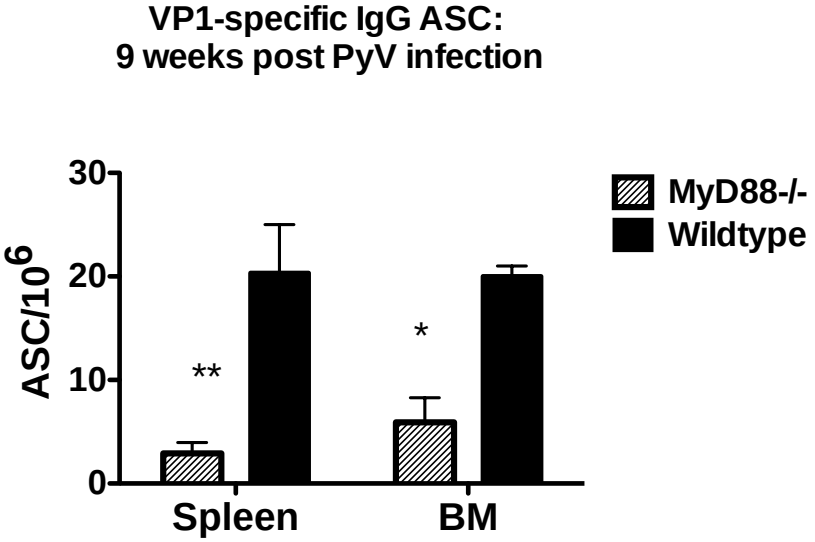
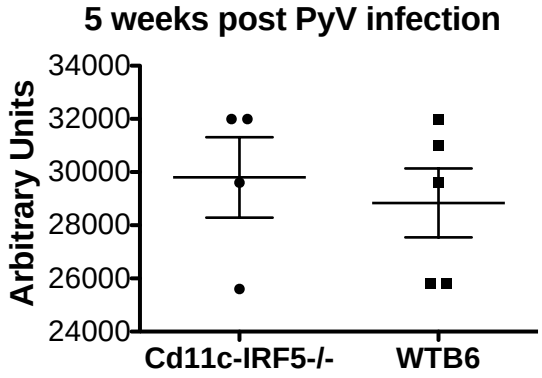


Figure 3.12- Defective VP1-specifc long-term IgG and fewer long-lived plasma cells in IRF5-/- mice

VP1- specific IgG responses were measured at various time points post infection in IRF5-/-, MyD88-/- and WTB6 mice. (a) PyV-specific IgG at the latest time point tested, 8 weeks post infection. (b-c) Splenic and bone marrow ASC were determined by VP1- specific ELISPOT in IRF5-/- mice at 8 weeks post infection and MyD88-/- mice at 9 weeks post infection. Mean +/- SD is shown. *p value <.05, **p value <.01, ****p value < .0001

Figure 3.13- CD11c-IRF5-deficient mice generate long-term B cell responses to PyV infection similar to WT mice

a.



b.

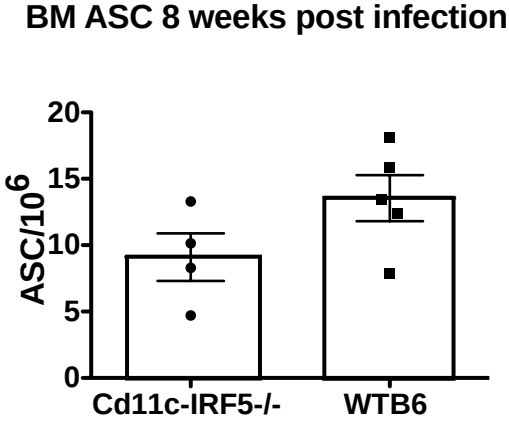


Figure 3.13- CD11c-IRF5-deficient mice generate long-term B cell responses to PyV infection similar to WT mice

(a) VP1-specific IgG ELISA on CD11c-IRF5-/- and WTB6 mice 5 weeks post infection. (b) VP1-specifc ASC in the bone marrow 7 weeks post infection. Mean +/- SD is shown

such as DCs may not play an important role in maintaining long-term B cell responses. It is important to mention that it was recently discovered that IRF5-/- mice, which are widely used in the field, also contain a spontaneous mutation in Dock2 (dedicator of cytokinesis 2). Dock2 is a guanine nucleotide exchange factor (GEF) involved in regulating Rac signaling pathways and marginal zone B cell-development (123). Rac signaling pathways are important in cell migration and adhesion (124). This means that the defects observed in IRF5-/- mice could be due to the absence of IRF5, or to the lack of Dock2. Of note, CD11c-cre-IRF5-floxed mice, used here, do not contain a mutation in Dock2 (116). The Dock2 mutation and its effect on the responses seen in IRF5-/- mice in response to PyV are further explained within the discussion section. The next few sections will discuss how the lack of MyD88-mediated pathways affects the maintenance of long-term antibody responses to PyV infection.

F. GC responses in MyD88-/- mice to PyV infection

Long-lived plasma cells and memory B cells are derived from GC (GC) B cells.. These long-lived populations are required to maintain long-term antibody responses. GC responses were examined in MyD88-/- and WT mice upon PyV infection. Eight days post infection, I stained for molecules highly expressed by GC B cells such as, GL-7, Fas and Bcl-6. Bcl-6 is an important transcription factor that is required for the generation of GC B cells. MyD88-/- mice can generate similar frequencies of GC B cells in the spleen as WT mice on day 8

post PyV infection, as previously observed in our lab (3), and data that I have repeated. (Figure 3.14a-b) Previously, our lab has also observed similar levels of AID enzyme expression in GC B cells of both WT and MyD88-/- mice (3). The GC B cells from WT mice up-regulate Bcl-6 at 40-60%. (Figure 3.14c) The GC B cells from MyD88-/- mice also up-regulate Bcl-6 to similar levels as WT mice. (Figure 3.14d) This suggests that GC B cells can form normally in MyD88-/- mice at early time points post infection. Furthermore, these data support the concept that MyD88-mediated signals are not required for initial GC responses during an acute PyV infection.

In acute viral infections or during protein immunizations the GC response usually peaks around 8-10 days post infection/immunization (22, 24). However, PyV causes a persistent viral infection and it is never cleared from the host. Perhaps the difference in the longevity of IgG responses in WT and MyD88-/- mice is due to short-lived GC responses in the MyD88-/- mice. Therefore, we investigated the longevity of the GC responses in MyD88-/- mice. GC B cells were stained, as described above, in MyD88-/- and WT mice 14 and 21 days post infection. MyD88-/- mice generated similar numbers of GC B cells as WT mice at day 14-post infection. (Figure 3.15a), but MyD88-/- mice generated lower numbers of GC B cells when compared to WT mice at day 21- post infection. At 21 days post infection, WT mice generated ~$1x10^6$ GC B cells/ spleen, whereas MyD88-/- mice had less than ~$5x10^5$ of GC B cells. (Figure 3.15b) GC B cell numbers were also analyzed at 7 months post

Figure 3.14- Normal formation of GC B cells in WT and MyD88-/- mice

a. WTB6

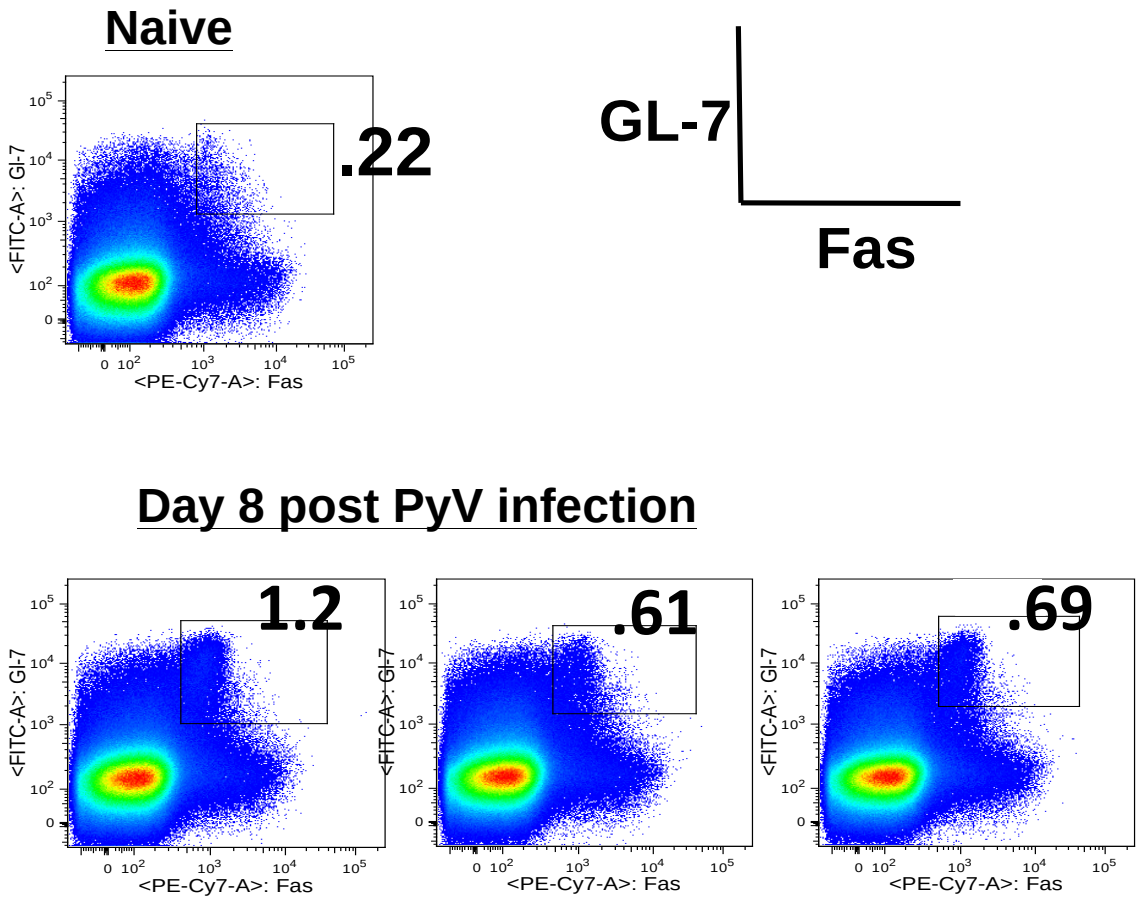


Figure 3.14- Normal formation of GC B cells in WT and MyD88-/- mice

b. MyD88-/-

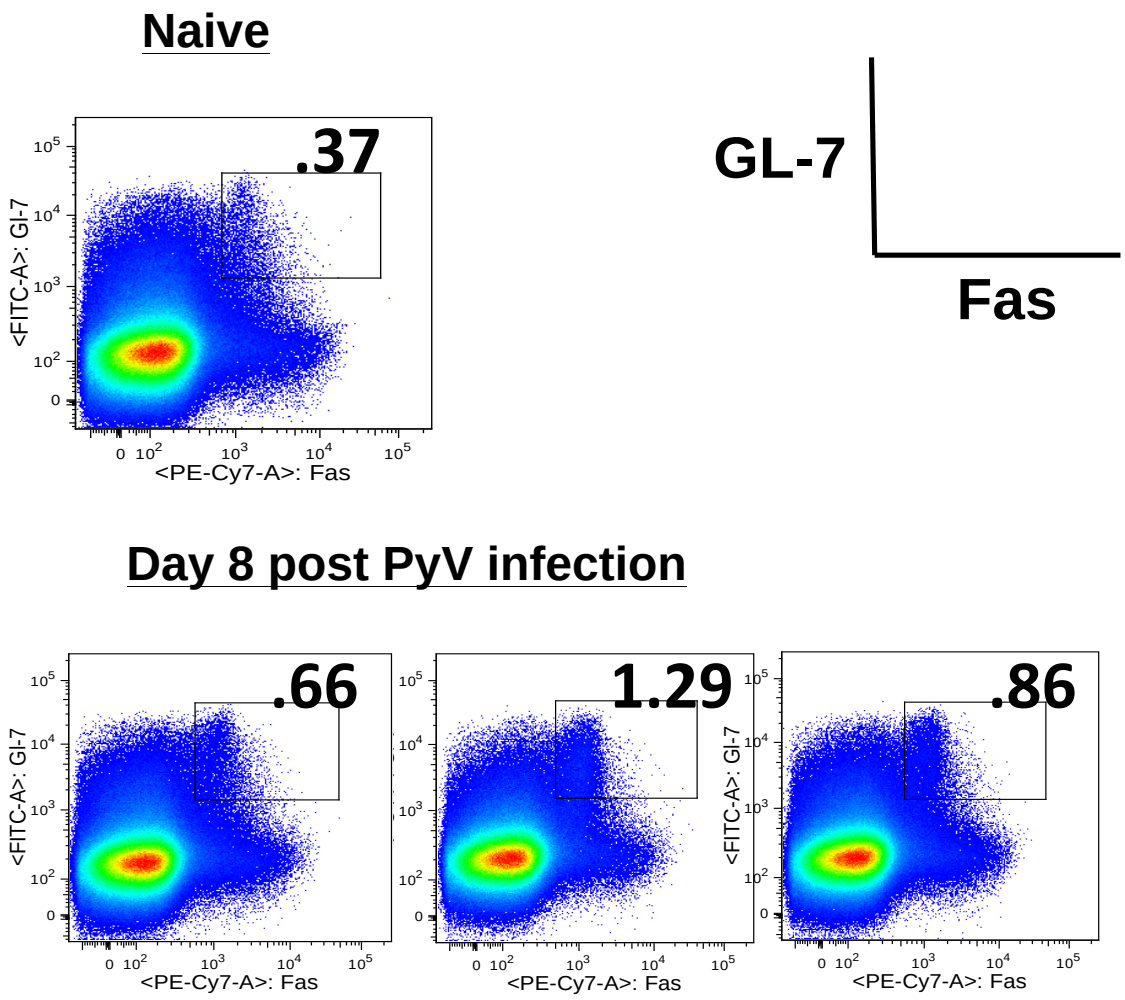


Figure 3.14- Normal formation of GC B cells in WT and MyD88-/- mice

c. Bcl-6 expression on GC B cells in WT mice

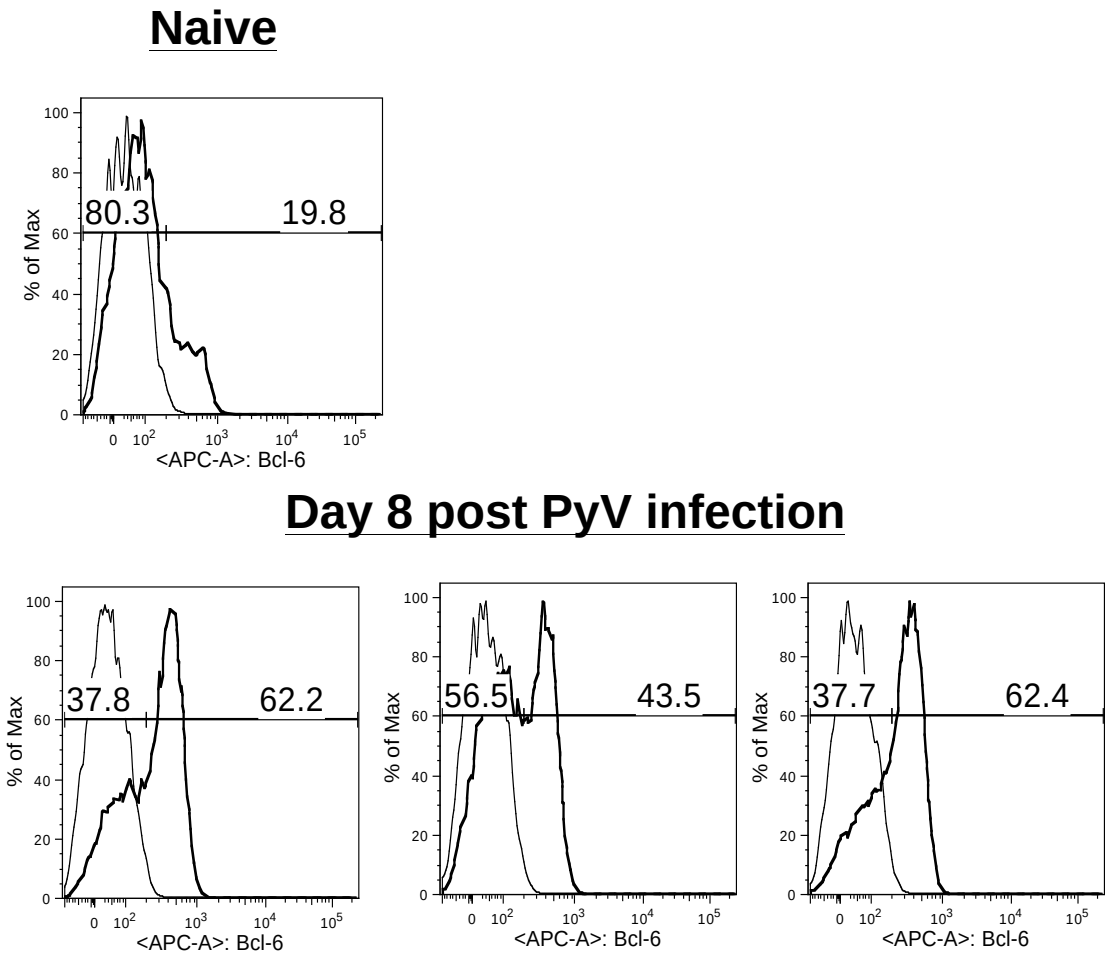


Figure 3.14- Normal formation of GC B cells in WT and MyD88-/- mice

d. Bcl-6 expression on GC B cells in MyD88-/- mice

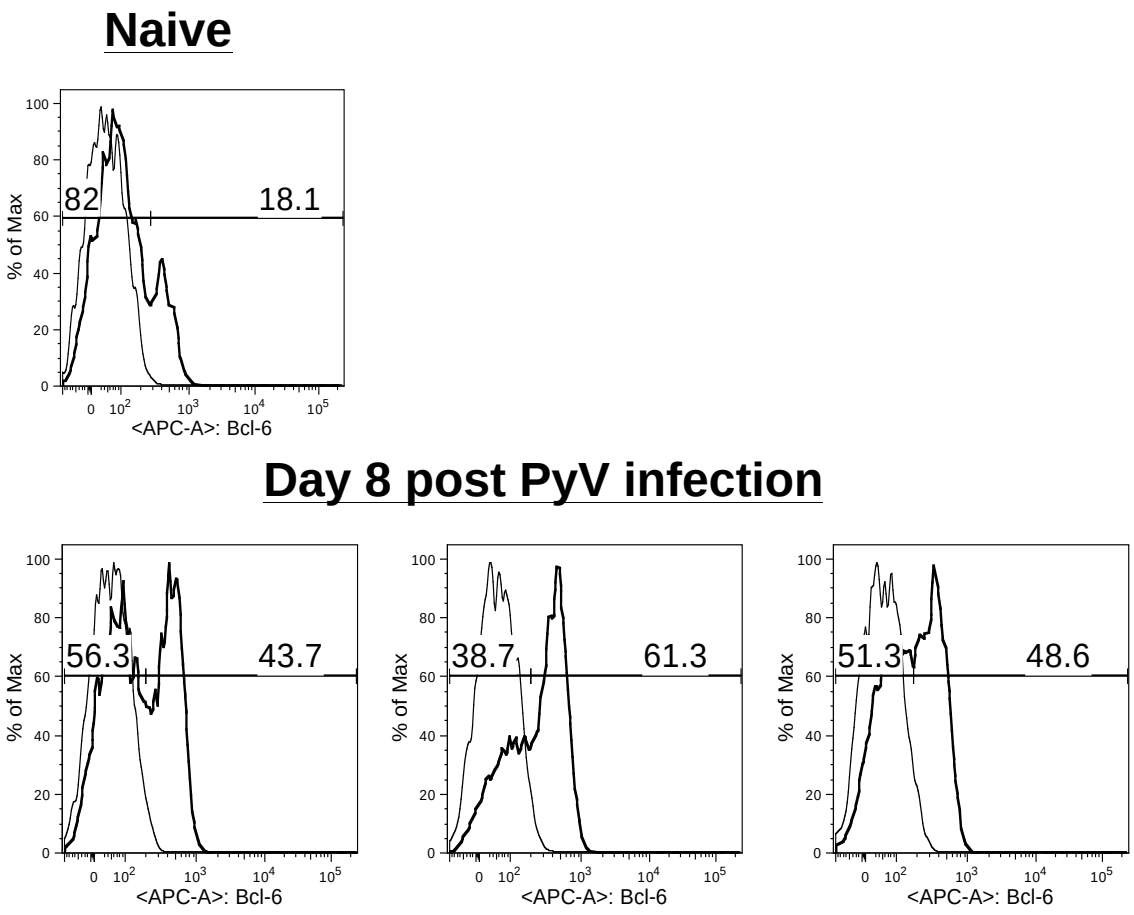
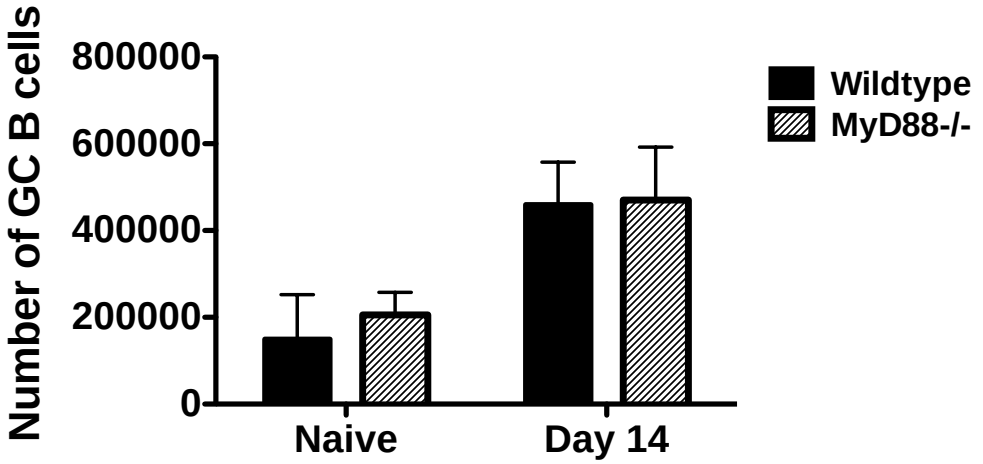


Figure 3.14- Normal formation of GC B cells in WT and MyD88-/- mice

GL-7^{hi}Fas^{hi} GC B cells gated on CD19+B220+ B cells in WT (a) and MyD88-/- mice (b) 8 days post PyV infection. Bcl-6 expression was detected in WT (c) and MyD88-/- mice (d) in the GL-7^{hi}Fas^{hi} GC B cells described in parts a and b. Darker, bold lines represent Bcl-6 expression. Lighter, dashed lines represent isotype control.

Figure 3.15- A decrease in the number of GC B cells in PyV-infected MyD88-/- mice overtime

a.



b.

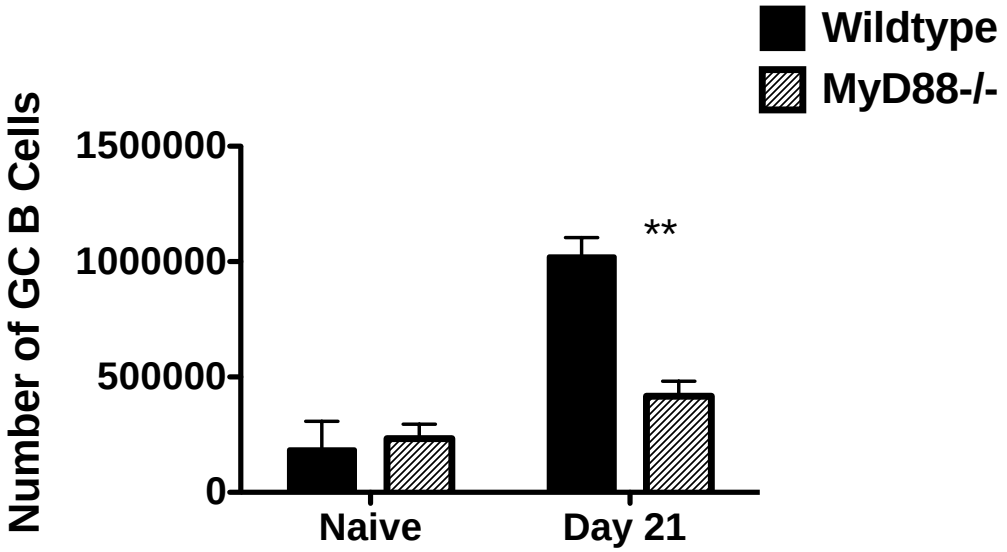


Figure 3.15- A decrease in the number of GC B cells in PyV-infected MyD88-/- mice overtime
c.

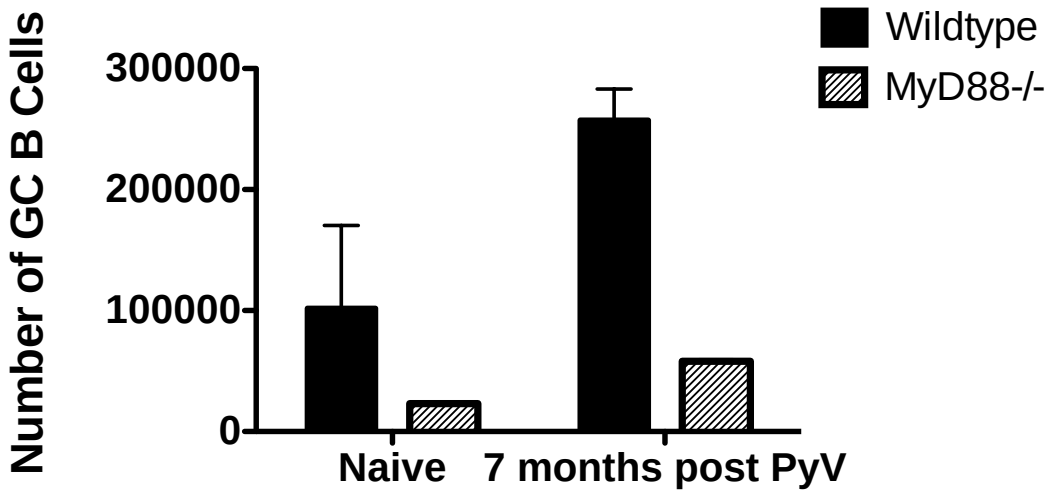


Figure 3.15- A decrease in the number of GC B cells in PyV-infected MyD88-/- mice overtime

Number of GL-7^{hi}Fas^{hi} GC B cells gated on CD19+B220+ B cells in PyV-infected MyD88-/- (n=3) and WT (n=3) mice (a) 14 days, (b) 21 days: **p value <.01, and (c) 7 months post infection. Mean +/- SD is shown

PyV infection to examine how long GC responses are maintained in this persistent virus model. At this late time point, the number of GC B cells was lower than the number of GC B cells at 21 and 7 days post infection in WT mice, but still above the naïve WT levels. WT mice, 7 months post infection, generated ~2.5x10^5 GC B cells. MyD88-/- mice generated ~ <100,000 GC B cells at this time point. (Figure 3.15c) Thus, overall, the phenotype that is being observed in MyD88-/- mice is that these mice can form GC B cells as well as WT mice initially, but overtime, MyD88-deficient mice are not able to maintain GC B cells in response to persistent PyV infection. The poor maintenance of the GC response may contribute to defects seen in long-term B cell populations in MyD88-/- mice to PyV infection.

G. TFH cells in MyD88-deficient mice in response to PyV infection

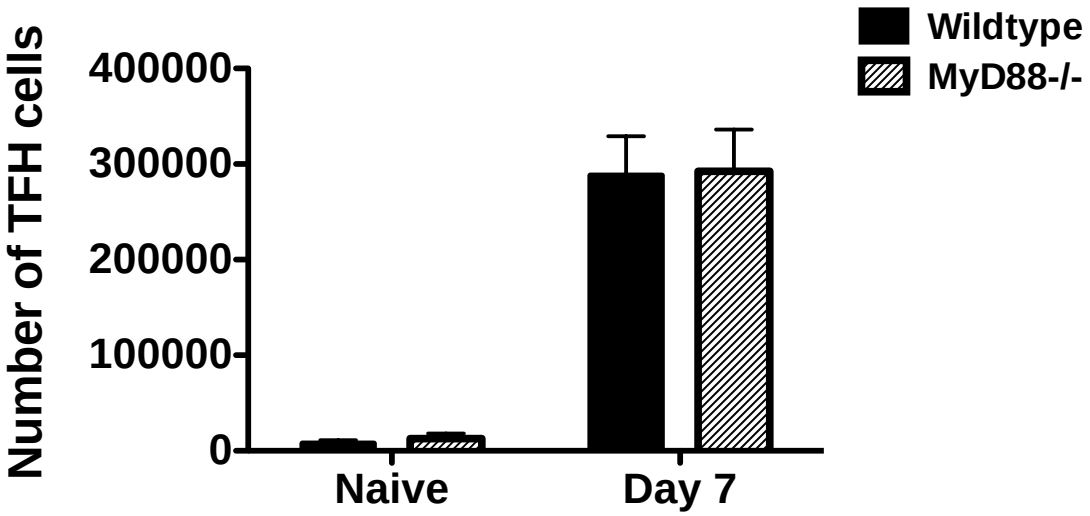
There are several molecules and cell types that are important in maintaining GC B cells. The defect in GC B cells in MyD88-/- mice is similar to defects observed in SAP-deficient animals (52). SAP is one of the several markers that define a subset of CD4 T cells known as TFH cells (21). TFH cells are also defined by their expression of CXCR5, ICOS, PD-1, SAP, Bcl-6, and several other molecules (46). TFH cells are specialized in helping B cells differentiate into GC B cells, and they are important in maintaining the GC response within the B cell follicle (21). Since we found that MyD88-/- mice are unable to maintain GC B cell numbers in response to persistent PyV infection, we examined whether this is

due to defects in TFH cells in our system. MyD88-/- mice and WT mice were infected with PyV and at 7 and 21 days post infection splenocytes were stained for the TFH cell markers CXCR5, ICOS, PD-1, GL-7 and Bcl-6. At 7 days post infection, MyD88-/- mice generated similar numbers (~300,000) of TFH cells (defined as CD4+T cells positive for CXCR5, PD-1, ICOS and Bcl-6) as WT mice. (Figure 3.16a) This is consistent with the fact that normal GC B cell responses are observed at this time point. However, at day 21-post infection, the MyD88-/- mice had lower numbers of TFH cells than WT mice. (Figure 3.16b) Similar defects in the number of TFH cells at 21 days post infection were observed in 3d mice. (Figure 3.17) To further validate the defects in TFH cell numbers, WT and MyD88-/- mice were stained with antibodies to PSGL-1 and Ly6C (53). Cells that are double negative for PSGL-1 and Ly6C have been shown to greatly express TFH cell markers such as CXCR5, PD-1 and ICOS (53). MyD88-/- mice, compared to WT mice at 21 days post PyV infection, expressed lower numbers of TFH cells which were defined by PSGL-1lo and Ly6Clo cells that were positive for CD4, CXCR5, PD-1, and Bcl-6 (Figure 3.16c), supporting data in figures 3.18b and 3.19.

Recent studies have shown a role for regulatory TFH cells (TFR cells) in controlling the GC response by directly controlling the number of TFH cells present and in the selection of high affinity, antigen-specific

Figure 3.16- Number of TFH cells reduced in MyD88-/-mice 21 days following PyV infection

a. TFH cells defined as CD4+CXCR5+ICOS+PD-1+Bcl-6+



b. TFH cells defined as CD4+CXCR5+ICOS+PD-1+Bcl-6+

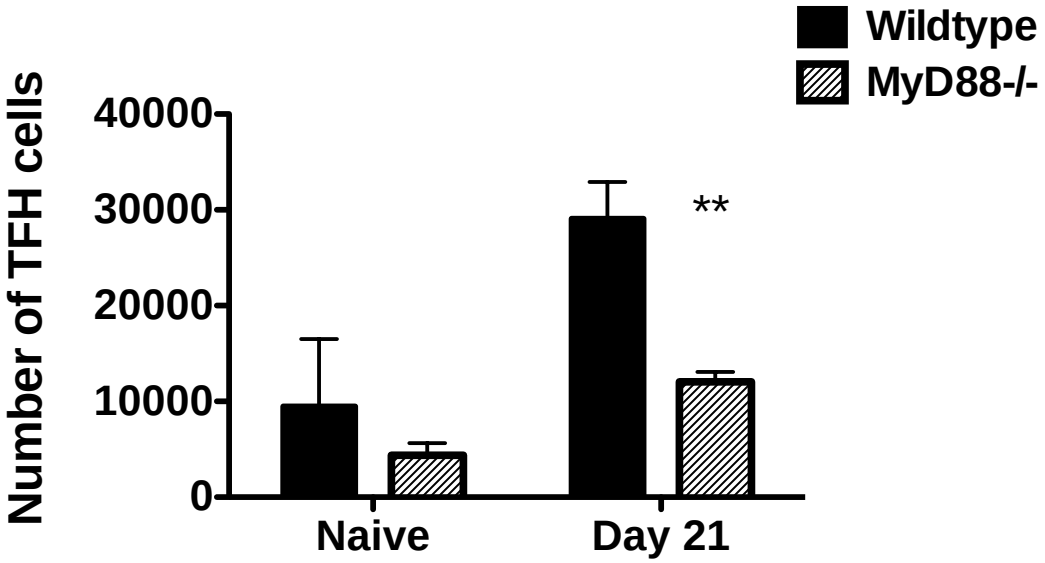


Figure 3.16 (cont.)- Number of TFH cells reduced in MyD88-/-mice 21 days following PyV infection

c. TFH cells defined as CD4+CXCR5+ICOS+Bcl-6+ from T cells that are double negative for PSGL-1 and Ly6C

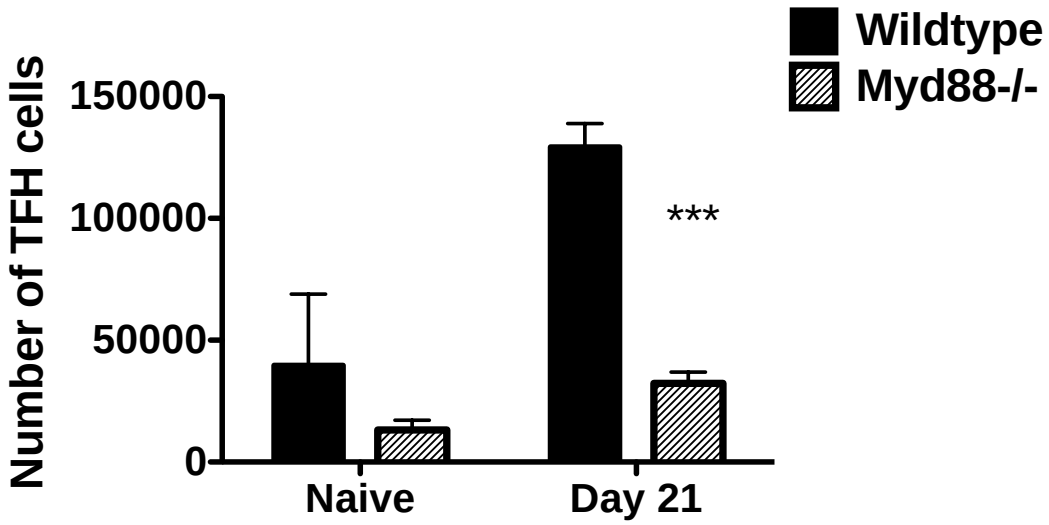


Figure 3.16- Number of TFH cells reduced in MyD88-/-mice 21 days following PyV infection

CD4+ T cells positive for CXCR5, ICOS, PD-1 and Bcl-6 were defined as TFH cells in (a) PyV-infected MyD88-/- (n=3) and WT (n=3) mice at 7 days post infection. (b) In MyD88-/- (n=4), and WT (n=3) mice at 21 days post PyV infection. (c) TFH cells defined as CXCR5+ICOS+Bcl-6+ CD4+ T cells that are double negative for PSGL-1 and Ly6C were shown in MyD88-/- (n=2), and WT mice (n=3) at 21 days post infection. Mean +/- SD is shown

**p value <.01, ***p value < .001

Figure 3.17- Number of TFH cells reduced in MyD88-/- and 3d mice at day 21 following PyV infection

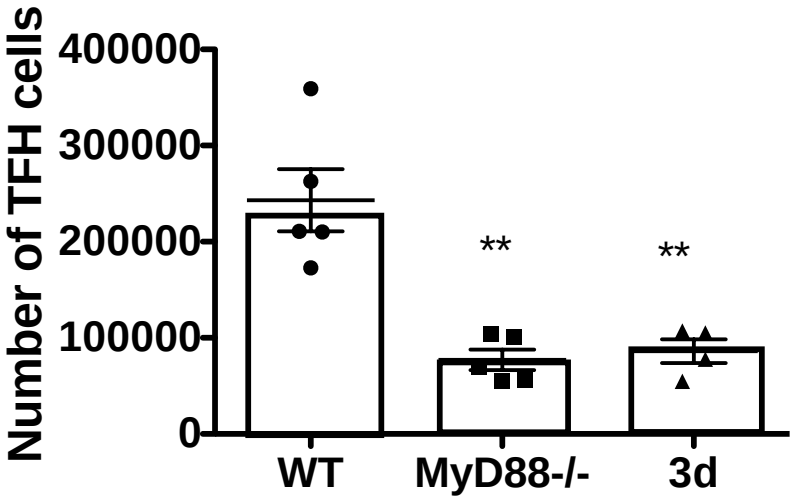


Figure 3.17- Number of TFH cells reduced in MyD88-/- and 3d mice at day 21 following PyV infection

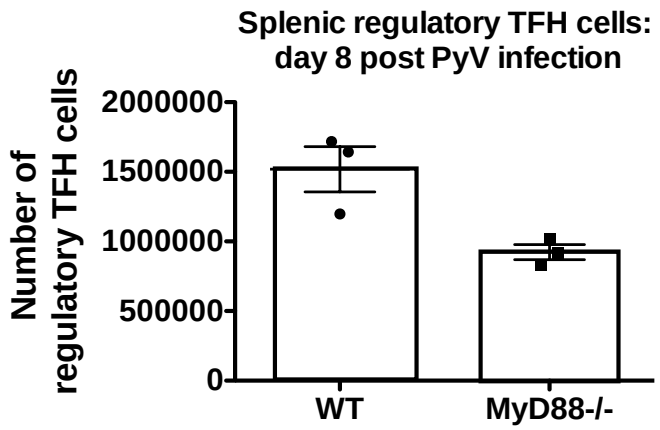
TFH cell numbers in WT (n=5), MyD88-/- (n=5), and 3d mice (n=4) 21 days post PyV infection. TFH cells were determined as CD4+ T cells that were also positive for CXCR5, Bcl-6 and PD-1. Mean +/- SD is shown. **p value <.01

GC B cell clones. (125) TFR cells express molecules that define TFH cells and regulatory T cells such as CXCR5 and FoxP3 respectively. TFR and TFH cells also require similar signals for their formation such as SAP and interactions with B cells (125). In order to determine whether TFR cells played a role in controlling the number of TFH cells generated in MyD88-/- and 3d mice, I stained for TFR cells in our system. TFR cells were determined as CD4+CXCR5+PD-1+FoxP3+ cells. At seven days post infection, there were slightly lower numbers of TFR cells present in MyD88-/- mice, but by day 21, TFR cell numbers in MyD88-/- mice were similar to WT levels. (Figure 3.18a-b) 3d mice also did not display a defect in TFR cell numbers at 21 days post infection. (Figure 3.18b) These data suggest that TFR cells do not influence defects in the maintenance of TFH cells in MyD88-/- mice upon PyV infection.

CXCR5 is a chemokine receptor that is crucial for TFH cells. CXCR5 helps in trafficking and locating CD4 T cells within the B cell follicle, where they can provide proper help to B cells. CXCR5 expression was examined on CD4 T cells in WT and MyD88-/- mice upon PyV infection. WT mice up-regulated CXCR5 to 9-13%, and MyD88-/- mice up-regulated CXCR5 to 4-8% 7-8 days post PyV infection. (Figure 3.19a) The difference in CXCR5 expression on CD4 T cells between MyD88-/- and WT mice was significantly greater on day 21-post PyV infection. (Figure 3.19a) One experiment also showed similar defects in CXCR5 expression in 3d mice. (Figure 3.19b) Lower expression of

Figure 3.18- No significant differences in the number of regulatory TFH cells in MyD88-/-, 3d and WTB6 mice post PyV infection

a.



b.

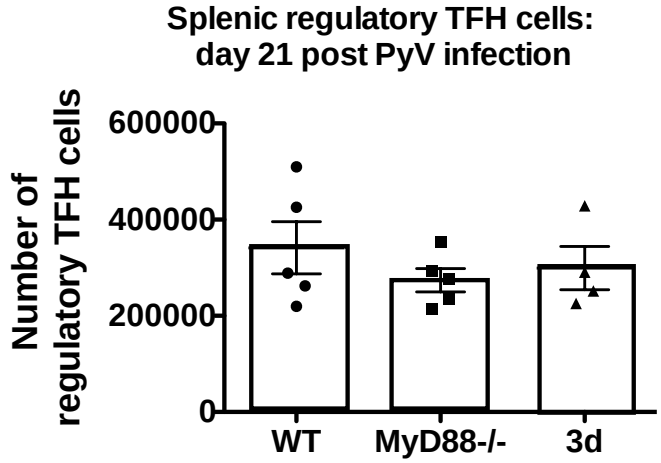
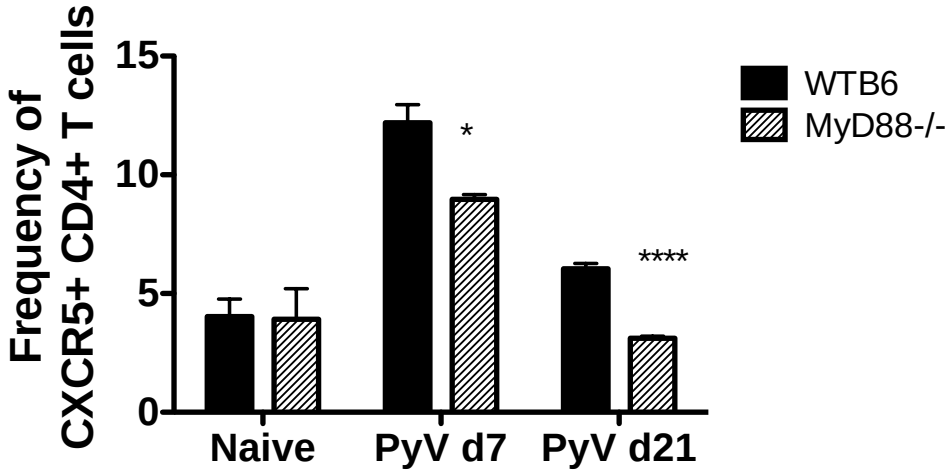


Figure 3.18- No significant differences in the number of regulatory TFH cells in MyD88-/-, 3d and WTB6 mice post PyV infection

CD4+ T cells positive for CD4, CXCR5, PD-1 and FoxP3 were defined as TFR cells at (a) 8 days post infection in MyD88-/- and WTB6 mice and (b) at 21 days post infection in MyD88-/-, 3d and WT B6 mice. Mean +/- SD is shown

Figure 3.19- Lower CXCR5+ CD4+ T cells in MyD88-/- and 3d mice post PyV

a.



b.

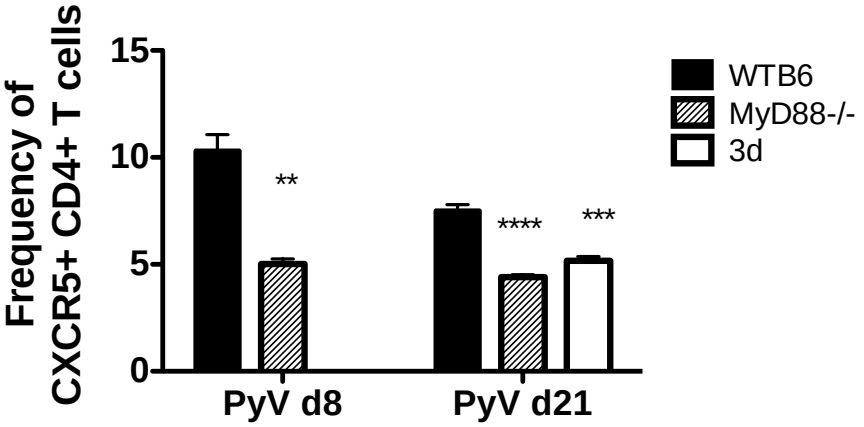


Figure 3.19- Lower CXCR5+ CD4+ T cells in MyD88-/- and 3d mice post PyV

CD4+ T cells from MyD88-/-, 3d and WT mice were analyzed for CXCR5 expression in separate experiments. (a) Frequency of CXCR5+ CD4+ T cells in MyD88-/- and WT mice at 7 and 21 days post infection. (b) Frequency of CXCR5+ CD4+ T cells in 3d mice, along with MyD88-/- and WT mice at 21 days post infection. Mean +/- SD is shown. *p value <.05, **p value <.01, ***p value < .001, ****p <.0001

CXCR5 at 7-8 days post infection may influence the number of CXCR5+CD4 T cells within the follicle, which can lead to lower numbers of CD4+ T cells differentiating into TFH cells and, subsequently, provide less B cell help. This potential defect in TFH cells may contribute to the reduction in GC B cell responses, which ultimately affects the long-term antibody response.

In addition to CXCR5, TFH cells express several different molecules such as ICOS and PD-1. ICOS and PD-1 signaling have been shown to be important not only in the formation of TFH cells, but also in preserving long-lived plasma cells (54, 126). The frequencies of ICOS and PD-1 on CXCR5+ CD4 T cells were equivalent in WT, MyD88-/- and 3d mice. (data not shown) All of these data suggest that not only defects in B cells, but also potential defects in T cell help could affect the GC response and ultimately the long-term antibody response in MyD88-/- mice upon PyV infection. Thus, it is difficult to determine whether the long-term antibody defects in response to PyV infection in MyD88-/- mice are completely B cell-intrinsic.

H. General Summary

In this chapter, the role of innate receptors in regulating humoral responses in response to PyV infection was examined. The absence of MyD88 resulted in low levels of virus-specific IgG, skewed isotype antibody responses, and defects in LLPC (3) and Bmem cell responses. 3d mice and IRF5-/- mice displayed similar humoral defects as MyD88-/- mice, suggesting that TLR-MyD88-IRF5 signaling

pathways are important in generating antiviral IgG2a responses and potentially in maintaining long-term anti-PyV responses.

The defect in VP1-specific IgG2a responses was shown to be a B cell-intrinsic defect. Adoptively transferring IRF5-/- B cells or WT B cells with WT T cells into RAG KO mice showed that only the presence of IRF5-/- B cells and WT T cells resulted in low levels of VP1- specific IgG2a and lower frequencies of IgG2a+ cells. Additionally, re-expressing IRF5 in IRF5-/- B cells restored levels of y2a transcripts. These studies, in collaboration with Paula Pitha, determined that IRF5 mediates IgG2a responses within B cells by mediating the expression of Ikaros.

3d mice also displayed similar defects in VP1- specific IgG2a as in MyD88-/- mice, but generated normal virus-specific IgG1. Studies in individual TLR 3,7, and 9 single knockout mice did not reveal a defect in PyV-specific IgG2a, suggesting that more than one of these endosomal TLRs have a redundant role in mediating this isotype response. TLR7/9 DKO mice showed defective VP1-specific IgG2a responses, further suggesting the redundant role of TLR7 and TLR9 in regulating PyV-specific IgG2a responses. TLR7 and TLR9 are known to signal through MyD88 and IRF5, thus based on the data shown, TLR7 and TLR9 are likely to be involved in regulating IgG2a responses to PyV.

Along with defects in IgG2a production, MyD88-/- mice, 3d mice, and IRF5-/- mice are unable to maintain long-term VP1-specific IgG and have low numbers of long-lived plasma cells in the bone marrow. Again, TLR 3, 7 and 9

single knockout mice did not show similar long-term B cell defects as 3d, MyD88-/- or IRF5-/- mice. These data with single TLR knockout mice support the idea that TLR7 and TLR9 are important in preserving long-term B cell responses to PyV. TLR7/9 DKO mice had low-levels of PyV-specific IgG, supporting the redundant role of TLR7 and TLR9 to mediate long-term anti-PyV B cell responses. It is not clear at the moment how the absence of MyD88 signaling in B cells, DCs, and T cells contributes to the defective long-term humoral response observed in MyD88-/- mice to PyV infection.

Further studies in this chapter showed that the defect in long-term antibody responses in MyD88-/- mice correlated with the poor maintenance of GC B cells. MyD88-/- mice could generate normal GC B cell numbers during acute PyV infection, but MyD88-/- mice could not maintain GC B cells at later time points post infection. It was also shown that MyD88-/- mice had lower number of TFH cells present, which could affect the GC B cell response and ultimately the long-term antibody response.

This chapter reveals how a persistent antigen maintains GC B cell responses longer and the pool of long-lived B cell populations in a MyD88-dependent manner. More importantly, this chapter provides evidence of how innate signaling pathways can help preserve long-term humoral protection to persistent virus infection.

CHAPTER IV: Role of PyV load and innate signaling pathways in sustaining long-term antiviral TI responses

The long-term maintenance of virus-specific antibodies prevents the recrudescence of viral infections and plays an important role in the control of persistent infections. Two types of long-lived antigen-specific B cell populations maintain sustained serum immunoglobulin levels: long-lived plasma cells and Bmem cells. Both of these long-lived B cell populations are thought to be dependent on T cell help, derive from GC responses and produce highly specific, class switched antibodies. In contrast, antibody responses generated without T cell help, the so called T cell-independent (TI) antibody responses are usually short-lived (127). The typical TI antigens, such as bacterial polysaccharides or NP (4-Hydroxy-3-nitrophenylacetyl)-Ficoll, are not proteins and thus cannot be processed by antigen presenting cells and presented as peptides to activate helper CD4 T cells. TI antigens, in general, do not induce GC formation and subsequent long-lived plasma cell and Bmem cell generation.

Persistent viral infections, such as infections with human or murine polyomaviruses, require all aspects of the immune system to keep the viral load in check for the host's lifetime. PyV is well controlled and does not cause disease in immunocompetent animals, but leads to tumor development after many months in completely T cell-deficient mice (2, 95). In addition to inducing efficient T cells and T cell-dependent B cell responses in normal hosts, PyV infection can induce a potent T cell-independent IgG response in T cell-deficient mice. These

TI antibody responses are protective (97), in that they reduce viral load and prevent virus-induced lethal acute myeloproliferative disease observed in PyV-infected T and B cell-deficient SCID mice (88). This suggests that in a partially immune compromised T cell-deficient host, TI antibody responses can help control the persistent viral infection. Also, transfer of sorted follicular or marginal zone B cells into SCID recipients prior to PyV infection resulted in protective TI IgM and IgG anti-PyV responses (97). Several other viruses, besides PyV, were shown to be also capable of inducing TI antiviral antibody responses. (1) This chapter uses PyV-infected mice as a model to further understand the mechanisms that are required to generate and maintain protective TI IgG responses.

TCRαβ KO mice (that lack αβ T cells) and TCRβδ/or TCRαβγδ KO mice (that lack both αβ and γδ T cells) survive PyV infection for many months, but maintain a significantly higher (~10-fold higher) persisting virus load than wild type B6 mice (95). The relatively long life span of T cell-deficient PyV-infected mice, which, in terms of adaptive immunity to PyV, have only TI B cell responses, suggests that the TI IgG responses may be long-lived, because mice which lack both T and B cells die rapidly after infection (88). This chapter provides evidence that PyV-infected T cell-deficient mice have long-lasting antiviral serum IgG responses, but the longevity of these responses is not due to the presence of long-lived bone-marrow residing plasma cells or Bmem cells. Instead, the sustained TI IgG is likely to be continuously generated by short-lived B cell

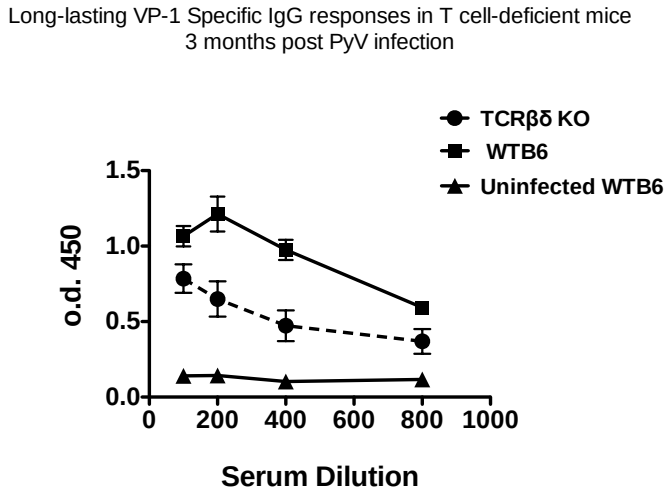
responses that require persisting virus and intact MyD88-mediated innate immune pathways.

A. Sustained antiviral IgG responses in the sera of PyV-infected T cell-deficient mice

To test the longevity of antibody responses in T cell-deficient mice, antiviral serum IgG levels of TCRβδ KO mice and control immunocompetent B6 mice were measured by VP1-ELISA at various time points post PyV infection. At 3 months post infection, TCRβδ KO mice maintained VP1-specific IgG levels in the serum, similarly to that in B6 wild type mice. (Figure 4.1a) Pooled serum samples taken from TCRβδ KO mice in an independent experiment also showed the maintenance of virus-specific IgG responses up until 6 months post infection, the latest time point tested. This was similar to the sustained long-term IgG in the pooled sera of B6 mice. (Figure 4.1b) The VP1-specific IgG titers were approximately 20 fold higher in B6 mice at 6 months post infection than in TCRβδ KO mice (data not shown), and these data on the magnitude of TI IgG responses are consistent with many experiments in our laboratory, including our previously published findings (4).

Figure 4.1- Long-lived serum IgG responses to PyV in T cell-deficient mice

a.



b.

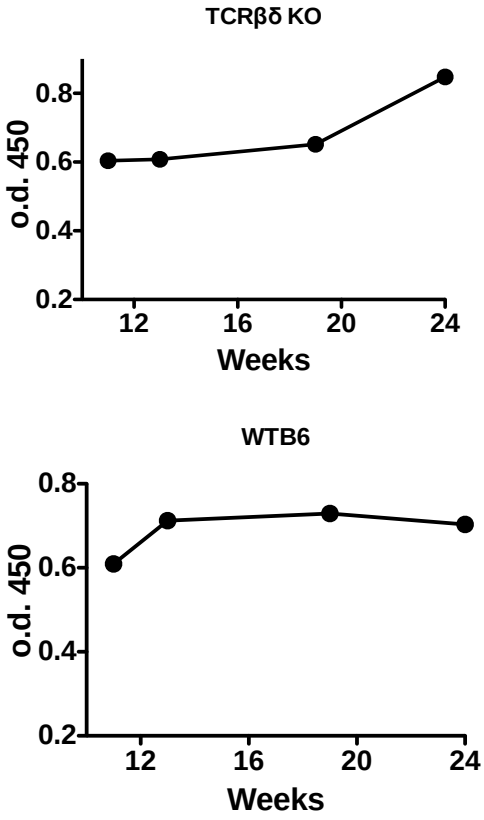


Figure 4.1- Long-lived serum IgG responses to PyV in T cell-deficient mice

(a) TCRβδ KO mice (n=5) and wildtype mice (n=2) were examined by ELISA for VP1- specific IgG at 3 months post infection. Mean +/- SD is shown. (B) VP1-specific IgG in pooled sera of TCRβδ KO mice (n=4) at 1:4000 dilution and of WTB6 mice (n=7) at 1:50,000 dilution.

B. Lack of GC B cells in T cell-deficient mice

Long-lived antibody responses in T cell-sufficient hosts usually derive from long-lived plasma cells or from the activation of Bmem cells, and both of these cell types are normally generated from T cell-dependent GC responses. Nevertheless, there are reports of short-lived TI GC responses and TI B cell memory in the literature (8). Moreover, unlike the conventional TI antigens, which are mostly polysaccharides or lipopolysaccharides, the chemical nature of the PyV capsid is protein, and PyV as a TI antigen can induce TI IgG2a, an isotype not common in responses to other TI antigens. The distinct nature of PyV as a TI-2 antigen suggests that TI responses to PyV infection may have features not characteristic for conventional TI-2 responses such as GC formation. Studies have shown that a TI-2 antigen, (4-hydroxy-3-nitrophenyl)acetyl-Ficoll, can generate short-term GCs (8). Therefore, we tested for the possibility that T cell-deficient mice form GC upon PyV infection. TCRβδ KO mice and wild type controls were infected intraperitoneally with PyV. Eight days post infection we harvested spleens from these mice and stained for GC B cells. Spleen cells gated on CD19+B220+ cells and double positive for GL-7 and Fas were considered as GC B cells (25, 26). The percentage of GC B cells in the spleens of wild type B6 mice increased from 0.11% in uninfected mice to 2.05% by day 8. (Figure 4.2) On the other hand, T cell-deficient mice did not have GC B cells in the spleen above the frequencies in

Figure 4.2- Lack of GC B cells in the spleens of PyV-infected T cell-deficient mice

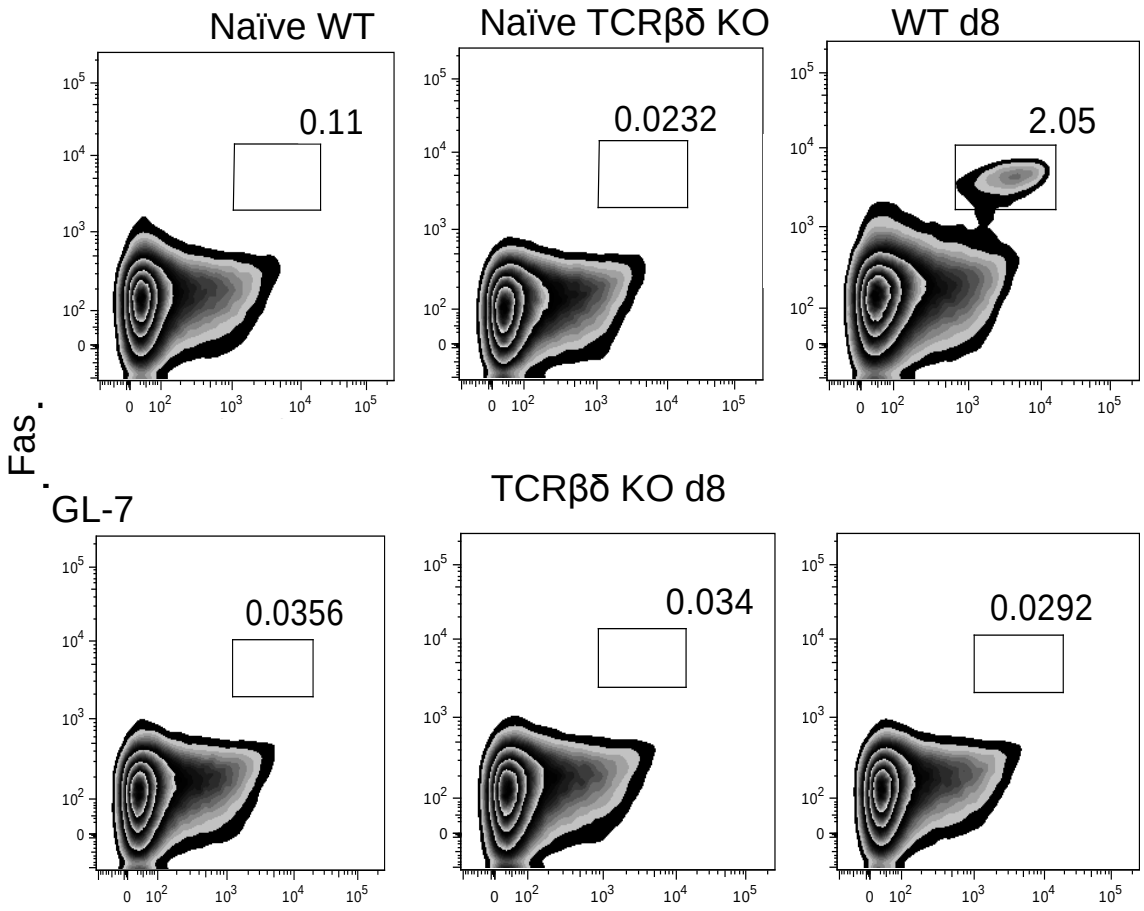


Figure 4.2- Lack of GC B cells in the spleens of PyV-infected T cell-deficient mice

Spleens of naïve and day 8 PyV-infected TCRβδ KO and WTB6 mice were examined by flow cytometry. The FACS plots are gated on B220+CD19+ B cells; the box/gate indicates Fas^{hi} $GL\text{-}7^{hi}$ GC B cells.

naïve controls (Figure 4.2). These data show that T cell-deficient mice, as expected, are not able to generate GC B cells in response to PyV.

C. Lack of bone marrow plasma cells and memory B cells in T cell-deficient PyV-infected mice

Since it was shown that T cell-deficient mice are unable to make GCs but still have long-term IgG antibodies in their serum, I investigated if long-term B cell populations could maintain the TI B cell response. It has been reported that immune responses to TI-2 antigens can involve Bmem cells (5). Since PyV is a TI-2 antigen, we wanted to test whether the presence of long-lasting antiviral IgG responses in T cell-deficient mice was due to long-lived plasma cells and/or Bmem cells. TCRβδ KO mice and wild type controls were infected with PyV and at various time points their spleens and bone marrows were tested for VP1-specific antibody-secreting cells (ASC) by ELISPOT. (Figure 4.3) Wild type B6 mice, as shown in figure 4.3, had 40-140/10^6 splenic ASC at all three time points after infection, and there was a similar increase in splenic ASCs in T cell-deficient mice. The number of virus specific ASCs in the bone marrow gradually increased in wild type B6 mice from 3 weeks to 6 months from 10/10^6 to ~80/10^6 bone marrow ASCs, respectively. On the

Figure 4.3- Accumulation of VP1-specific ASC in the bone marrow of B6 but not of TCRβδ KO mice after PyV infection

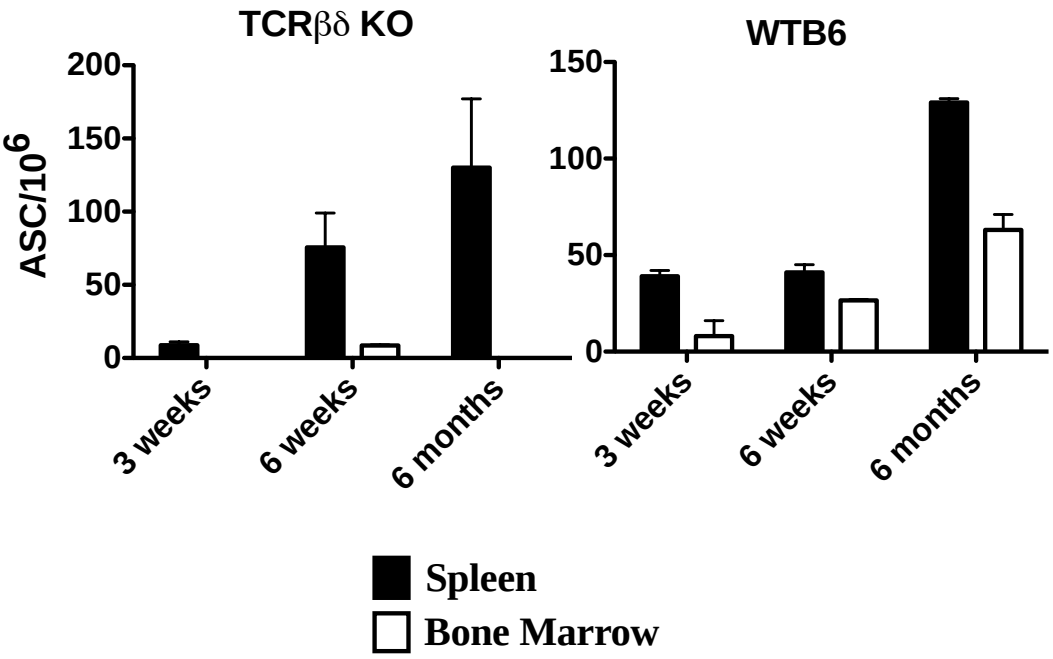


Figure 4.3- Accumulation of VP1-specific ASC in the bone marrow of B6 but not of TCRβδ KO mice after PyV infection

TCRβδ KO (left) and B6 mice (right) were examined for VP1- specific IgG ASC by ELISPOT at 3 weeks, 6 weeks and 3 months post PyV infection. Black bars: spleen ASC, open bars: BM ASC. Mean +/- SD is shown.

other hand, there was no increase in ASCs in the bone marrow of T cell-deficient mice, suggesting that T cell-deficient mice are not able to generate long-lived plasma cells in response to PyV infection.

Bmem cells are the other population of B cells that contribute to long-term humoral immunity. In order to study mouse memory B cell responses, we used a functional adoptive transfer assay as described in Chapter III. In this functional memory B cell assay, SCID mice were reconstituted with either of the following groups of splenocytes: naïve or PyV-immune splenocytes from TCRαβ KO mice, naïve or PyV-immune splenocytes from TCRγδ KO mice, naïve or PyV-immune splenocytes from TCRαβγδ KO mice, and naïve or PyV-immune splenocytes from WTB6 mice. Following cell transfer, some mice from each experimental group were infected with PyV. SCID mice given virus-immune wild type B6 cells or TCRγδ KO cells, followed by a PyV infection, generated good recall (Bmem cell) responses to PyV. (Figure 4.4) Alternatively, SCID mice reconstituted with virus-immune TCRαβ KO or TCRαβγδ KO splenocytes, followed by a PyV challenge did not generate Bmem cell responses, demonstrating that Bmem cell recall responses to PyV infection depends on αβ T cells.

Therefore, these observations taken together provide evidence that the long lasting serum antibody responses observed in T cell-deficient mice are not due to the presence of long-lived plasma cells or Bmem cells.

Figure 4.4- The generation of B cell memory, measured as VP1- specific IgG recall response, requires αβ T cell help

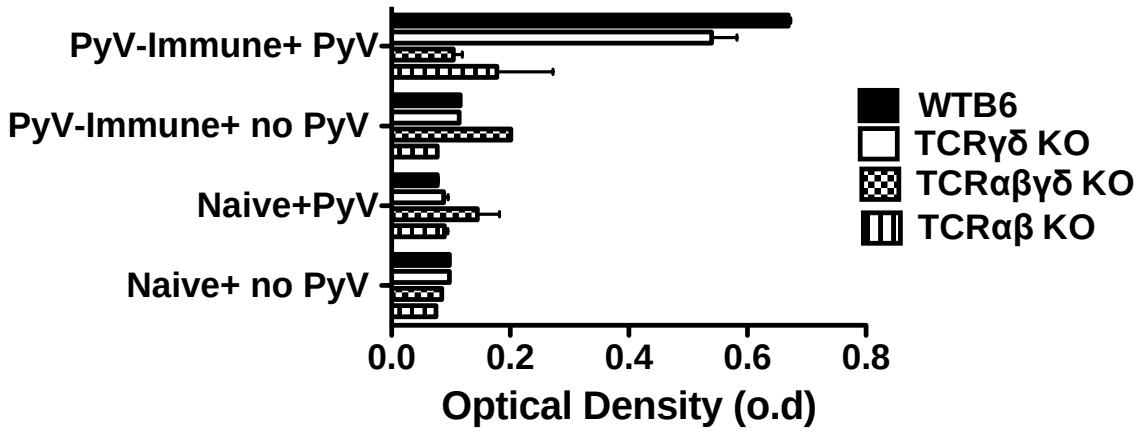


Figure 4.4- The generation of B cell memory, measured as VP1- specific IgG recall response, requires αβ T cell help

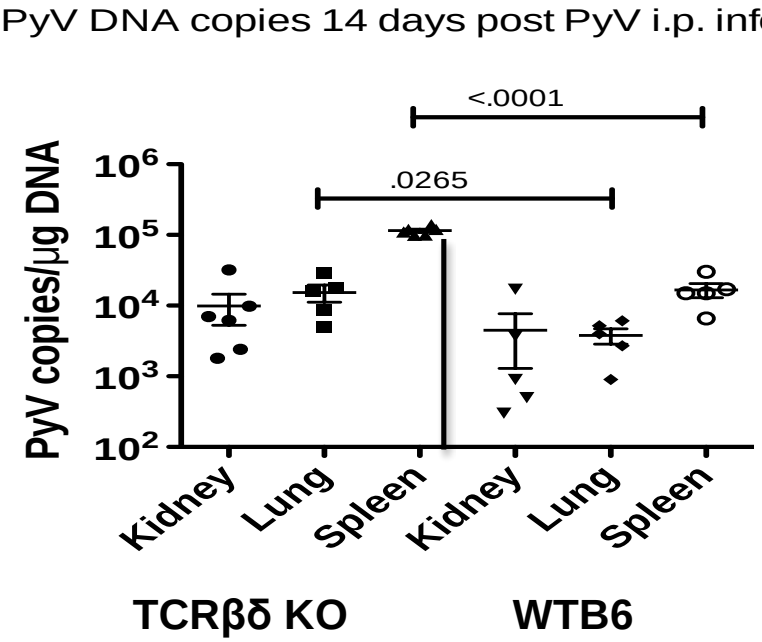
Splenocytes (10^7) from previously PyV infected (PyV immune) or naive TCRαβ KO, TCRγδ KO, TCRαβγδ KO or WT mice were intravenously transferred into SCID recipients. One day-post transfer, 2 out of 3 SCID recipients received a secondary PyV infection. VP1- specific IgG was determined by ELISA from sera of SCID recipients 7 days post infection.

D. Persisting viral load is higher in T cell-deficient mice compared to wild type

CD4 T cell-deficient mice (I-$A^{b-/-}$) infected with PyV generate only short-term antibody responses to PyV and lack long-lived plasma cells in the bone marrow (94). Therefore, TI antibody responses are not maintained long-term in mice that lack CD4 T cells, but have functional CD8 T cells (94). This contrasts with mice that lack both CD8 and CD4 T cells and maintain persistent TI antiviral serum IgG responses. PyV is a persistent virus infection, and CD8 T cells are known to be effective in controlling the virus load (89, 90). We hypothesized that an elevated virus load in mice lacking all T cells, including CD8 T cells, compared to I-$A^{b-/-}$, which lack CD4 T cells but have CD8 T cell responses to PyV, may play a role in maintaining long-lived TI antibody responses, and possibly explain the profound differences found in the longevity of TI antiviral IgG responses in I-$A^{b-/-}$ and TCRβδ KO mice. PyV levels in the kidney, lung and spleen of TCRβδ KO mice and wild type B6 controls were determined on day 14 following intraperitoneal or intranasal infection. In both types of infections, the TCRβδ KO mice had 10-fold higher levels of PyV than wild type mice (Figure 4.5a-b). Thus, in contrast to CD4 T cell-deficient mice, which have persistent PyV in their organs at the same levels as wild type B6 mice (94), TCRβδ KO mice have higher

Figure 4.5- Higher PyV viral load in T cell-deficient mice than in B6 mice

a.



b.

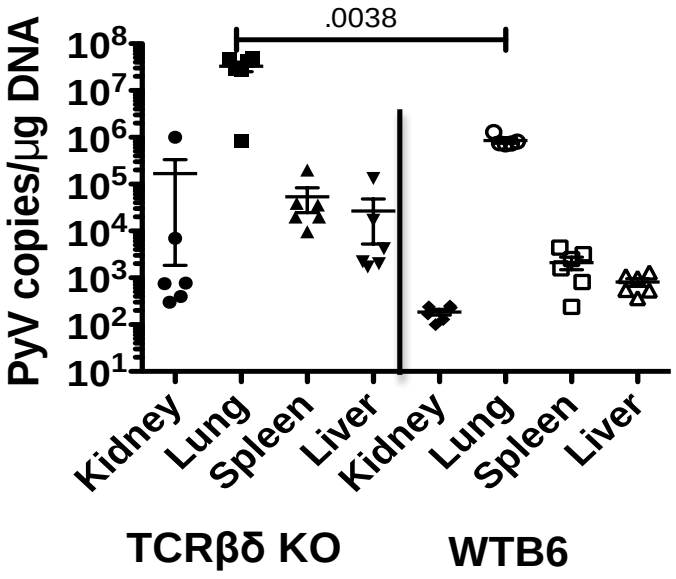


Figure 4.5- Higher PyV viral load in T cell-deficient mice than in B6 mice

PyV loads were determined as PyV genome copies/µg of tissue DNA by real time q-PCR in (a) kidney, lung and spleen 14 days post infection i.p. of TCRβδ KO and WT mice, (b) in lung, kidney, spleen and liver 14 days post i.n. infection of TCRβδ KO and WT mice.

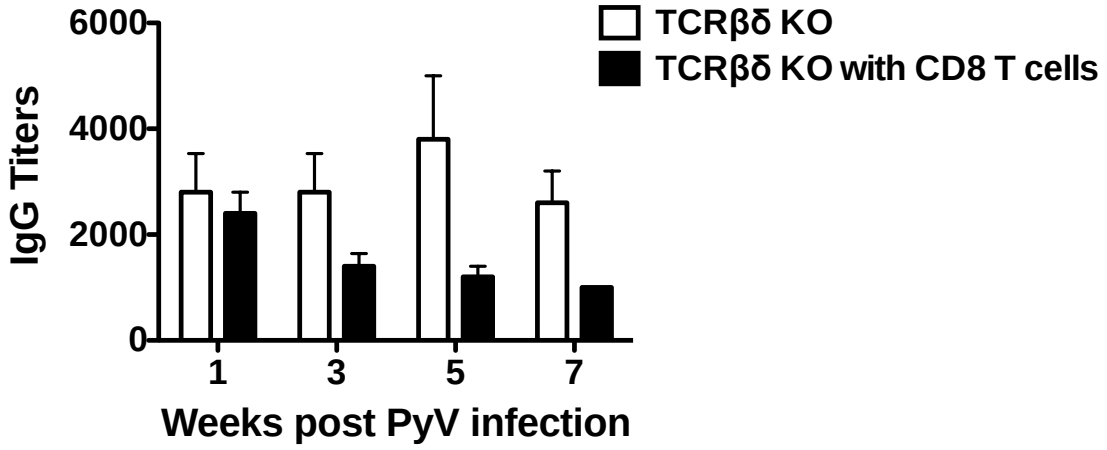
levels of PyV, which may contribute to the maintenance of long-lasting TI antibody responses in these mice.

E. Decrease in virus load after transfer of CD8 T cells into TCRβδ KO mice is associated by rapidly declining antiviral serum IgG levels

To test whether high levels of persistent PyV are responsible for the long-lasting antibody responses in T cell-deficient mice, naïve CD8 T cells, obtained by MACS enrichment from spleens of uninfected wild type B6 mice, were transferred into TCRβδ KO mice. The following day (day1), the CD8 T cell-reconstituted TCRβδ KO mice were infected with PyV, similarly to a control group of T cell-deficient mice that received no cell transfer. PyV-specific IgG levels in the serum were determined by VP1-specific ELISA at 1, 3, 5 and 7 weeks post infection.(Figure 4.6a) At 1-week post infection, the VP1- specific IgG response was similar between the two groups. In spite of this, over time, TCRβδ KO mice that had received CD8 T cells generated lower levels of virus-specific IgG. PyV viral loads were also measured at the last time point of the study (7 weeks post infection) (Figure 4.6b). This experiment established a correlation between the presence of CD8 T cells, lower persisting viral loads, and lower antibody responses.

Figure 4.6- Transfer of CD8 T cells prior to PyV infection reduces serum IgG in T cell-deficient mice and decreases lowers PyV load

a.



b.

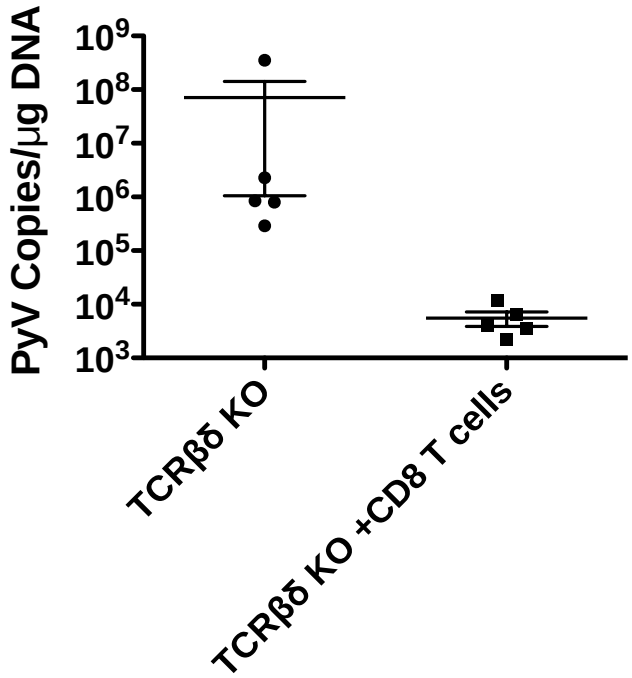


Figure 4.6- Transfer of CD8 T cells prior to PyV infection reduces serum IgG in T cell-deficient mice and decreases lowers PyV load

TCRβδ KO mice (n=5) were injected i.v. with $7x10^6$MACS-purified WT CD8 T cells, while another group of TCRβδ KO mice (n=5) were given no cells. Following cell transfer, all mice were infected with PyV i.p. (a) At 1,3, 5, and 7 weeks post infection, virus-specific IgG levels were determined by VP1-specific ELISA (b). PyV loads were determined by q-PCR at the latest time point tested; 7 weeks post infection and were expressed as PyV genome copies/mg tissue DNA.

F. Lack of long-lasting antiviral IgG responses in T cell-deficient MyD88-deficient mice

Based on our finding thus far, we hypothesized that a high level of persistent PyV antigen in TCRβδ KO mice may activate naïve B cell populations that emerge from the bone marrow continuously, thereby maintaining the long-lasting TI IgG response. This envisioned mechanism poses the question of why B cells that are likely to encounter PyV antigens during their development in the bone marrow resist becoming tolerized? Bone marrow cells from TCRβδ KO mice 3-4 months post infection indeed contained high levels of PyV genomic DNA, as detected by qPCR, indicating the presence of high viral load at the site of B cell development (Figure 4.7). Therefore it is highly probable that developing B cells encounter PyV in the bone marrow, but, this encounter does not seem to lead to tolerance because newly emerging mature B cells keep generating antibody after they interact with PyV in the periphery. A report by Berland et al. (128) suggested that antigens with “dual specificity”, which ligate the BCR and endosomal TLRs such as TLR7/8 and TLR9 may break B cell tolerance. In that study, B cells with a transgenic B cell receptor specific for autoantigens showed a TLR7-dependent loss of B cell tolerance. If we define “dual specificity antigen” as antigens that activate both BCR and TLRs, PyV can be considered as a “dual specificity antigen”. PyV infection, indeed, results in the activation of endosomal MyD88-mediated TLRs as shown by studies in Unc93B1 mutant mice that cannot signal through the endosomal TLRs 3, 7 and 9 (Chapter III). Therefore, we

Figure 4.7- High levels of persisting PyV in the bone marrow of T cell-deficient mice

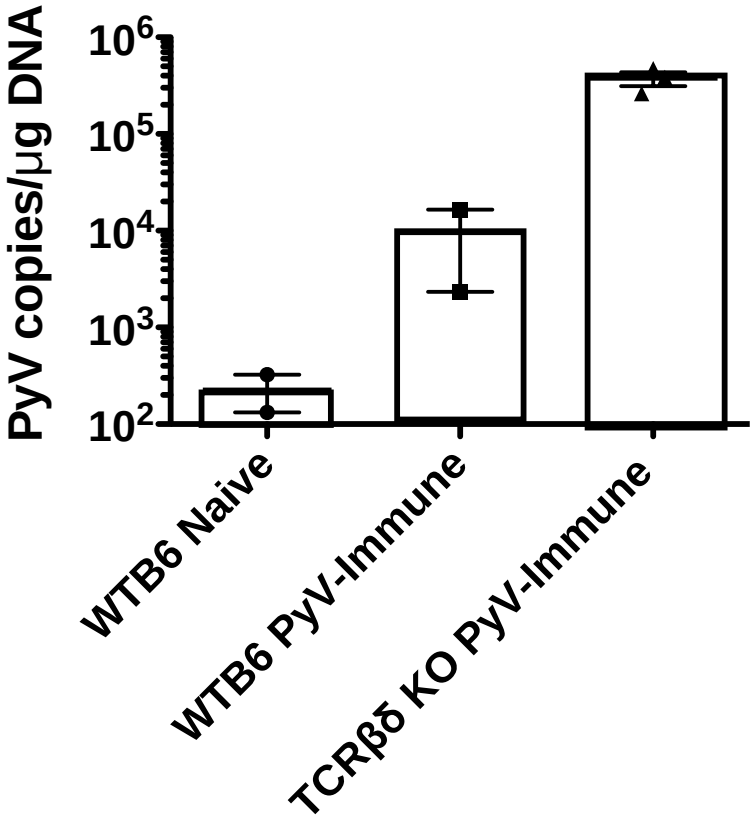


Figure 4.7- High levels of persisting PyV in the bone marrow of T cell-deficient mice

PyV loads were determined as PyV genome copies/µg of tissue DNA by real time q-PCR in the bone marrow of TCRβδ KO mice and WT mice 3-4 months post infection i.p.

hypothesized that B cells in TCRβδ KO mice, with high levels of PyV in the bone marrow, bypass tolerance to PyV in a TLR-dependent manner. This disruption of tolerance may be also dependent on MyD88, a cytoplasmic signaling protein important for the action of many TLRs. Thus, it would follow that in TCRβδ KO mice that are also deficient in MyD88 signaling tolerance would be developed and maintained; and new B cells coming out from the bone marrow would not respond to persisting PyV, leading to decreased antiviral serum IgG with time. To test this hypothesis, we infected TCRβδ KO /MyD88-/- triple knockout mice and mice that were T cell-deficient but MyD88+/+ (TCRβδ KO) with PyV. VP1-specific IgG responses were tested at 1, 2 and 4 weeks post PyV infection. (Figure 4.8a) The TCRβδ KO/MyD88-/- mice had similar levels of virus-specific IgG as TCRβδ KO mice at 1-week post infection, but, the long-lasting antibody response was not maintained in these mice at 2 and 4 weeks post infection. TCRβδ KO/MyD88-/- and TCRβδ KO mice generated similar levels of VP1-specific IgM. (Figure 4.8b) This shows that the absence of MyD88 signaling did not just merely block switching to IgG in TCRβδ KO /MyD88-/- mice and further supports the tolerance hypothesis. These results suggest that MyD88 signaling is essential in maintaining long-lasting antibody responses in T cell-deficient mice after virus infection.

Figure 4.8- VP1-specific IgG and IgM responses in T cell-deficient MyD88 +/+ and T cell-deficient MyD88 -/- mice

a.

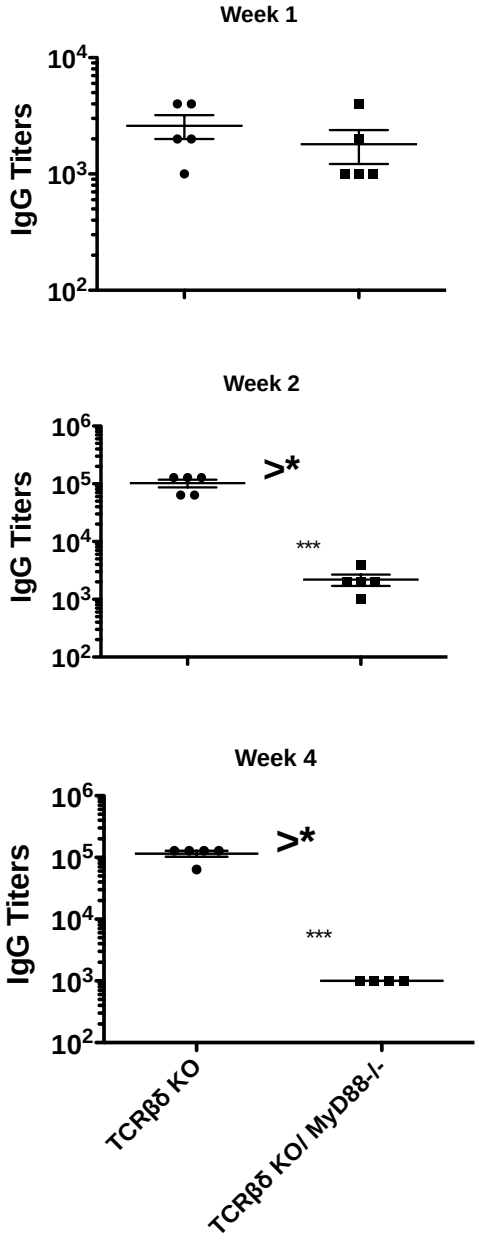


Figure 4.8- VP1-specific IgG and IgM responses in T cell-deficient MyD88 +/+ and T cell-deficient MyD88 -/- mice

b.

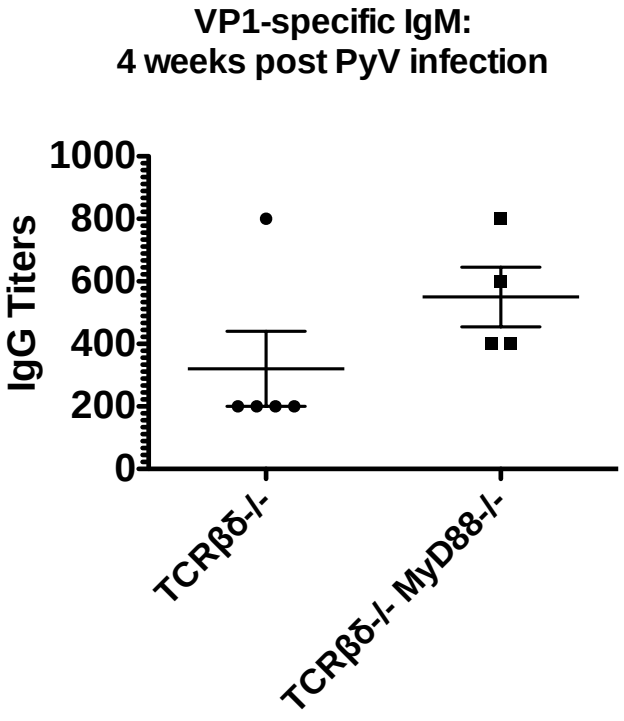


Figure 4.8- VP1-specific IgG and IgM responses in T cell-deficient MyD88 +/+ and T cell-deficient MyD88-/- mice

TCRβδ KO MyD88-deficient (n=5) and TCRβδ KO MyD88 sufficient mice (n=5) were infected with PyV i.p. (a) VP1- specific IgG was determined by ELISA at 1,2 and 4 weeks post PyV infection. (b) VP1-specific IgM was determined at 4 weeks post PyV infection. >* The IgG titers are equal or greater than 128000. ***p value < .001

G. General Summary

This chapter provides evidence that PyV-infected T cell-deficient mice have long-lasting antiviral serum IgG responses, and the longevity of these responses is not due to the presence of long-lived bone marrow-residing plasma cells or Bmem cells. Instead, the sustained TI IgG is likely to be continuously generated by short-lived B cell responses that require high persisting virus load and intact MyD88-mediated innate immune pathways.

TI IgG responses were maintained up to 6 months post PyV infection in the absence of T cells. Even though other TI models have suggested the occurrence of 'TI B cell memory' by the presence of antigen-experienced, quiescent, IgG memory B cells, (5)the long-term TI IgG responses seen in TCRβδ KO mice were not a result of GC formation and GC-derived long-lived plasma cells or the activation of recall memory B cells.

Previously, it has been shown that CD4+ T cell-deficient mice generate short-lived antibody responses to PyV and maintain a PyV load that is similar to WT mice (94). In this chapter, I have shown that TCRβδ KO mice (which lack both CD4 and CD8 T cells) had long-lasting TI IgG responses and higher PyV loads than WT mice. This suggests that the virus load plays a role in sustaining long-term TI IgG responses. Adoptively transferring WT CD8 T cells into TCRβδ KO mice prior to PyV infection resulted in gradually decreasing levels of VP1-specific IgG, associated with a lower PyV load than TCRβδ KO mice without CD8

T cells, again supporting the idea that the high virus load was required for sustained TI antibody responses.

Based on the viral load data, it was hypothesized that that a high level of persistent PyV antigen in TCRβδ KO mice may activate naïve B cell populations that emerge from the bone marrow continuously, thereby maintaining the long-lasting TI IgG responses. As high PyV loads were also detected in the bone marrow where B cell lymphopoeisis occurs, I questioned if developing B cells encounter PyV antigens, why weren't B cells that enter the periphery, then, tolerized? As a dual specificity antigen, it is reasoned that PyV can activate both BCR and TLR-signaling pathways. The activation of both of these signaling pathways by high levels of an autoantigen has been shown to lead to the breaking of tolerance and autoantibody production (128). Using TCRβδ KO mice that were also deficient in MyD88 signaling, I showed that overtime these mice generated lower levels of PyV-specific IgG. Thus, MyD88-meditated signaling can affect the tolerization of B cells which influences long-term TI IgG production in response to PyV infection.

CHAPTER V: Discussion

Our lab has previously shown that MyD88-/- mice have defects in long-term humoral immunity in response to PyV infection. MyD88-/- mice had low levels of PyV-specific IgG, skewed isotype responses and a lack of in long-lived plasma cells in the bone marrow (3).

There has been controversy in the literature over the importance of direct TLR signaling on B cells in the maintenance of humoral responses. Pasare et al had shown a direct and indirect role of TLRs in B cell responses. In their studies, MyD88-deficient mice immunized with OVA-LPS displayed defects in total IgG, lower levels of IgG1, and an abolishment of IgG2a/c OVA-specific responses. Studies that depleted regulatory T cells in MyD88-/- mice restored helper T cell activation and function. However, the restoration of helper T cell activity did not reestablish TD B cell responses in MyD88-/- mice (66). These data suggested that direct TLR signaling in B cells might be important for normal TD antibody responses.

To the contrary, Gavin et al used MyD88-/-;Trif$^{LPS2/LPS2}$ (deficient in all TLR signaling) mice to show that TLR signaling is not important for antibody responses to protein antigens delivered with various adjuvants (129). Gavin et al suggested the use of LPS as an adjuvant in the studies by Pasare et al influenced the outcome of that study. Another study showed that the transfer of MyD88-/- B cells into B cell-deficient mice that were subsequently immunized with NP-CGG generated similar NP-antibody titers as WT B cells. The addition of

LPS enhanced the antibody response to NP-CGG, but no defects in antibody responses were observed from MyD88-/- B cells in these studies (130).

Nevertheless, several other studies in the literature have corroborated a humoral defect in MyD88-/- mice, confirming observations made in our lab. MyD88-/- mice infected with murine gamma herpesvirus 68 (MHV68) generated low numbers of activated B cells, GC B cells and class-switched B cells 16 days post infection (131). To investigate the role of MyD88 in B cells to maintain WT antibody responses, WT mice were irradiated and reconstituted with a mixture of WT and MyD88-/- bone marrow cells. Chimeric mice with MyD88-/- B cells generated similar defective anti-MHV68 humoral responses as MyD88-/- mice which confirmed a B cell-intrinsic role of MyD88 in maintaining antibody responses and GC reactions (131). Intranasal infection of MyD88-/- mice with vesicular stomatitis virus (VSV) infection resulted in impaired IFNα production, defective neutralizing antibodies, and low levels of anti-VSV IgG2a (132). Additionally, MyD88-/- mice infected by influenza A virus showed lower levels of total IgG and defective levels of virus-specific IgG2a 21 days post infection (133). MyD88-/- mice challenged i.p. with a non-viral pathogen, heat-killed *Streptococcus pnuemoniae*, also showed defects in IgG3, IgG2b and IgG2a (134). In vitro studies have also shown that different B cell subsets can secrete various cytokines, antibodies and proliferate in response to TLR ligands, further showing a role of TLRs in mediating B cell responses (116, 135). All of these studies provide evidence for the role of MyD88-mediated signaling to be

important in B cells. The work in Chapter III elucidated the role of innate signaling pathways in CSR and in the regulation of long-term antiviral TD humoral immunity to PyV infection.

Chapter III describes the role of MyD88-mediated signaling in regulating IgG2a responses, GC B cell reactions, long-lived plasma cells, and memory B cells to PyV infection. Previous studies in our lab have shown that MyD88-/- mice, upon PyV infection, generate low-levels of virus-specific IgG, attenuated levels of virus-specific IgG2a, and lower numbers of long-lived plasma cells (3). The work in this thesis and our collaboration with Dr. Paula Pitha provided a mechanism for the TLR-MyD88-activated transcription factor IRF5 in mediating IgG2a responses within B cells. My thesis work also determined that TLR7 and TLR9 (through studies in 3d mice and TLR7/9 DKO mice) are important in the generation and maintenance of long-term antiviral humoral responses. The defect in long-term IgG responses and long-lived B cell populations in MyD88-/- mice may result from defective innate pathways in multiple cell types, as MyD88 signaling is important in several cell types, not just B cells.

A. Role of B cell-intrinsic IRF5 in isotype switching to IgG2a

Chapter III discussed a role for TLR-MyD88-IRF5-mediated mechanism in class switching to IgG2a. In addition to IRF5, other molecules have been shown to be important in generating IgG2a such as the transcription factor, T-bet, IRF4 and AID. T-bet is a transcription factor that is generally induced in B cells upon LPS

and CD40 stimulation in vitro and is primarily important in IgG2a induction during TI B cell responses rather than TD B cell responses. (136) IFNy secretion by non-T cells, such as NK cells, can activate IgG2a production by LPS-activated B cells in vitro (137). Also, it has been shown that the induction of IgG2a GLTs in B cells requires IFNy-mediated up-regulation of T-bet. (102) Therefore, it was important to investigate T-bet expression in IRF5-/- B cells. T-bet levels were not affected in IRF5-/- B cells stimulated with TLR ligands, suggesting that the defect in IgG2a in IRF5-/- mice is not dependent on T-bet (116). It is of note to mention that another study did observe defects in T-bet expression in CpG (TLR9 ligand)-stimulated IRF5-/- B cells (115). The difference in T-bet expression in these studies could be due to the kinetics of T-bet expression. Savitsky et al examined T-bet expression 6 hours post CpG stimulation and observed a defect in T-bet expression. However, T-bet expression in TLR-stimulated IRF5-/- B cells was not affected when observed two days post stimulation (116). T-bet expression is delayed upon CpG-stimulated IRF5-/- B cells(116), which can contribute to the discrepancy in T-bet expression in the two studies described.

AID is an important enzyme that is required for CSR, as AID-deficient B cells are not able to class switch (19). Studies looking at AID transcripts in TLR-stimulated IRF5-/- B cells showed that AID was not decreased when compared to WT-stimulated B cells (116). This suggests that the defect IgG2a production in IRF5-/- B cells is not a result of lower levels of AID.

IRF4 plays an essential role in B cell differentiation into plasma cells and in CSR (111) (116). Studies show that upon TLR-activation, IRF4 and IRF5 compete for binding to MyD88 (138). IRF5-/- B cells stimulated with LPS, LPS and IL-4 or TLR7 and TLR9 ligands, showed no reduction in the expression of IRF4. This supports the idea that the absence of IRF5 does not affect IRF4, and IRF4, in turn, is not responsible for the defect in CSR observed in IRF5-deficient mice (116).

IRF5, in its activated form, is known to form homodimers or heterodimers with other IRF proteins such as IRF3 and IRF7. IRF5/IRF3 have different function than IRF5/IRF7 in regards to type I IFN production (139). Studies in Chapter III describe another mechanism of IRF5. IRF5 can negatively regulate Ikaros expression by binding to the *Ikzf1* promoter region and control transcriptional activity (116). In addition to IRF5, IRF4 and IRF8 were shown to be able to bind to the IRF binding site in the promoter region of *Ikzf1* (116). Other studies have shown that IRF4 and IRF8 can stimulate *Ikzf1* expression to negatively regulate pre-B cell proliferation. (140)Yet, only IRF5 and IRF8 were able to control *Ikzf1* transcriptional activity. (116) This discrepancy may be due to necessity of an association of IRF4 with other lymphoid cell-specific transcription factors to facilitate transcriptional activity of *Ikzf1* (141).

A recent finding of a spontaneous genomic duplication and a frameshift mutation in *Dock2* in IRF5-/- mice has complicated the interpretation of experiments conducted in IRF5-/ mice (123). Dock2 is involved in regulating Rac

signaling pathways, marginal zone B cell-development, plasmacytoid DC homing(124) and B cell development. All studies with IRF5-/- mice with PyV infection in this thesis were done using IRF5-/- mice that also contained the Dock2 mutation. Nevertheless, Fang et al demonstrated that B cells obtained from a Dock2 mutation-free, IRF5-deficient mice showed the same IgG2a class switching defect as did the IRF5-/- and Dock2 mutation-carrying mice (116). Dock2 mutation-free, IRF5-deficient mice were generated by crossing IRF5 floxed mice with OzcCre-expressing mice. The cre gene, in OzCre mice, is under the control of the phosphoglycerate kinase-1 (PGK) promoter. Additionally, a spontaneous Dock2 mutation was found in apoptosis-associated specklike protein containing a caspase recruitment domain (ASC) KO mice as well. ASC is an adaptor protein found downstream of inflammasome receptors. Infection of ASC KO mice with PyV did not result in any humoral defects (data not shown) as observed in MyD88-/-, 3d-/ or IRF5-/- mice, indicating that Dock2, solely, is not responsible for the B cell defects we found in IRF5-/- mice. At this point we can conclude that (i) the IgG2a defect is due to IRF5 mutation and not merely caused by Dock2 defect, and (ii) mice lacking both IRF5 and Dock2 have long-term antibody defects in response to PyV. The individual roles of IRF5 and Dock2 needs to be sorted out in studies with IRF5-deficient, Dock2 sufficient mice.

We also started to conduct studies using IRF5 floxed mice that were crossed with CD11c-Cre mice. These mice are selectively deficient in IRF5 in CD11c positive populations and do not carry a Dock2 mutation. As described in

Chapter III, CD11c-IRF5-deficient mice did not display defects in IgG2a production. These studies are consistent with a B cell-intrinsic role of IRF5 in mediating IgG2a responses.

B. Role of TLR pathways in mediating anti-PyV igG2a responses

3d mice have similar defects in virus-specific IgG2a responses as MyD88-/- and IRF5-/- mice, which suggests that TLR7- and TLR9-MyD88 mediated pathways are important in the PyV infection model. 3d mice are deficient in TLRs 3, 7, and 9 signaling because of a point mutation in Unc93B1 that disrupts the translocation of TLRs 3,7 and 9 from the ER to endosome. The point mutation also affects exogenous antigen presentation through MHC class I and MHC class II. Severe defects are observed specifically with cross presentation. (142) Antibody defects observed in the 3d mice are not likely to be due to these other non-TLR-related defects, as similar defects are observed in MyD88-/- mice. Single TLR3-/-, TLR7-/-, and TLR9-/- mice did not have a defect in virus-specific IgG2a as MyD88-/-, 3d, TLR7/9 DKO, and IRF5-/- mice. IRF5 displays a B cell-intrinsic role in regulating IgG2a responses and IRF5 signals downstream of TLR7, TLR9, and MyD88. Therefore it is likely that TLR7 and TLR9-MyD88 pathways directly act in B cells to regulate PyV-specific IgG2a levels.

While PyV is not known to replicate in lymphocytes (T and B cells), it is of interest to understand how PyV infection is sensed by endosomal TLRs in B cells. It is a possibility that PyV can enter B cells, but not infect them, but, so far,

there is no direct evidence to support this. It is not clear how endosomal innate receptors such as TLR7 and TLR9 are recognizing PyV directly. PyV can cause a lytic infection leading to the death of the infected cell. B cells are efficient at taking up antigen or apoptotic debris from the environment. Through this mechanism various antigens or TLR ligands can end up within endocytic pathways in B cells and interact with endosomal TLRs (121, 121, 121). Further studies are required to investigate this potential mechanism.

C. Role of TLR pathways in long-term humoral responses to PyV

The work in this thesis implicates MyD88-dependent TLRs in generating antiviral long-term B cell responses. MyD88-/-, 3d, and IRF5-/- mice generate low-levels of long-term PyV-specific IgG and defective numbers of long-lived plasma cells in the bone marrow. Experiments with individual TLR3, 7, and 9 deficient mice did not show the defective antibody responses seen in MyD88-/- mice. TLR7-/- mice, in some cases, displayed mild antibody defects. The mild defects in TLR7-/- mice were not always consistently significant. There are compensatory mechanisms that can exist in single TLR deficient mice.

Studies with 3d mice and TLR7/9 DKO mice suggest a role for both TLR7 and TLR9 in long-term B cell responses and in generating virus-specific IgG2a during a PyV infection. MCMV infection induces high levels of type I IFN and pro-inflammatory cytokines, but the absence of both TLR9 and TLR7 abolished cytokine production against MCMV. The absence of only TLR7 or TLR9 did not

have severe defects (121). Therefore, these studies suggest that TLR7 and TLR9, together, are important in the IFN responses induced by the recognition of MCMV, a DNA virus. Similarly, both TLR7 and TLR9 play a role in antibody maintenance in response to PyV infection. Furthermore, it is possible that the paucity of VP1-specific IgG2a is affecting total IgG responses, and this may play a role in the observed long-term humoral defect in MyD88-/- mice.

In addition to the role of TLRs in maintaining long-term B cell responses, TLR signaling has been shown to be important in vaccine studies (143). Immunizing mice with nanoparticles containing antigen along with ligands that activate both TLR4 and TLR7, synergistically, increase antigen-specific neutralizing antibodies (144). An understanding of TLR signaling pathways in mediating humoral responses is important for the generation of vaccine efficacy.

D. Possible mechanisms involved in the long-term B cell defects in MyD88-deficient mice upon PyV infection

It is not clear if the defect in long-term B cell populations in MyD88-/- mice is entirely B cell-intrinsic. Our studies on TD B cell responses were done in MyD88-/- mice, which lack MyD88 in every cell type. MyD88 signaling is important in B, T and antigen presenting cells such as DCs and macrophages. All of these cell populations play a part in generating and maintaining GC and long-term B cell responses. Previously, our lab has generated data suggesting that the long-term antibody defect is a B cell-intrinsic issue through adoptive transfer studies.

Adoptively transferring MyD88-/- B cells or WT B cells into B cell deficient mice or transferring MyD88-/- T cells or WT T cells into T cell-deficient mice followed by PyV infection, we showed that mice with MyD88-/- B cells had defects in generating VP1-specific IgG one-month post infection. Mice with WT B cells, MyD88-/- T cells or WT T cells generated normal levels of virus-specific IgG (3). A caveat in this experiment is that within either T cell-or B cell-deficient recipient mice, the adoptively transferred MyD88-/- B cells or T cells were interacting with WT DCs and antibody responses were only observed for 30 days post infection. To further understand which cell type is important in maintaining long-term B cell responses, mixed bone marrow chimeras were generated. WT mice were irradiated and reconstituted at a 9:1 ratio of either TCRKO:MyD88-/- or µMT:MyD88-/- bone marrow cells. In WT mice reconstituted with TCRKO:MyD88-/- bone marrow cells the only source of T cells were from MyD88-/- mice, which can interact with WT B cells. On the other hand, in WT mice with µMT:MyD88-/- bone marrow cells the only source of B cells is from MyD88-/- mice, which can interact with WT T cells. Two months after the generation of chimeras, reconstitution of the cells was examined, followed by PyV infection of some mice in both groups. I used the chimeric mice in the adoptive transfer memory B cell assay described in this thesis. PyV-immune chimeric splenocytes were transferred into SCID recipients, along with control groups that received uninfected chimeric splenocytes. A VP1-specific ELISA was done at 7 days post secondary infection of SCID recipients following cell transfer.

SCID mice that received splenocytes from TCRKO:MyD88-/- chimeras, generated WT memory B cell responses. However, SCID mice with µMT:MyD88-/- chimeric splenocytes had defective memory B cell responses, suggesting a B cell-intrinsic role of MyD88 in maintaining memory B cell responses to PyV infection. The interpretation of these experiments is difficult because the µMT:MyD88-/- chimeras did not reconstitute B cells to similar numbers as seen in the TCR KO:MyD88-/- chimeras. This caveat can affect B cell responses observed in the memory B cell assay. The transfer experiments and chimeric mice studies, so far, suggest that T cell-intrinsic MyD88 is not essential for normal B cell responses and a B cell-intrinsic role of MyD88 may be important in maintaining long-term antibody responses. It is possible that a B cell-intrinsic defect can be involved in the GC and TFH cell impairments observed in MyD88-/- mice. As previously mentioned, interactions with B cells are required for the differentiation of activated CD4 T cells into TFH cells. (31, 145) B cell-intrinsic defects in MyD88-/- mice can affect the interaction between B cells and CD4 T cells, which can cause impairments in TFH cell development resulting in poor GC responses.

The absence of MyD88 signaling can affect T cell and DC activation, generate improper co-stimulatory signals or cytokine help to B cells (56). Studies with CD11c-IRF5 floxed mice show no significant differences in PyV-specific long-term IgG and long-lived plasma cells in the bone marrow. This suggests that an IRF5 defect only in DC, by itself, is not responsible for the altered long-term

humoral responses to PyV infection. Many other cell types, among them, FDCs, can influence GC and long-term B cell responses (24) (146). FDCs are important for antigen capture and presentation of the antigen via immune complexes to GC B cells for survival and selection (24). FDCs may play a role in maintaining long-term GC responses. It is possible that, in this PyV model, the lack of MyD88 signaling can affect the function of FDCs, which could result in defective GC responses and lower numbers of long-lived B cell populations. Further studies are necessary to determine the role of FDCs in long-term B cell responses to PyV infection.

In addition to different cell types, there are various factors important for the maintenance and survival of long-lived plasma cells such as CXCL12, BAFF, APRIL, TACI, and cytokines such as IL-6 and TNF. (18) CXCL12 is a chemokine that attracts CXCR4+ plasmablasts to the bone marrow, the survival niche for long-lived plasma cells. I have examined CXCR4 expression levels on PyV-infected MyD88-/- and WT mice 14 days post infection. No significant differences were observed in CXCR4 expression between the two groups. (data not shown) Signaling through TACI, a receptor for BAFF and APRIL, is important in the maintenance of long-lived antibody secreting cells, as shown in a mouse model of influenza infection (37). Furthermore, TACI was shown to interact with MyD88 and trigger CSR by activating NF-kB through a TLR-like signaling pathway (147). I investigated antibody isotypes and long-lived PC in PyV-infected TACI deficient mice. The absence of TACI had no effect on VP1-specific IgG2a or IgG1 levels.

The isotype response to PyV infection was similar to WTB6 mice (data not shown) Thus, in a mouse model of PyV infection, TACI signaling did not influence CSR. However, TACI deficient mice had lower numbers of long-lived PCs in the bone marrow when compared to WTB6 mice. (data not shown) This suggests that TACI may be important in preserving long-lived plasma cells, and further studies would be required to determine whether TACI signaling through MyD88 or a MyD88-independent pathway affects the maintenance of long-lived PCs.

From the data presented in Chapter III, it seems that in MyD88-/- mice the defect in long-lived plasma cells is more severe than the defect in memory B cells. It may relate to the observation that long-lived plasma cells form later during the GC reaction than memory B cells (24), and this is consistent with the defects seen in the maintenance of GC in MyD88-/- mice. All of this suggests that generation and maintenance of long-term B cell responses in MyD88-/- mice, in response to PyV infection, involves various cell types and molecules. Based on the data in Chapter III, it is likely that MyD88-mediated pathways are required in B cells for normal responses, but the MyD88-mediated pathways in other cell types may also contribute.

The effects of innate signaling in the maintenance of antiviral TI humoral responses were examined in Chapter IV. Chapter IV shows that mice maintain long-lasting serological memory to PyV in the absence of T cells. This is achieved without the formation of GC, long-lived plasma cells in the bone marrow

or Bmem cells. The maintenance of this “TI serological memory”, however, requires high virus load and intact MyD88-mediated pathways in the host. As an infectious virus acting as a TI antigen, PyV differs from many well-characterized and frequently used TI model antigens such as bacterial polysaccharides or NP-Ficoll. The chemical nature of the viral capsid of PyV is protein, and not polysaccharide. Moreover, PyV is not inert, but a live, replicating virus, that can elicit innate, inflammatory or danger signals. Therefore, it was of interest to systematically investigate the mechanisms that allow the maintenance of potentially life-saving long-term IgG levels in persistently virus-infected T cell-deficient mice.

E. GC responses in T cell-deficient mice

The formation of GC is a prerequisite for Bmem cell and long-lived BM- residing plasma cell formation in TD responses. GC are normally not generated in the absence of T cells, but under unusual circumstances, such as in BCR transgenic mice with a high frequency of high affinity antigen-specific B cells, or after immunization with TI antigens (NP Ficoll) at very high antigen doses, short lived GC, defined as clusters of PNA+ and GL-7+ B cells, can be induced in a T cell-independent manner (8, 9). However, the TI GC are not functional, as no SHM was detected in the TI GC B cells (8), and they don’t last past day 5 following immunization. In this chapter, I showed that TCRβδ KO mice infected with PyV did not show an increase in GC B cell frequencies in the spleen on day 8-post

infection (the peak of the GC response) compared to the negligible levels of GC B cells in uninfected T cell-deficient mice. Therefore the long-lasting TI serum IgG responses to PyV are not products of an unusual, pathogen-induced TI GC reaction.

F. Generation of long-lived plasma cells and memory B cells in TI B cell responses

The non-secreting form of B cell memory resides in antigen-experienced quiescent Bmem cells, which can be rapidly activated in recall responses, giving faster and larger responses to re-challenge than naïve B cells which see the antigen for the first time. Memory B cells are usually formed in a TD response, but there are some reports of TI Bmem cell responses. One report by Alugupalli et al (6) showed that the B1b cell population that expands during *B. hermsii* infection can survive for months in a host free of pathogen and provides long-lasting, protective effect by secreting IgM in response to Borrelia (6, 148,). Immunization with the TI type 2 antigen, NP-Ficoll, also leads to B cell responses lasting for months. Long-lasting NP-Ficoll-specific IgM and IgG3 responses are generated by extrafollicular B1b plasmablasts, not by the conventional memory B cell populations generated from B2 cells in a usual TD antibody response. Another example of TI B cell memory, using the definition of antigen-experienced, resting, non-secreting B cells for Bmem cell, Obukhanych et al.

reported the formation of such memory B cells, some of them isotype-switched to IgG, in response to the TI antigen NP-Ficoll (5).

Studies in this chapter tested whether spleen cells of PyV-immune T cell-deficient mice can mount a fast and enhanced recall IgG response to PyV re-challenge in a functional adoptive transfer assay. Using this assay we found that recall IgG responses arose only from the splenic B cells of PyV-immune mice that had αβ T cells, such as wild type B6 and TCRγδ KO mice, but not from TCRαβKO or TCRαβγδ KO mice. Therefore, αβ T cell help is essential for the formation of recall Bmem cells even when immunized with PyV, an infectious viral TI antigen.

Long-lived plasma cells can maintain serological memory even in the absence of any other B cells, as shown by studies using CD20 depletions (149). The CD20-specific depleting antibodies eliminate both naïve B cells and Bmem cells, but spare fully differentiated plasma cells. It was determined that mice without T cell help, including both the CD4 T cell-defective I-$A^{b-/-}$ mice (94) and the αβ T cell-defective TCRαβ KO or TCRαβγδ KO mice, did not accumulate plasma cells in their bone marrow. Therefore, long-lived plasma cells were not responsible for the maintenance of TI antiviral serum IgG.

G. High viral loads influencing TI antibody responses

Chapter IV demonstrates the existence of high antiviral serum IgG for as long as 6 months after infection (the length of our study) in TCRαβ KO and TCRαβγδ KO

mice. While long-lasting TI IgG is observed in TCRαβγδ KO mice, our lab has previously published that TI IgG responses in I-$A^{b-/-}$ mice were short-lived and greatly decreased after day 23 post infection (19, 94). How can we reconcile these seemingly contradictory results? A major difference between PyV-infected I-$A^{b-/-}$ and TCRαβ KO or TCRαβγδ KO mice is the amount of persisting virus they harbor. Consistent with the antiviral role of CD8 T cells, I-$A^{b-/-}$ mice have CD8 cytotoxic T cell responses and a low level of PyV persistence, undistinguishable from that of wild type B6 mice. On the other hand, TCRαβ KO or TCRαβγδ KO mice lack CD8 T cell responses and have about ten-fold higher persisting PyV load compared to B6 mice. We propose that the high virus load continuously activates naïve B cells, maintaining the serum antibody levels by IgG secretion by short-lived PC. In the CD4 T cell-deficient I-$A^{b-/-}$ mice the virus load may not be sufficiently high to assure continuous B cell activation and sustained IgG responses. This scenario is supported by our data showing that transfer of CD8 T cells into T cell-deficient mice decreases virus load and is also associated with a reduction of antiviral IgG responses with time, similarly to what was seen in I-$A^{b-/-}$ mice.

H. Role of innate pathways in maintaining TI antibody responses

The high virus load in TCRαβ KO or TCRαβγδ KO mice is not restricted to the periphery; it is also present in the bone marrow, where B cells continuously develop. This brings up the question of how can B cells that develop and

differentiate in the presence of PyV antigens maintain their reactivity and not become tolerized to PyV? Most viral infections activate innate immune responses and induce multiple inflammatory signals, which serve as the first line of defense against an infection. These innate responses may be important for the early control of pathogens, but they also shape the adaptive immune responses. We have found previously that PyV infection activates MyD88-mediated pathways, and these pathways play a crucial role in the maintenance of long-term IgG responses to PyV infection in immunocompetent (T cell sufficient) mice (3). MyD88 mediates signaling from most TLRs (except TLR3) and the cytokine receptors IL-1R and IL-18R. For the induction of long-lasting IgG responses to PyV the cytokine receptors were dispensable (3). These experiments show that PyV can be considered as a “dual specificity” antigen, which can simultaneously engage B cell receptors and TLRs. Remarkably, in transgenic B cell models the presence of an antigen with this “dual specificity” was shown to lead to a breach of anergy (128). Self-antigens that could bind the B cell receptor and engage TLR7 or TLR9 generated autoantibodies (20, 150). PyV, which is present in the bone marrow, can engage the BCR, become endocytosed and potentially be recognized by endosomal TLR7 or TLR9-MyD88 mediated pathways. This recognition of PyV by both the BCR and the endosomal TLRs could be a key step in allowing continuous PyV-specific antibody production. While investigating the role of innate MyD88-mediated pathways in the maintenance of long-term IgG in T cell-deficient mice, we found that the lack of sustained IgG responses in

mice deficient in both T cells and MyD88 signaling contrasted with long lasting TI serum IgG in MyD88 sufficient T cell-deficient mice. This suggests that the continuous activation of naïve B cells emerging from the bone marrow by the high virus load is successful only if the antigen also activates the innate immune pathways. MyD88 signaling, as shown in Chapter III, is important in antibody isotype responses and in the maintenance of long-lasting antibody responses in T cell-sufficient mice.. Thus, a possible alternative scenario is that the defect in TI IgG responses in TCR β δ KO/MyD88-/- is due to a requirement for MyD88 in TI switching to IgG. However, our data showing similar low levels of IgM in both TCRbdKO and TCRbdKO/MyD88-/- mice argue against this possibility. Our study shows that although short-lived TI antibody secretion does not require MyD88-mediated pathways in virus-infected T cell-deficient mice, the long-term maintenance of the antiviral serum antibody levels, which is often life-saving, in the absence of T cells, requires the activation of innate pathways.

In summary, studies described in Chapter IV are consistent with the hypothesis that innate immune activation and high antigen load, together, can break tolerance of developing B cells. This proposed tolerance breaking mechanism may act as a safeguard to assure protective humoral immunity against a viral pathogen even under the conditions of partial immunodeficiency (lack of T cells). Furthermore, this mechanism would greatly reduce the long-term production of irrelevant or auto-reactive antibodies and the occurrence of pathogenic autoantibody-induced conditions.

I. Conclusions

The PyV mouse model has allowed me to ask how innate signaling contributes to the maintenance of life-long antiviral antibodies in immunocompetent hosts and T cell-deficient mice infected with this persistent virus.

We have reported previously that long-term humoral immunity to PyV was defective in mice that lacked MyD88, as the MyD88-/- mice failed to form long-lived plasma cells (3). The results in Chapter IV taken together with the previous findings suggest that there are important mechanisms that assure that long-lived antibody responses, both TI and TD, are maintained only to those antigens that turn on innate immunity by their pathogen-associated molecular patterns, which can represent “danger” to the host.

Results from Chapter III show the importance of MyD88-mediated TLR pathways in regulating isotype-switched, long-term B cell responses to PyV infection. In spite of that, I have shown that MyD88 signaling is important in maintaining TFH cell and GC B cell numbers, assuring the relative longevity of GC reaction, which contributes to the normal generation of long-term B cell responses. Studies in Chapter IV demonstrate how the presence of antigen at high levels and innate signaling pathways preserve TI antibody responses. The key aspect in these studies was the amount of antigen present within the bone marrow. An antigen that could reach a threshold that could trigger both BCR and TLR pathways leading to the break in B cell tolerance. Thus, this mechanism is

of biological importance as it can provide long-term humoral protection against foreign pathogens, such as virus infections that are uncontrolled and grow in immune compromised mice.

TD and TI B cell responses were studied here in a PyV mouse model, but our findings on the role of MyD88-mediated TLR signaling pathways in promoting both TD and TI long-term B cell responses are likely to apply to other viral infections as well (116, 151).

CHAPTER VI: References

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