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TLR7 in Innate and Adaptive Immunity to Influenza

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B.S., University of Illinois Urbana-Champaign, 2006

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An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University in partial
fulfillment of the requirements for the degree of Doctor of Philosophy in
Graduate Division of Biological and Biomedical Science,
Immunology and Molecular Pathogenesis

2012

Abstract

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By Victoria Jeisy Scott

Toll-like receptor 7 (TLR7) is a major pattern recognition receptor involved in the recognition of influenza A virus (IAV) infection due to its ability to sense ssRNA. However, the role of TLR7 in the shaping of the humoral response to IAV infection and vaccination is not well understood. I demonstrate that the absence of TLR7 signaling leads to the induction of a Th2 polarized memory response to IAV infection. This imbalance is likely due to the increased number of myeloid derived suppressor cells (MDSCs) recruited to the site of infection, influencing the programming of the subsequent adaptive response. Furthermore, I also demonstrate that this Th2 bias in TLR7$^{-/-}$ mice is maintained long-term with a decrease in memory IgM antibody secreting cells (ASCs). However, this Th2 polarization does not inhibit their ability to defend against a secondary IAV challenge. In contrast, TLR7-mediated signaling during influenza vaccination was important. In the absence of TLR7-mediated signaling, insufficient HA-specific antibodies were induced and left mice vulnerable to IAV challenge. Hence, my findings suggest that the addition of RNA or other TLR7 ligands to influenza vaccines will improve their immunogenicity.

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Acknowledgements

I couldn't have done this work without the love and support of my family and husband. My parents, John and Alice Jeisy, as well as my grandparents Don and Jean Broden, and Bob and Joyce Jeisy, have been a constant support to me and have always made me feel like I could accomplish anything. Justin Scott is a wonderfully loving man that has been the person I've relied on the most to get through my graduate work. Thanks to my brother Kyle Jeisy for putting up with my torments. My church family and friends have kept me grounded. My advisors, Prakash Sambhara and Jackie Katz, have guided me to my fullest potential. My committee helped shape the direction of my project. My undergraduate advisor, Deborah Berthold, helped build the foundation of becoming an effective researcher. Also to thank those teachers at Stewardson-Strasburg who inspired my love of biology, particularly Sheryl Rincker.

Chapter 1 : Introduction

Innate Immunity

Life is defined by boundaries. Skin separates a person from their environment and membranes separate cells from each other. These boundaries separate self from non-self. Pathogens seek to invade the boundaries of their host to consume their resources. Humans protect themselves against these threats utilizing both an innate and adaptive immune system to keep pathogenic non-self separate from one's self.

Innate immunity is the primary defense against pathogens. Every living organism has some form of pathogen sensing mechanisms as a component of innate immune defenses [8-12]. The earliest example of innate immunity was the use of phagocytosis by unicellular organisms which utilized random sampling by pinocytosis [10, 13]. Additionally, they produced soluble mediators to specifically recognize foreign pathogens [14, 15]. As organisms evolved in complexity, so did their innate immune systems. When new abilities evolved, old capabilities were maintained, creating an innate immune system that is evolutionarily conserved throughout phylogeny. Evolution has selected innate receptors that recognize conserved microbial motifs, known as pathogen associated molecular patterns (PAMPs). These receptors, termed pattern recognition receptors (PRRs) are non-clonally distributed with fixed germline sequences and enact immediate effector mechanisms following recognition of PAMPs [16].

Although innate immunity is essential for defense early in infection, highly specific PRRs and the cells of the innate immune system are not always adequate

for pathogen clearance. As newly emerging microorganisms evolve, the lack of flexibility in the PRRs leaves the host susceptible. To overcome this inevitability, vertebrates have evolved a secondary component of immunological defense known as adaptive immunity. The receptors used by the cells of the adaptive immune system are defined by their ability to somatically mutate to specifically target individual components derived from pathogens. A major function of innate immunity, in addition to initial pathogen recognition, is to modulate the activation and polarization of the subsequent adaptive immune response [8, 17, 18]. Together, these two components of immunity provide protection from pathogens utilizing both conserved PAMPS and microbe-specific motifs for their detection and elimination.

PRRs of the Innate Immune System

Pathogens have tropisms for different cell types throughout the body and have the ability to replicate in various locations either locally or systemically. This can cause tissue damage by the destruction of infected cells or through secretion of toxins by certain pathogens. In response, the innate immune system has evolved PRRs to monitor each one of these possibilities.

The first major barrier a pathogen must overcome is the epithelial surfaces of their host. This external barrier of the skin and mucosal surfaces are constantly exposed to non-self, whereas the body interior is sterile. Epithelial cells express a diverse array of PRRs to aid in their recognition of pathogens [19-21]. Of the epithelial surfaces, the skin is the most difficult route of entry for a pathogen. The skin alone will protect the host from many potential pathogens, as it is made up

of several layers with many passive and active immunological barriers [20, 22-25]. More easily breached are the mucosal epithelial surfaces. These surfaces are merely one cell layer thick to allow for the exchange of air in the lung or the absorption of nutrients from within the lumen of the gut, for example. Mucosal membranes, however, are not without protection. These surfaces are protected by the constant flow of mucous, antimicrobial peptides, surfactant proteins, as well as commensal bacterial [19, 26-32]. In addition to these passive defenses, mucosal membranes are protected by organized centers of immune cell activity known as mucosa-associated lymphoid tissue (MALT) [33-36]. Here, innate immune cells sample antigens entering the epithelia, as well as those within in the lumen. These cells will initiate an inflammatory response after their PRRs become activated in response to pathogen invasion, facilitating the induction of the adaptive immune responses to these antigens. If their PRRs are not engaged, tolerance to the specific antigen is induced [37-39]. Although epithelial surfaces protect animals from most challenges, pathogens can break this dynamic barrier. Once an epithelial barrier is breached, the pathogens are sensed by the resident cells of the hematopoietic compartment, including Langerhans cells, dendritic cells, and macrophages. Other cells of the innate immune system, such as monocytes and neutrophils, also circulate throughout the body. Aside from immunologically privileged sites, there is no location a pathogen can infect where they can evade detection by the innate immune system.

Innate immune receptors exist not only in nearly every location of the body, but are also found in a variety of cellular compartments. PRRs can be produced as secreted factors, located within the cell as cytosolic sensors,

membrane-bound on the plasma membrane, or exist in vesicular compartments. Soluble factors such as mannose-binding lectin (MBL), pentraxins, ficolins, and surfactant proteins recognize conserved microbial motifs [29, 40-45]. Examples of conserved bacterial molecules recognized by these receptors include lipopolysaccharide (LPS), peptidoglycan (PGN), and mannose containing carbohydrates. The main function of these secreted PRRs is to bind, agglutinate, opsonize, and neutralize extracellular microorganisms. The binding of secreted PRRs often leads to the activation of complement and/or phagocytosis of the pathogen by a phagocytic cell [40, 46, 47]. Within the plasma membrane are several families of receptors that also sample microbial motifs common to extracellular pathogens. These PRRs include C-type lectins, scavenger receptors, integrins, and Toll-like receptors (TLR) [48]. Within the cell, cytosolic PRRs can bind microbial molecules such as PGN and nucleic acid. Unlike the receptors present on the plasma membrane, these receptors mainly target intracellular pathogens [5, 48, 49]. Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) recognize PGN, where protein kinase R (PKR) and retinoic acid-inducible gene 1 (RIG-I) bind to ribonucleic acid (RNA) [50-53]. One additional multimeric oligomer found in the cytosol responsible for the recognition of double-stranded RNA (dsRNA) is the inflammasome [54-56]. The inflammasome is comprised of an apoptosis-associated speck-like protein containing a caspase activation and recruitment domain, caspase 1, and a NOD-like receptor family, pryin domain containing 3 (NLRP3) [56]. Unlike the other classes of PRR, endosomal PRRs exclusively recognize nucleic acid and are most active in professional antigen presenting cells (APCs) and phagocytic cells [57, 58]. So far, only TLRs 3, 7, 8,

and 9 have been found to be restricted to the endosomal compartment [59]. Together, PRRs are not only found in nearly every location of the body, but also on and within various subcellular compartments.

PRR signaling pathways

Many innate PRRs have similar downstream effector functions, mainly in the induction of inflammatory cytokines and type 1 interferons (IFNα/β) [58] (Figure 1-1). One of the major transcription factors activated during an innate immune response is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB was first discovered by David Baltimore in 1986 [60]. He found that NF-κB was important for the transcription of the immunoglobulin (Ig) κ light chain in B cells. NF-κB has subsequently been found to be essential for the transcription of many genes [61]. In the context of PRR signaling, NF-κB is usually a heterodimer of RelA/p65 with one of its four other subunits [62]. NF-κB is prevented from entering the nucleus by the inhibitor of kB (IκB). The IκB kinase (IKK) complex comprised of IKKα, IKKβ, and IKKγ, phosphorylates IκB. This phosphorylation event targets IκB for ubiquitination and degradation by the proteasome, allowing for the translocation of NF-κB and activation of inflammatory cytokines. TLRs, NLRs, RIG-I, and inflammasomes all activate NF-κB.

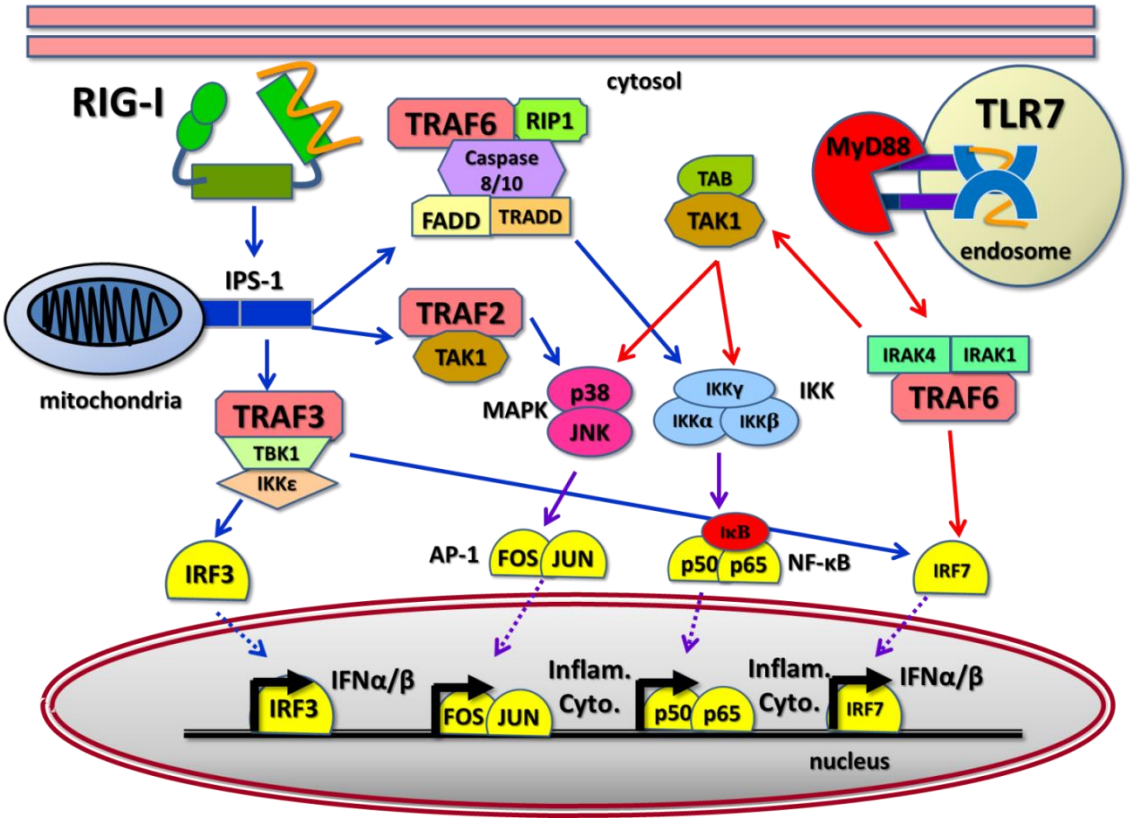

Figure 1-1: RIG-I and TLR7 signaling

Cytosolic RIG-I and endosomal TLR7 have significant redundancy in their downstream effectors in their recognition of RNA. The signaling pathways displayed are a consensus from the following papers: Kato 2011, Mikkelsen 2009, Gilliet 2008, Moynagh 2005 , Lee 2007 [1-5]

In addition to NF-κB induction, the transcription factors activator protein 1 (AP-1) and interferon regulatory factors (IRFs) are activated upon PRR stimulation of RIG-I and the endosomal TLRs. AP-1, like NF-κB, is a heterodimer of one of the Fos, Jun, or activating transcription factor (ATF) family of proteins [63]. AP-1 translocation is activated by the mitogen-activated protein kinase (MAPK) signaling pathway, utilizing its p38 and c-Jun N-terminal kinase (JNK) subunits. Like NF-κB, AP-1 activates inflammatory cytokine transcription. However, only IRFs are able to transcribe IFNα/β [64]. IRF translocation is

mediated by TNF receptor associated factors (TRAFs) in combination with a kinase. TLR7 activation of IRF7 is dependent on Interleukin-1 receptor-associated kinase (IRAK) 1 and 4 [3, 4]. Myeloid differentiation primary response gene 88 (MyD88) dependent TLR7 signaling through IRAK4/IRAK1/TRAF6 not only activates IRF7, but also signals through AP-1 and NF-κB utilizing a phosphorylation cascade through TGFβ-activated kinase 1 (TAK1) and TAK1-binding protein (TAB) [3] (Figure 1-1). Unlike TLR7, RIG-I uses three separate pathways to activate the IRF, AP-1, and NF-κB transcription factors [65]. First, Interferon-beta promoter stimulator 1 (IPS-1) dependent RIG-I signaling requires the use of TRAF2 and TAK1 to activate the MAPK pathway [2]. Second, IPS-1 assembles a complex of TRAF6, receptor interacting protein-1 (RIP1), Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD), Fas-Associated protein with Death Domain (FADD), and caspases 8/10 to activate the IKK complex required for NF-κB translocation [1]. Finally, RIG-I utilizes the kinases TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKKε in addition to TRAF3 to activate translocation of both IRF3 and 7 [64]. Although PRRs activate different downstream signaling pathways, they lead to the translocation of similar transcription factors. This overlap is important for innate host defense against pathogens; if a microorganism blocks one signaling pathway, another can compensate for the necessary signaling.

IFNα/β is one of the most important early mediators of antiviral immunity during infection, as mice lacking the IFNα/β receptor (IFNAR1/2) are unable to control viral infection [66]. It has long been established that viral RNA is a potent inducer of IFNα/β [67-69]. In 1957, Isaacs and Lindenmann first described the

phenomena where a virus could infect one cell, and have its secreted components inhibiting viral replication in neighboring cells not infected with virus [70]. They called these soluble components “interferons”. The “antiviral state” induced by IFNα/β is the consequence of the transcription of IFN stimulated genes (ISGs) [64, 71-75]. Of the many genes regulated by IFNα/β signaling, four are well known to have specific antiviral properties [72, 74, 76]: Mx1, RNA-specific adenosine deaminase (ADAR1), 2’,5’–oligoadenylate synthetase (OAS)-dependent RNase L, and protein kinase RNA-activated (PKR). These ISGs mediate viral infection by blocking and modifying RNA transcription, degrading mRNA transcripts, and halting protein translation respectively [77-88]. Although many of these effects are dually destructive to host cells, sacrificing a relatively small number of cells to limit the spread of virus infection is beneficial to the host. In addition to the induction of ISGs, IFNα/β is also important for the modulation of the subsequent adaptive immune response to infection [18, 89-92].

Toll-like Receptors

TLRs were one of the first families of PRRs identified and remain the most widely studied type of PRR [16]. The *Toll* gene was first identified in drosophila in 1985 as being essential for embryonic dorsal-ventral axis development [93, 94]. Additionally, it was discovered in 1996 that Toll had antifungal properties [95]. The mammalian *Toll* homologues, TLRs, were identified in 1994 [96]. Unlike flies, mammals do not rely on their TLRs for development, but retained the function of pathogen recognition [57, 97]. Both drosophila *Toll* and

mammalian TLRs utilize NF-κB for immunological effector mechanisms [98, 99]. Currently, there have been 13 mammalian TLRs identified [57]. Human cells express TLRs 1-10, where mice express TLRs 1-7, 9, and 11-13.

The TLR protein structure is highly conserved from drosophila to humans. These type-1 transmembrane proteins bind to a wide variety of PAMPs with their leucine rich repeat (LRR) ectodomain (ECD), and send signals to the cell through their cytosolic Toll/IL-1 receptor (TIR) domain [100]. The structure of the ECD of TLR3 was solved in 2005 [101, 102]. The LRRs follow the typical consensus motif of 19-25 residue repeats, having conserved hydrophobic residues as positions 2, 5, 7, 12, 15, 20, and 23. These hydrophobic residues point inward, forming the hydrophobic core of the individual LRR. A series of approximately 24 LRRs combine to form a β-sheet structure, creating a crescent shape [103-105]. One face of the crescent is highly glycosylated, while the ascending lateral surface lacks N-linked glycans [101, 104]. TLR3 was shown to bind its dsRNA ligand as a homodimer, recognizing the sugar phosphate backbone of the RNA rather than its individual base-pairs [106]. Two monomers of a TLR bind its ligand with its ascending lateral surface and stabilize the complex with protein-protein interactions at with the C-terminus portions of their LRRs [106-109]. The shape of this complex resembles an “m”, where the N-termini of the ECDs face outward with the C-termini interacting in the middle (Figure 1-2). This protein-ligand-protein interaction brings together the two TIR domains to interact with TLR3’s adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) and allows downstream signaling to occur [103, 106]. This type of dimer/ligand interaction was also shown to be true for the TLR4 homodimer and the TLR1,2

heterodimer [100]. TLRs 7, 8, and 9 are predicted to bind their ligands in a similar fashion based on their amino acid sequence, although their crystal structures remain unsolved [110]. The ability of the TLRs to recognize different ligands comes in their variations in the N and C-terminal caps, as well as the variations in length and insertions in their LRR loops [104]. TLR ECDs can recognize a wide variety of PAMPs. TLRs located in the plasma membrane generally bind conserved microbial motifs expressed by extracellular pathogens, including glycolipids, diacyl and triacyl-lipopeptides, lipoteichonic acid, zymosan, LPS, flagellin, and profilin [45]. Endosomal TLRs exclusively recognize nucleic acid such as double-stranded deoxyribonucleic acid (dsDNA), dsRNA, and ssRNA [59, 111]. Remarkably, the TLR ECDs have evolved to recognize nearly every potential microbial threat to the host utilizing a simple LRR crescent motif.

The C-terminal ends of the TLR ECDs continue with a 20 amino acid stretch of uncharged, mostly hydrophobic residues that comprise the protein's trans-membrane domain [104]. All TLR cytoplasmic tails end with an evolutionarily conserved Toll/Interleukin-1 receptor (TIR) domain. As the name implies, both TLRs and IL-1 receptors utilize TIR domains for signal transduction [112, 113]. The TIR domain requires cytosolic adaptor proteins for downstream signaling. Like IL-1, most TLRs signal though MyD88 [114]. However, TLR4 and TLR3 can signal independently of MyD88 through their interaction with TRIF [115, 116]. TLRs residing in the plasma membrane require the additional adaptors MyD88-adapter-like (MAL) or TRIF-related Adaptor Molecule (TRAM) to signal through MyD88 and TRIF respectively [57, 104].

Currently, no adaptor molecules have been identified for use by the endosomal TLRs. As described previously, TLR signaling leads to the translocation of NF-κB, AP-1, and IRFs to activate inflammatory cytokines and IFNα/β. The TLR family of PRRs is a sophisticated example of how a family of innate sensors has evolved to recognize a wide array of ligands, yet lead to similar down-stream effector functions.

While cytoplasmic membrane sense conserved microbial motifs that are not present in eukaryotic cells, TLRs residing in endosomes bind to nucleic acids. Because of the danger for sensing self nucleic acids which could lead to autoimmunity, endosomal TLRs are more tightly regulated than those on the surface [59]. First, endosomal TLRs preferentially bind to nucleic acid motifs regularly found in pathogen genomes or replication intermediates as opposed to nucleic acids typically found in eukaryotic cells. TLR9 binds to unmethylated CpG rich DNA found in bacterial genomes, but less frequently in mammals [117]. TLR3 binds to double stranded RNA, a common viral replication intermediate, which is rarely found in eukaryotic RNA [106]. TLR7/8 binds to single-stranded GU-rich RNA, found commonly in viral genomes [118-121]. Although these nucleic acid motifs are common in microorganisms, they are not necessarily restricted to pathogens.

A second safeguard against self nucleic acid recognition by TLRs 3, 7, and 9 is their restrictive endosomal localization. Self nucleic acids outside cells are quickly degraded by DNases and RNases, where pathogenic nucleic acids are protected by bacterial cell walls and viral capsids [122]. Outside of an inflammatory response or other tissue destruction, it is uncommon for APCs to

phagocytose large amounts of self nucleic acid due to this extracellular degradation. Because of this, phagosomal compartments of APCs are largely restricted to exogenous antigens as opposed to self-antigens [122, 123]. TLRs 3, 7, and 9 are transported from the endoplasmic reticulum (ER) to the endosomes via UNC93B1 [124-126]. In an uninfected cell, TLR9 and TLR7 are corralled within the ER. Upon stimulation or viral infection, UNC93B1 traffics these TLRs to the endosomal compartment. UNC93B1 preferentially transports TLR9 to the endosomes as opposed to TLR7 [127-129]. If this balance is altered where more TLR7 is present in the endosomes, severe autoimmune disease can occur [130].

One final safeguard against self recognition of DNA/RNA by endosomal TLRs is the requirement for post-translational modification of their ECDs. The functionality of TLR3, 7, and 9 are restricted to an acidified endolysosomal compartment due to a two-step proteolytic cleavage event of their ECDs [123, 131-135]. The first step involves the partial removal of the ECD, which can be mediated by either asparagine endopeptidases or cathepsin proteases. The second step, which is completely cathepsin dependent, trims the newly exposed N-terminus [131]. The proteases required for this posttranslational modification are restricted to the endolysosome, hence these TLRs can only become activated there. Ewald *et al.* demonstrated that TLRs forced to localize to the plasma membrane cannot signal through MyD88 because this post-translational processing does not occur [133]. With the following safeguards in place for the endosomal TLRs, the majority of self-nucleic acid recognition is averted.

Cellular mediators of immunological responses

All cell types express a few different TLRs, with the broadest array expressed by the hematopoietic cells of the innate immune system [136, 137]. As described previously, pathogens initiate infection through contact with epithelial cells; these cells express an array of TLRs to aid in their recognition of microorganisms. Studies of human cell lines have shown that epithelial cells have the ability to express all ten TLRs, although the levels of expression vary [21, 138-141]. Using the example of influenza A virus (IAV) infection, respiratory cells are the primary site of initial infection and viral replication [32]. Human respiratory epithelial cells express high levels of TLRs 2-6 and respond most robustly to TLR3 ligand [139, 140, 142]. Because they are equipped with these TLRs and other PRRs, respiratory epithelial cells are able to trigger the first signals of infection by secreting chemokines and vasoactive factors. Alveolar macrophages (AMs) are resident in respiratory tissues and are among the first responders to IAV infection in addition to the epithelial cells [143, 144]. AMs express TLRs 1, 2, 4, 6-8 and are the primary source of IFNα early in infection [144, 145]. The epithelial cells and resident immune cells are the first responders to pathogenic infection of the lung.

Within the first 2-3 days of infection, neutrophils and macrophages (MØ) are recruited to the site of infection [146-149]. Neutrophils express mRNA for all TLRs except TLR3 [141, 150] whereas MØs are able to express all TLRs [136, 151, 152]. Neutrophils and MØs recruited during infection are known to play a protective role against IAV morbidity. Depletion of neutrophils after a sublethal dose of IAV leads to uncontrolled virus growth and mortality in mice, as well as

decreased expression of cytokines in the lung [149, 153], suggesting that these cells are required for the control of primary IAV infection. Neutrophils' main effector functions involve the release of their granular contents, including peroxidases, lysozyme, collagenase, and lactoferrin [154]. They are also phagocytic but are not considered professional APCs [155]. MØs are professional APCs, express several families of PRRs, and mediate inflammation through their secretion of a variety of cytokines and chemokines [156, 157]. At the site of infection, monocytes can become differentially polarized to become M1, M2, or regulatory macrophages (Mregs) that polarize subsequent adaptive immune responses [158-160]. Myeloid cell precursors can also become myeloid-derived suppressor cells (MDSCs) [161]. Although it has not yet been shown which PRRs MDSCs express, it is known that MDSCs can be activated in response to TLR 3, 4, 7, and 9 ligands, demonstrating that these receptors are functional in MDSCs [162-164]. MDSCs are a heterogeneous population of $Gr1^{+}CD11b^{+}$ immature myeloid cells that have immune suppressive ability [161, 165, 166]. They can be further classified as granulocytic or monocytic MDSCs based on their Ly6G or Ly6C expression respectively [167]. The Gr1 antibody recognizes both Ly6G and Ly6C [165]. Additionally, the marker for F4/80 can be used to differentiate MDSCs from $Gr1^{+}CD11b^{+}$ neutrophils [168, 169]. MDSCs are known to inhibit Th1 responses at the site of inflammation by secreting IL-10 and induce Th2 polarization of the adaptive immune response by secreting nitric oxide (NO) and reactive oxygen species (ROS) [170, 171]. Finally, dendritic cells (DCs) are recruited to the site of infection. The major functions of DCs are to transport antigen to secondary lymphoid tissue and to secrete immunomodulatory

mediators. Conventional DCs (cDCs) express high levels of CD11c, and may or may not express CD11b [172]. These cells mainly utilize TLRs 1-4 [173]. $CD11c^{+}CD11b^{-}$ cDCs are the most efficient at transporting antigen from the site of infection and presenting epitopes to CD4 and CD8 T cells in the draining lymph node (LN) [172]. Plasmacytoid DCs (pDCs) express lower levels of Cd11c and are positive for the B-cell marker B220. These cells express high levels of TLR7 and 9, as well as moderate levels of TLR1 and 4 [173]. In addition, pDCs constitutively express high levels of IRF7, the downstream transcription factor for IFNα used by TLR7 and 9 [174]. Although cDCs can express IFNα/β, pDCs are known as "professional IFNα producing cells" due to their ability to rapidly secrete large amounts of IFNα in response to viral infection [173-176]. Together, this second wave of innate immune cells amplifies and polarizes the ongoing innate immune response.

The final step of an effective response to primary infection is the initiation of the antigen-specific adaptive immune response. Unlike the PRRs of the innate immune system, lymphocyte B-cell receptors (BCRs) and T-cell receptors (TCRs) are generated through somatic gene-segment rearrangements [177, 178]. This results in a virtually unlimited repertoire of possible antigenic targets for these lymphocytes. A side effect of receptor variation is the possibility of producing receptors that are specific to self-antigens, which would result in autoimmune disease. In the primary lymphoid tissues, lymphocytes expressing receptors that recognize self-antigen die in the process of clonal deletion [179, 180]. T cells must undergo an additional round of positive selection in the thymus to ensure their ability to recognize their cognate peptide antigen in the context of either major

histocompatibility complex (MHC) I or II [181]. After lymphocyte maturation is complete, naïve lymphocytes leave the primary lymphoid tissues and enter the circulatory and lymphatic systems. Lymphocytes encounter their cognate antigen in the secondary lymphoid tissues of the LNs, spleen, or the organized lymphoid tissues of the MALT. Activated APCs at the site of infection transport antigen to the secondary lymphoid organs where B and T cells are concentrated [182]. APCs process proteins of pathogenic origin into peptides that are presented on their surface bound to MHC-I and II. As the T cells circulate through the LN, for example, they sample peptides presented by the DCs. If the T cell recognizes their cognate antigen and receives help by means of B7-CD28 interaction, they proliferate and become activated leading to the initiation of an adaptive response [94]. The antigen-specific effector functions employed by lymphocytes are required to completely resolve the ongoing infection.

There are two components of the adaptive immune response: cell-mediated and humoral immunity. T cells comprise the cell-mediated component, consisting of different T-cell subsets. CD8 T cells are primarily responsible for the cell-mediated destruction of cells infected with intracellular pathogens. CD4 T cells secrete cytokines and aid in the activation of B cells and CD8 T cells, and are referred to as T-helper (T_h) cells. There are several kinds of T_h cells that have been identified based on their ability to differentially affect the outcome of an immune response. These T_h cells are typically defined by the types of cytokines they secrete. Currently, T_h1, T_h2, T_{reg}, T_h17, T_h9, and T_h22 have been identified based on their ability to secrete IFNγ, IL-4, IL-10, IL-17, IL-9, and IL-22 respectively [183]. However, this list is by no means exhaustive, as new T_h subsets

are being described on a regular basis. Two of the earliest and most clearly defined subtypes are T_h1 and T_h2 CD4 T cells [184, 185]. T_h1 cells are known to secrete large amounts of IFNγ and TNFα, activate M∅s, and induce the immunoglobulin (Ig) G polarization of murine B cells towards $IgG2_{a/c}$ [94]. T_h1 cells are most effective at defending against intracellular pathogens. Conversely, T_h2 cells are most effective at defending against extracellular pathogens, particularly against helminths [94]. T_h2 cells are also the predominant cells implicated in allergic reactions to non-pathogenic antigens [186]. These cells secrete IL-4, IL-5, and TGF-β, causing the IgG1 polarization of murine B cells. The mediators that promote a T_h1 response reduce the magnitude of a Th2 response, and vice versa. Regardless of the polarization, CD4 T cells are important for the establishment of an effective B-cell response and absolutely essential for the generation of long-term memory B cells. Once activated, CD4 T cells migrate from the T-cell zone of the LN to the B-cell zone. Upon arrival, they initiate the humoral immune response.

The humoral immune response is dependent on the secretion of Ig by B cells. In absence of CD4 T-cell help, B cells can only produce low-affinity IgM antibody. Unlike TCRs restriction to peptide/MHC complex recognition, BCRs bind to soluble antigens, usually in their native protein conformation. Because of this, it is possible that B cells can secrete antibody in response to a T-independent (TI) antigen. B cells respond to TI antigens if several BCRs are cross-linked simultaneously, like carbohydrates, or if B cells recognize antigen in the presence of a strong TLR ligand [187-189]. B cells are unique in that they are not only adaptive immune mediators, but are also professional APCs [190, 191]. They

express TLRs 1, 2, 4, 6, 7, 9 and 10 to aid in the activation of their APC functions [7, 192-196]. Once a BCR recognizes antigen, receptor-mediated endocytosis allows for the processing and presentation of the antigen on their surface MHC-II. This presentation enables the B cell to communicate with CD4 T cells [7, 194, 197, 198]. With T cell help, B cells can recognize T-dependent antigens, which are normally protein antigens. T-cell help also allows B cells to enter germinal center (GC) reactions. In a GC, activation-induced cytidine deaminase (AID) is turned on and mediates somatic hypermutation and class-switching of the Fc-portion of the BCR [199, 200]. Mutating the BCR changes the affinity of the BCR to its antigen. B cells compete for positive signals received from BCR engagement; those that have higher affinity survive and are clonally selected to proliferate, where those with decreased affinity are neglected and undergo apoptosis [94]. This facilitates BCR affinity maturation of the remaining B-cell clones, which then secrete high affinity antibodies against the cognate antigen. A secondary outcome is the class-switch recombination of the Fc portion of the secreted antibody. Initially, B cells secrete pentameric IgM. Although IgM has low affinity for its antigen, it has high avidity because it is a multimer. Because of these properties, IgM is very effective at opsonizing pathogens and activating the complement cascade. In mice, CD4 T_h1 cells expression of IFNγ induces the B cells to class-switch to IgG2a/c. This antibody is able to fix complement, opsonize pathogens and is efficient at binding protein antigens [201]. This isotype of IgG is the most effective at activating antiviral effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and binding to activating Fcγ receptors on myeloid cells [202]. CD4 T_h2 cells' expression of IL-4 induces class switch to

IgG1. IgG1 is effective at binding to Fc receptors on mast cells, as well as preferentially binding to inhibitory Fcγ receptors on myeloid cells [201, 202]. Additionally, IgG1 is not as effective at activating complement as IgG2a [203, 204]. This further demonstrates that Th1 polarization is more suited for viral infections, whereas Th2 responses evolved to mediate parasitic infections. The induction of the proper adaptive immune response is important for both the effective clearance of the primary infection and the establishment of a long-lasting protective memory response specific to the specific pathogens.

Influenza Virus

Influenza and its complications are the leading cause of death in the United States due to acute infectious disease [205]. The World Health Organization has estimated that annual epidemics of influenza are responsible for approximately three to five million cases of severe illness and 250,000 to 500,000 deaths globally [206, 207]. The annual burden of influenza disease in the U.S. varies considerably, due in part to the characteristics of the circulating virus. Using data from the past 3 decades, the CDC estimates that approximately 21,600 (range, 3,300-48,600) annual deaths occur due to influenza-like illness and its complications [208]. This translates to an annual economic impact of $87.1 billion in the US alone [209]. IAVs are also the cause of occasional pandemics, which have been occurring since the first recorded incidence in 1510 [210]. The public health impact of influenza pandemics also vary considerably; the 1918-19 pandemic being the worst with an estimated 20-50 million deaths [211, 212]. Because of the seasonal burden created by influenza and its

continuous pandemic potential, development of improved preventative strategies remains an urgent public health need.

Influenza viruses belong to the Orthomyxoviridae family of viruses, defined by their segmented, negative-sense ssRNA (-ssRNA) genome [213]. The genome is packaged in a host-derived lipid bilayer envelope spiked with viral glycoproteins [214]. There are three types of influenza viruses: A, B, and C. IAVs have the broadest host range, capable of infecting multiple animal species [215, 216], whereas influenza B and C mainly infect humans. Influenza A and B viruses account for the majority of the annual burden of disease. However, IAVs have an expanded host range, and consequently are the only type of influenza with the capacity to cause pandemics. Due to the yearly seasonal burden and their pandemic potential, IAV is the focus of this dissertation.

The IAV viron contains 8 gene segments encoding 11 known viral proteins [217]. The most abundant protein on the surface is hemagglutinin (HA). The HA homotrimer mediates viral entry by binding to host receptors, glycoconjugates on the cell surface that contain terminal sialic acids (SA) [218]. Also prevalent on the surface of the viron is neuraminidase (NA). The NA homotetramer possesses sialidase activity that is thought to cleave SA from the infected cell surface and facilitates the viron's egress from the host cell [219]. There is also *in vitro* evidence that NA may play a role during viral infection [220-222]. Within the viral envelope is the matrix protein 2 (M2) homotetramer proton channel which aids in the uncoating of the viral particle [223, 224]. The gene-segment encoding M2 also encodes matrix protein 1 (M1). M1 lines the inside of the viral envelope to provide structure to the viron and binds to the virus ribonucleoproteins

(vRNP) [225, 226]. The vRNP complex is comprised of the eight gene segments coated with nucleocapsid protein (NP) complexed with the viral RNA polymearase (vPol) subunits of PB1, PB2, and PA [227]. Within the viron is the nuclear export protein (NEP) responsible for vRNP transport out of the nucleus. The genome of IAV also encodes two virulence factors, non-structural protein 1 (NS1) and PB1-F2. These proteins counteract the anti-viral capabilities of IFNα/β [228-230]. Mutations in these IAV proteins have been associated with host range as well as pathogenicity.

Because of its critical role in viral entry, antibodies that recognize the globular head of HA are capable of neutralizing virus infectivity. Neutralizing antibodies against HA are the primary immune correlate of protection against influenza and also play an important role in viral clearance [231-233]. However, the HA, and to a lesser extent the NA, undergo continual variation through the process of antigenic drift [213]. Mutations arise in the viral proteins through low-fidelity replication of the RNA genome. The accumulation of amino acid mutations within the HA enable variant viruses to evade neutralizing antibodies prevalent in a population due to prior exposure or vaccination to related viruses. Antigenic drift variant viruses replace previously circuiting viruses and are the source of seasonal epidemics. A second, more drastic form of IAV variation is antigenic shift. This typically occurs when animal and human IAV infect the same host, resulting in a reassortment of gene segments, or by direct transfer of a wholly avian or animal virus to humans [213, 234]. Antigenic shift creates variant viruses that are entirely distinct from the previously circulating IAV strains. If the

novel strain can transmit efficiently and cause disease among an immunologically naïve population, a pandemic may ensue.

To date, 17 subtypes of HA as well as 9 types of NA have been identified in IAV [216, 235, 236]. The recent discovery of H17 in Guatemalan little yellow-shouldered bats suggests that additional subtypes remain to be discovered [236]. The HA and NA of this bat influenza virus are significantly divergent from other IAV subtypes, and the NA is so entirely different from avian NA that it is classified as an NA-like protein rather than a new NA subtype. With the exception of the H17 subtype which may have evolved separately, all other IAV subtypes (H1-16) and (N1-N9) are found to circulate in wild water birds [216, 237]. These birds are the natural reservoir of IAV and are the source of subtypes that have established stable lineages in mammals including humans, horses, swine, and dogs.

In water birds, IAV replicates primarily in the intestinal epithelium resulting in asymptomatic infections. Some viruses within the H5 and H7 subtype can acquire the ability to cause severe systematic disease, usually when introduced to terrestrial domestic avian species [216]. The SA linkage to galactose is a determinant of species specificity for IAV [238-240]. The human upper respiratory tract expresses predominantly α-2,6 linked SA, the SA preferentially recognized by human influenza viruses. In contrast, avian influenza viruses preferentially recognize α-2,3 linked SA which is highly expressed in the avian intestinal tract [239, 241]. The upper respiratory tract of pigs, however, express both α-2,6 and α-2,3 linked SA. Because of their dual SA expression, swine are susceptible to both human and avian viruses, making them the perfect vessels for

the creation of avian/human reassortant viruses [240, 242]. The ferret model is considered to be the best animal model for influenza virulence and transmission studies due to a similarity with humans in their α-2,3 linked SA, patterns of viral infection, and disease symptoms [243]. Although mice are not natural hosts of influenza, they are a suitable animal model for IAV; their lungs have mostly 2,3 linked SA, support the replication of avian and pandemic viruses, and generally reflect the pathogenicity seen in humans [243].

Only IAV of the H1, H2, H3 and N1 or N2 subtypes have established stable lineages with the potential for sustained transmission amongst humans to date. Since the beginning of the 20th century, the world has experienced four influenza pandemics: 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (H1N1) [210]. In 1918-19, the most devastating IAV pandemic in recent history was caused by an H1N1 avian-like virus [211, 212]. This virus arose after genes from an avian source adapted to infect humans [244]. The pandemics of 1957 and 1968 emerged as a result human and avian influenza virus rassortment, resulting in novel viruses [245]. The 2009 pandemic was caused by a triple-reassortant virus, possessing genes from human, avian, and swine-origin influenza viruses [246-248]. At present, only A/H3N2, A(H1N1)pdm09, and influenza B viruses of two sub-lineages circulate in a sustained manor in humans [249, 250].

Avian IAV of the H5, H7, H9, and H10 subtypes have infected humans and caused disease, although these subtypes have not acquired the ability to transmit efficiently among humans [235, 240, 251, 252]. Of these avian subtypes, H5N1 is still considered to pose the largest threat to human health due to its virulence [253-255]. H5N1 viruses were first reported to infect humans in 1997 when 18

human cases (6 fatalities) were identified in Hong Kong following wide-spread outbreaks in poultry [256, 257]. As of April 15, 2012 there have been over 600 cases with a fatality rate of 59% [258]. Currently H5N1 viruses have not acquired the ability for sustained human-to-human transmission. However, two independent studies have identified pathways of genetic change that confer enhanced transmissibility by respiratory droplet in the ferret model, further underscoring the pandemic potential of H5N1 viruses [259] (Fouchier, R. *et al.* Science publication forthcoming). Therefore, continued monitoring of avian IAV infections in humans and poultry and the development of effective pandemic countermeasures are essential components of preparedness.

Virus Lifecycle

Before influenza can effectively infect a host cell, the HA protein must first be processed. HA is initially translated as HA0 and must be cleaved into HA1 and HA2 by a protease. The sequence of the HA0 cleavage loop contributes to the pathogenicity of the virus strain due to the location of the appropriate proteases able to perform that cleavage [260, 261]. If the cleavage loop contains a single Arg residue, a trypsin-like protease (TLP) will cleave HA0. These proteases are restricted to the respiratory and gastrointestinal tract of humans and birds respectively [262]. Because of the location of the TLPs, low-pathogenic IAVs can only cause local infections. However, if HA0 contains multiple basic residues in their cleavage loop, they can be processed by furin or other serine endoproteases. These proteases are ubiquitously expressed throughout the host [261, 262]. The ability of HA0 to be cleaved in multiple cell types allows these viruses to spread

systemically and, as a result, are highly pathogenic [263, 264]. The cleavage of HA0 into HA1 and HA2 exposes the HA2 N-terminus fusion peptide, which is required for HA-mediated membrane fusion [218].

Once HA binds to host SA on the cell's surface, clatherin-coated pits induce receptor-mediated endocytosis [214, 218, 265] (Figure 1-2). In the late endosome, the pH drops and clatherin disassembles. When the acidity of the endosome reaches pH5, HA undergoes a conformational change, inserting its HA2 fusion peptide into the lipid-bilayer of the endosome [218]. This event triggers the fusion of the viral envelope with the endosome. Simultaneously, M2 transports protons into the viron, enabling the disassembly of M1 from the vRNPs [218, 224]. Fusion and disassembly of the viron allows for the release of vRNPs into the cytoplasm for their transport into the nucleus utilizing their nuclear localization signal (NLS) [214, 266]. Once inside the nucleus, the –ssRNA must be translated into a positive-sense mRNA [6, 267]. Addition of a 5' cap is accomplished by PB1 binding to poly-U-rich 5' end of the –ssRNA [268], PB2 binding the m7GpppXm-containing cap structures at the 5' ends of host nuclear mRNAs [269, 270], and PB1 binding the 3' end of the –ssRNA. This binding induces endonuclease activity, cleaving the 5' end cap off of the host RNA. This "new" 5' end serves as the beginning of the viral mRNA transcript. During mRNA synthesis, the 5' end of the –ssRNA stays bound to the vPol, while the 3' end is transcribed [268]. PB1 catalyzes transcript elongation until it reaches the 5' end. At this point, the vPol adds multiple copies of the poly-U 5' end onto the 3' end of the mRNA. The result is an mRNA that is translatable by host RNA polymerase II.

The host RNA polymerase begins translation all of the viral proteins in the cytoplasm. The viral envelope proteins are translated in the endoplasmic reticulum (ER) similar to other cellular trans-membrane proteins. In the ER, the surface proteins oligomerize and are glycosylated. As they leave through the golgi, their oligosaccharides are further modified. Completed surface proteins then assemble at plasma membrane lipid rafts [214, 271, 272]. After their translation in the cytoplasm, M1, NP, and NEP are transported back into the nucleus for vRNP assembly [273]. After all late genes have been made, vPol is modified so that PA can replicate the complementary RNA (cRNA) back into genomic viral (vRNA) [214]. NP coats the cRNA in order to facilitate access of base-pairs for replication of the –ssRNA by vPol [274]. Once replicated, NP and M1 coat the new vRNA, reforming the vRNPs. Then, NEP binds the vRNPs to act as a nuclear export protein [275]. M1 then binds the vRNPs to the cytoplasmic tails of NA and HA that are localized at the plasma membrane [276, 277]. The assembly of all the necessary components induces plasma membrane bending that ultimately initiates particle budding [278]. Finally, it is essential that NA cleaves SA during particle egress; disruption of NA results in the accumulation of virus particles on the cell's surface [279, 280]. Free virus particles are then able to infect neighboring cells, continuing virus replication at the site of infection.

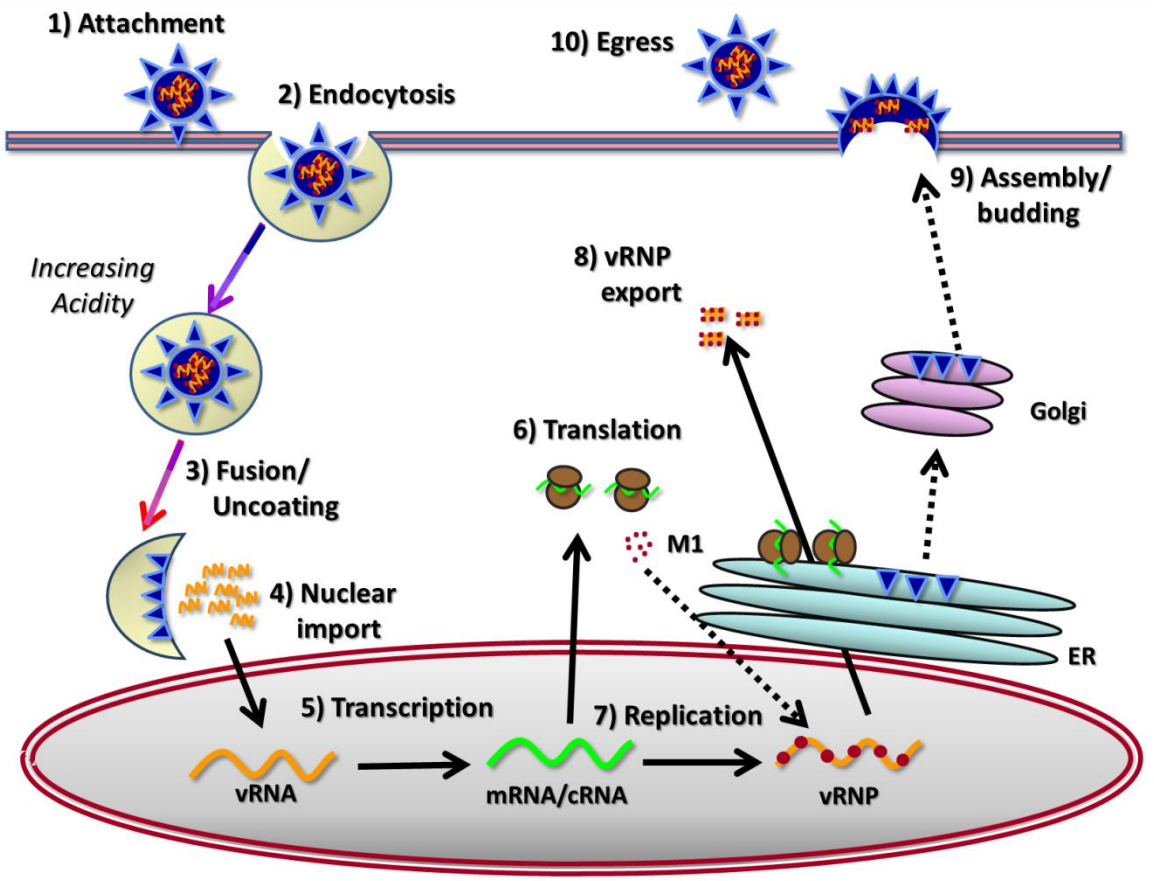

Figure 1-2: Influenza Virus Lifecycle

Figure based on Shaw 2011 [6]

Vaccines

In the U.S., the Advisory Committee on Immunization Practices (ACIP) recommends annual vaccination for individuals six months and older as the major public health tool to reduce the impact of influenza and its complications [281]. The first efforts to develop an influenza vaccine was in the late 1930's and 40's [282, 283], soon after the virus's discovery in 1933 [284]. The first commercially available influenza vaccine was a whole-virus inactivated vaccine (WIV), licensed in 1945 [285, 286]. However, since the 1970's the US has stopped

using WIV and instead predominantly uses split or subunit seasonal influenza vaccines due to their overall improved safety profile [287-290]. Currently licensed influenza vaccines are trivalent, containing antigens from influenza A(H1N1)pdm09, H3N2, and an influenza B virus [291]. Because of continual antigenic drift, all seasonal influenza vaccines must be updated regularly since vaccine efficacy is reduced against mismatched viruses [147].

Two general types of influenza vaccine are commercially available today [292-294]. The first type of vaccine is the trivalent inactivated influenza vaccine (TIV) [295]. To improve the yield of production, vaccine candidate IAVs are made into reassortant viruses, possessing the HA and NA of the wild-type (WT) virus with internal genes from the high-growth virus, A/Puerto Rico/8/34 (PR8) [296, 297]; to produce an inactivated vaccine, recombinant virons are treated with either formalin or β-propiolactone. After the virus is fully inactivated, the particles are purified to remove impurities and the inactivating chemicals added during the manufacturing process [250]. Split vaccines (SV) are produced with the addition of a detergent to disrupt the viral envelope. Additional purification steps are used in the production of a subunit vaccine to remove other viral components and enrich for HA and NA content [298]. Vaccine potency is based on the HA content, a normal dose containing approximately 15μg of HA [289, 299-301]. Although other viral components are known to be present in these vaccines, their levels are not routinely quantified [250]. A second type of vaccine, live attenuated influenza vaccines (LAIV), has been in use in Russia since the 1960's and was first licensed in the US in 2003 [295, 302]. LAIV is based on the development of cold-adapted influenza A and B master donor viruses which

confer attenuating properties [303-306]. LAIV vaccine viruses are reassortants bearing the relevant WT HA and NA with the attenuated internal genes of the master donor virus. Intranasal delivery of LAIV is thought to mimic natural influenza virus infection, and therefore have the advantage over inactivated influenza vaccines in eliciting stronger mucosal antibody and T-cell responses [307]. A quadrivalent LAIV vaccine containing antigens representing two influenza B sublineages has recently been licensed and expected to be available in 2013 [308]. However, TIV remains the most widely administered influenza vaccine worldwide.

As mentioned previously, neutralizing antibody recognizing HA is the main immune correlate of protection against infection. The level of serum anti-HA antibody titers induced in an individual is the typical criteria used to determine a vaccine's effectiveness. Healthy adults benefit the most from TIV vaccination, as reported in randomized controlled trials. It is estimated that adults younger than 65 years of age respond to influenza immunization with 50-90% vaccine efficacy [250, 309-311]. In years where the vaccine strain is mismatched, efficacy is lower than well matched years, although immunization still provides some measureable benefit [311-314]. Many aspects contribute to the efficacy of influenza vaccines, the major factors being previous viral antigen exposure, age, and underlying medical conditions. Although TIV is immunogenic in healthy adults, it is not as immunogenic in young children or the elderly [315-320]. Unfortunately, these populations also have a higher risk for serious complications as a result of IAV infection [250, 294]. Elderly individuals do not respond as well to TIV as younger adults; this is partially a result of their

impaired immune systems [317, 321]. During a randomized, controlled trial of persons older than 60 not living in long-term care facilities, it was found that influenza vaccine efficacy was 58% in this age group [322]. During a controlled study of children 1-15 years old, TIV vaccine efficacy was estimated to be 77% against H3N2 and 91% against H1N1 laboratory confirmed IAV infection [323]. However, when younger children (aged 6-24 months) were evaluated, TIV was found to be only 66% effective in preventing influenza infection during the one year study [324]. To improve the immunogenicity in young children and other unprimed individuals, two doses of TIV are required to elicit sufficient HI titers [325]. Also experiencing lower HI titers to TIV are individuals with chronic medical conditions or those who are immunocompromised [326-328]. A case-control study of individuals aged 50-64 compared persons having high-risk medical conditions to healthy age-matched adults and found that vaccine effectiveness averaged 48% for preventing laboratory confirmed influenza infection and only 36% effective at preventing influenza related hospitalization in those at high risk compared to 60% and 90% vaccine effectiveness in healthy adults [312]. Because these groups do not respond as well to TIV, ongoing research is needed to improve the immunogenicity of influenza vaccines in these susceptible populations.

Vaccines are not only used in the prevention of seasonal influenza epidemics, but are also the most effective means of protection from pandemic viruses. Unfortunately, there are limitations to the development of pandemic influenza vaccines. The first major challenge is the time required to produce a new influenza vaccine. Using current technology, it takes approximately 6

months from the identification of the vaccine seed strains to vaccine administration [329]. Currently, most influenza vaccines are produced in embryonated hen eggs. The disadvantage to using this method is that manufacturers are limited by the number of pathogen-free eggs they can receive each season as these numbers are relatively inflexible and may vary from year to year [330-332]. In addition, egg-derived vaccines cannot be administered to those with egg allergies. Although cell-culture based technology is in development to address the limitation of egg-derived influenza vaccines [331, 333], there are concerns with micro-organism contamination or acquisition of cancer causing agents as exemplified with the case of SV40 contamination of the polio vaccine [332, 334-336]. A final concern is that vaccines against avian viruses which pose a public health threat, such as the H5 and H7 subtypes, have proven to be less immunogenic than human subtype influenza vaccines [337-340]. The placebo-controlled clinical trial by Treanor *et al.* demonstrated that 90μg of HA was required to elicit seroconversion in 28% of individuals after the first dose, and only 57% converted after a second dose [341]. This high dose of antigen is not ideal for a pandemic situation, where availability of vaccine will be limited. The addition of an adjuvant to influenza vaccines would allow for the greater availability of doses to the population and could potentially subvert vaccine shortages, as occurred during the 2009 pandemic [293, 342-346]. For example, squalene-based oil-in-water emulsion adjuvants have already proven to be effective at increasing the immunogenicity of H5N1 vaccines [346, 347]. The continued pursuit of new methods that improve immunogenicity and dose-

sparing of influenza vaccine remains a public health priority to prepare for future pandemic threats.

TLR7 & IAV

IAV RNA, in the form of either vRNPs or mRNA transcripts, is recognized by a variety of PRRs. TLR7 and 3 recognize IAV RNA in the endosome, whereas RIG-I, OAS, and PKR recognize it in the cytoplasm [74, 228, 348-353] (Figure 1-2). The NLRP3 dependent inflammasome is also activated in the cytoplasm by IAV RNA [54-56, 354]. Although influenza virus infection activates these PRRs utilizing similar downstream signaling pathways (Figure 1-1), studies of IAV infection in PRR-specific knockout (KO) mice highlight their different roles and importance.

TLR3 was shown to be expendable for the control of primary infection and the initiation of adaptive immune response during IAV infection [355, 356]. However, TLR7/MyD88 and RIG-I/IRF1 signaling were necessary for the induction of innate and adaptive immune responses following IAV infection [355, 357, 358]. A study by Koyama *et al.* demonstrated that during an *in vitro* infection of embryonic fibroblast (MEF) cells, only IPS1 was shown to be required for IFNβ transcription [357]. In contrast, either IPS1 or MyD88 signaling was sufficient during IAV infection *in vivo* for IFNβ expression in the lungs of mice 24h post-infection; this indicates that these pathways are redundant in their ability to initiate innate immune responses against influenza [357] (Figure 1-1). The same study also found only MyD88 signaling was required for the Th1 polarization of the immune response to IAV [357]. The humoral immune

response requirement for MyD88 signaling has since been confirmed by other groups [355, 358, 359]. Together, these data suggest that IRF1 may play a role in the very early IFNα/β response, while MyD88 signaling is more important for the modulation of the adaptive response [358, 360-362].

TLR7 is the innate immune receptor that recognizes endocytosed, uridine-rich, ssRNA, making it one of the major PRRs responding to influenza infection [118, 120, 302, 363]. PDCs were the first cell type recognized to use TLR7 as a major PRR [176]. Unlike pDCs, cDCs do not constitutively express high levels of TLR7 [3, 173, 174]. As mentioned previously, pDCs constitutively express high levels of both TLR7 and IRF7 in order to quickly respond to viral infections with IFNα/β secretion. In addition, a study by Di Domizio *et al.* demonstrated that TLR7 signaling in human pDCs could activate ISGs independently of IFNα production utilizing a phosphoinositide 3-kinase (PI3K)/MAPK pathway activating signal transducers and activators of transcription-1 (STAT1) [175]. pDCs are a major producer of IFNα/β early in infection [3, 174], and play a critical role in antiviral defense [74, 173, 364]. TLR7 activated pDCs promote viral clearance, stimulate CD4 T_h1 polarization, and aid with the modulation of humoral response [173, 364, 365]. However, TLR7 signaling is not only important for the function of DCs, but is also intrinsically important for the function of B cells.

Like other professional APCs, B cells are able to utilize TLR signaling for their activation and differentiation. Human naïve B cells express low levels of endogenous TLR7 and do not become activated after TLR7 stimulation alone [7, 192, 366-369], although one group has shown that naïve human B cells can

produce low levels of IgM in response to resmiquimod, a TLR7 ligand [370]. Human B-cell activation in response to TLR7 agonists increases when cells are exposed to IFNα derived from pDCs [369, 371, 372]. Naïve B cells in mice, however, constitutively express relatively high levels of TLR7 and can respond polyclonally to TLR7 ligand in the absence of BCR engagement [355, 368, 373]. Like murine B cells, human memory B cells express high levels of TLR7 and respond directly to TLR7 stimulation with activation and proliferation [7, 366, 369, 370, 374]. Based on these observations, it is likely that B cells are differentially regulated by TLR7 in humans and mice.

Using KO mouse experiments, it has been established that TLR7/MyD88 signaling is important for the control of GC formation and isotype switching of B cells [355, 373, 375-378]; it is not known which cell types require TLR7 signaling for this to occur: DCs or B cells. A study by Browne demonstrated that conditionally knocking out MyD88 signaling in either DCs or B cells inhibits an animal's response to retrovirus infection, although this effect was greater in mice having MyD88$^{-/-}$ B cells [375]. Hou *et al.* also used conditional DC and B cell MyD88$^{-/-}$ mice to test humoral responses against inactivated whole PR8 vaccine. Mice with DC's lacking MyD88 signaling had no significant defect in the resulting humoral response post vaccination, where mice having MyD88$^{-/-}$ B cells had decreased levels of total IgG, IgG2c, and IgG2b with increased levels of IgG1 in response to vaccination [197]. These limited data suggest TLR7 expression by either B cells or DCs is required in response to viral infection, while only B cell expression of TLR7 is important in in the context of vaccination.

Current Study

The exact role of RNA sensing by TLR7 in the immune response to IAV infection and vaccination remains unclear. Identifying essential PRRs and their role in the immune response to influenza viruses may help in the design of next generation influenza vaccines with improved immunogenicity for all ages. My specific aims for addressing this issue were to investigate the contribution of TLR7 on the innate and adaptive immune response to IAV infection and vaccination using a $TLR7^{-/-}$ mouse model. Since beginning this project in 2007, newly published work suggests TLR7 and RIG-I play redundant roles in the induction of the antiviral responses to IAV infection [357, 362]. However, only TLR7/MyD88 signaling has been found to influence the polarization of the adaptive immune response [355, 357-359, 362]. In light of these findings, I hypothesized that TLR7 was essential for certain innate immune cell functions and proposed a mechanism behind the observed T_h1/T_h2 polarization imbalance. In addition, I investigated the role TLR7 played in the establishment of an effective memory response to IAV infection. In particular, it was not known if a T_h2 biased adaptive response caused by lack of TLR7 signaling would adversely affect the long-term memory response to IAV infection. Finally, I wanted to determine if TLR7 signaling was important for the establishment of an effective memory response against a commercially available influenza SV, as this aspect had not been addressed within current literature.

Chapter 2 : "Increased MDSC Accumulation and Th2 Biased Response to Influenza A Virus Infection in the Absence of TLR7 in Mice"

PLoS ONE 2011; 6(9): e25242. Epub 2011 Sep 23.

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Contributions: VJS harvested all samples, preformed experiments resulting in data for figures 2.1-2.11, and wrote the paper. WGD, JRP, and JBB harvested samples and edited the paper. W-JS prepared, stained, and scored the H&E histology samples. SRZ, JMK, and SS edited the paper.

Abstract

Toll-like receptors (TLRs) play an important role in the induction of innate and adaptive immune response against influenza A virus (IAV) infection; however, the role of Toll-like receptor 7 (TLR7) during the innate immune response to IAV infection and the cell types affected by the absence of TLR7 are not clearly understood. In this study, we show that myeloid derived suppressor cells (MDSC) accumulate in the lungs of TLR7 deficient mice more so than in wild-type C57Bl/6 mice, and display increased cytokine expression. Furthermore, there is an increase in production of Th2 cytokines by $TLR7^{-/-}$ compared with wildtype CD4+ T cells *in vivo*, leading to a Th2 polarized humoral response. Our findings indicate that TLR7 modulates the accumulation of MDSCs during an IAV infection in mice, and that lack of TLR7 signaling leads to a Th2-biased response.

Introduction

The innate immune system is the frontline for host defense against pathogens and is evolutionarily conserved among many organisms [16, 379, 380]. The innate immune system recognizes a diversity of conserved motifs, referred to as pathogen associated molecular patterns (PAMPs), through a number of immune sensors, known as pattern recognition receptors (PRRs). Mammalian PRRs are found in nearly every cell of the body and are distributed throughout the cell, thus casting a wide net to detect invading pathogens [5, 48, 57, 381]. Some well-studied families of PRRs include collectins, pentraxins, Toll-like receptors, C-type lectin receptors, and retinoic acid inducible gene I (RIG-I)-like receptors [5, 40, 48, 57, 380, 381]. These PRRs recognize PAMPs such as

bacterial cell wall components, flagellar proteins, and nucleic acids. Viral replication produces distinct nucleic acids not commonly found in mammalian cells, such as double stranded, uridine-rich or unmodified RNA, as well as DNA found outside of the nucleus. These unusual nucleic acids are recognized by TLRs 3, 7, and 9, or RIG-I [48, 119, 120].

Influenza A virus (IAV) remains a major public health burden. Hence, it is important to understand how the innate immune response programs the resulting protective adaptive immune response to IAV [382, 383]. IAV has a single-stranded, negative-sense, segmented RNA genome. TLR3, which recognizes double stranded RNA (dsRNA), has been shown to either play a minor role or contribute negatively to the inflammatory response to IAV infection [355, 356]. TLR7 senses single-stranded RNA (ssRNA) within an endosome, whereas RIG-I detects ssRNA in the cytoplasm; both have been shown to be instrumental in the induction of a protective immune response to IAV infection [228, 302, 353, 357, 358, 384, 385]. Myeloid differentiation primary response gene 88 (MyD88) and interferon-β promoter stimulator 1 (IPS-1), the adapter proteins downstream of TLR7 and RIG-I, are redundant in their ability to activate type I interferons (IFNα/β) in response to acute IAV infection both *in vitro* and *in vivo* [357]. These adaptors, however, play different roles in the resulting adaptive immune response. While mice lacking MyD88 had decreased levels of the Th1 polarized antibody IgG2a as well as a decreased CD4+ T cell IFNγ response, IPS1$^{-/-}$ mice and wild type mice had normal levels of IgG2a and IFNγ production [357]. Similar to MyD88$^{-/-}$ mice, TLR7$^{-/-}$ mice are deficient in IgG isotype switching of the humoral response [55, 143, 355, 358, 386]. Furthermore, MyD88 signaling is

important for T cell polarization of lymphocytes *in vitro*, as its absence leads to a Th2 bias four weeks post-infection (p.i.) [358]. Although changes in the adaptive response are evident, it is not clear whether the altered immune responses seen in the MyD88$^{-/-}$ model are due to the inhibition of pathogen recognition by a TLR or to the inhibition of IL-1 signaling. The specific contribution of TLR7 to the regulation of the innate immune response to IAV infection is still unclear.

To answer this question, we investigated the innate immune response in B6 and TLR7$^{-/-}$ mice during acute IAV infection and observed subsequent differences in the adaptive immune response. We found that lack of TLR7 leads to the accumulation of Gr1^{+}CD11b^{+}F4/80^{+} monocytes, otherwise known as myeloid derived suppressor cells (MDSC) in the lungs [169, 387]. The accumulation of MDSCs in TLR7$^{-/-}$ mice during IAV infection was associated with the Th2 polarization of CD4+ T cells and IgG isotypes.

Materials and Methods

Animals

C57Bl/6 (B6) wild-type mice were purchased from Charles River Laboratories (Wilmington, MA). *TLR7$^{-/-}$* mice on B6 background were a gift from Akiko Iwasaki (Yale University, New Haven, CT) [363] and Regeneron Pharmaceuticals, Inc (Tarrytown, NY) and were bred at Charles River Laboratories. *B6.Cg-Tg(TcraTcrb)425Cbn/J* mice (OT-II) were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were age matched and housed under pathogen-free conditions. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an

Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Influenza virus

Influenza A/Puerto Rico/8/34 virus (PR8) was propagated by allantoic inoculation of 10-day old embryonated chicken eggs. The viral plaque titer, 50% mouse infectious dose (MID_{50}), 50% egg infectious dose (EID_{50}), and 50% lethal dose (LD_{50}) were determined using methods described previously [388]. One MID_{50} was equivalent to 40 PFU, 10 EID_{50}, and 0.01 MLD_{50}.

Infections of mice and harvesting tissues for flow cytometric analysis and viral titer quantification

All mice were infected intranasally (i.n.) with 25 MID_{50} of PR8 under anesthesia in a volume of 50μL or mock infected with 50μL of phosphate buffered saline (PBS). Animals were monitored daily for 14 days post-infection (p.i.) for body weight changes and other clinical signs of morbidity. On the indicated days p.i., animals were sacrificed to harvest lungs, spleens, mediastinal lymph nodes (MLN), and sera. To characterize cellular infiltrates, lungs were digested with collagenase A (Sigma-Aldrich, St. Louis, MO) to release cells. Spleens, MLNs, and digested lungs were passed through 70nm cell strainers (BD Biosciences, San Jose, CA) to make single cell suspensions, treated with ammonium chloride (Sigma-Aldrich, St. Louis, MO) to lyse red blood cells. Cell numbers were then counted using a hemocytometer. Cells were later stained with fluorochrome-conjugated antibodies for flow cytometric analysis, using an LSR-II (BD Biosciences, San Jose, CA). Percent of total cells for various cell types were calculated. Percent total numbers were multiplied by total cell numbers counted

to obtain total cell numbers for each cell type indicated. To determine viral titers and cytokine concentrations, lungs were homogenized in 1ml of cold PBS. Clarified homogenates were titrated in 10-11 day old eggs to determine the EID_{50} viral titers in the lungs as previously described [389].

Hemagglutination Inhibition (HI) Assay

Serum samples were treated with receptor-destroying enzyme (Denka Seiken Co., Tokyo) overnight at 37°C, followed by heat inactivation (56°C for 30 min). Serially diluted sera in V-bottom 96-well plates were tested in duplicate for their ability to inhibit the agglutination of 0.5% turkey red blood cells by 4 HAU PR8 in a standard hemagglutination inhibition (HI) assay as described previously [390].

Histopathology

Mice were either infected with $25mID_{50}$ PR8 or mock infected with PBS. Mouse tissues were harvested either 3 or 7 days p.i. For histological analysis, lung tissues from euthanized mice were fixed in 10% neutral buffered formalin **(**Fisher Scientific, Pittsburg, PA) for two days and embedded in paraffin. Three-micrometer sections from formalin-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin (H&E) for histopathological evaluation. Inflammation scores were determined by a pathologist.

Intracellular cytokine staining (ICCS)

Lungs were processed as described above. Cell suspensions were prepared in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, Hudson, NH), 100ng/mL Penicillin/ streptomycin (Invitrogen, Carlsbad, CA), 50mM β Mercaptoethanol (Invitrogen,

Carlsbad, CA). Cells from lungs were cultured with PR8 at a multiplicity of infection of 1 overnight and treated with Brefeldin A (BD Biosciences, San Jose, CA) for 6 hours. Cells were then stained for surface markers, placed in permeabilization/fixation buffer (BD Biosciences) and stained for fluorochrome conjugated antibodies against mouse cytokines to assess the number of cells expressing cytokines.

Cytokine/Chemokine analysis by Bio-Plex assay

Following the manufacturer's protocol, a panel of 23 inflammatory cytokines and chemokines was measured using a Bio-Plex suspension array system (Bio-Rad, Hercules, CA). Briefly, clarified lung homogenates collected from PBS control and infected groups were diluted 1:2 in PBS. Samples and assay standards were added to a 96-well filter plate, followed by anti-cytokine antibody-coupled beads, biotinylated bead detection antibodies and, finally, phycoerythrin (PE)-conjugated streptavidin. Plates were read using a Bio-Plex suspension array system and data were analyzed using Bio-Plex Manager 4.0 software (Bio-Rad, Hercules, CA).

***In vitro* functional analysis of MDSCs**

B6 and TLR7$^{-/-}$ mice were infected with PR8, and lungs were harvested on day 7 p.i. Lungs were processed for a single cell suspension as described above, and stained with fluorochrome-conjugated antibodies. Gr1^{+}CD11b^{+} cells were collected using FACS Aria (BD Biosciences, San Jose, CA). Sorted cells were plated at 1x10^{5}cells/well in a 96 well flat bottom plate. Splenocytes from B6 mice were harvested as described above and incubated with 10nM of OT-II peptide (OVA 329 - 337) (AnaSpec Inc., San Jose, CA) for 1 hour at 37^{0}C, washed three

times with RPMI-10 +10% FBS, and plated with the MDSCs at $1X10^5$ cells/well. Untouched CD4+ T cells from the spleens of OT-II mice were purified with MACS Microbeads (Miltenyi Biotec Inc, Auburn, CA) using negative selection. Transgenic CD4+ T cells were plated with the other two cell types at $1x10^6$ cells/well. Plates were incubated for 24 hours at 37°C in a CO_2 incubator. 18 hours after plating, Brefeldin A was added and cultures were incubated for the remaining 6 hours. Cells were then stained with blue-fluorescent reactive live/dead dye (Invitrogen, Eugene, OR), surface markers, and intracellular cytokines as described above and analyzed using BD LSR-II flow cytometer.

Antibody isotype determination by ELISA

ELISA plates were coated with 100HAU/well of PR8 overnight at 4°C. Plates were washed 3X with PBS-Tween (PBST) and then blocked with 5% bovine serum albumin (BSA) for 2 hours at room temperature. Plates were washed 3X with PBST. Mouse sera were diluted 1:10, added to wells, and incubated overnight at 4°C. Plates were washed 3X with PBST, and horseradish peroxidase (HRP) conjugated isotype specific anti-mouse Ig (Southern Biotech, Birmingham, AL) was added at 1:5000 dilution to each well for 2 hours at room temperature. Plates were washed 3X with PBST and TMB substrate (Southern Biotech, Birmingham, AL) was added to each well, followed by stop solution. Absorbance was read at 450nm. A standard curve of purified mouse IgM, IgG, IgG1, or IgG2a was used as a measurement of antibody concentration.

Antibodies for flow cytometric analysis

Fluorochrome-conjugated anti-mouse antibodies against CD3(Pacific Blue, APC), DX5 (FITC), NK1.1(PerCP Cy5.5), CD19 (PE, APC-Cy7, PerCP Cy5.5) , B220 (PE-

Cy7, Pacific Blue), CD11c (FITC), Gr1 (APC-Cy7), CD11b (Alexa Fluor 700, PerCP Cy 5.5), IFNγ (Alexa Fluor 700), IL-17 (FITC), CD4 (PE, Alexa Fluor 700), CD8 (FITC, PE-Cy7), CD95 (PE-Cy7), and GL7 (FITC) were purchased from BD Biosciences, San Jose, CA. Flurochrome conjugated antibodies against murine F4/80 (APC), IL-10 (APC), TNFα (eFluor 450), and IL-4 (PE-Cy7) were obtained from eBiosciences, San Diego, CA. The PR8 nucleoprotein(NP)-tetramer (H-2D^b ASNENMETM) (PE) was purchased from ProImmune, Sarasota, FL. Samples were run on a BD LSRII and data were analyzed using FlowJo 7.5.5 (Treestar, Ashland, OR).

Statistical analysis

Statistical significance was determined by a two tailed student's t-test unless otherwise mentioned. Asterisks indicate the levels of statistical significance relative to B6 control: *= $p<0.05$, **=$p<0.01$,***=$p<0.001$. Error bars represent the standard error of the mean (SEM).

Results

TLR7$^{-/-}$ mice show increased morbidity, but similar lung viral titers following IAV infection compared to wild type mice.

To examine the role of TLR7 in the innate immune response to IAV infection, B6 and TLR7$^{-/-}$ mice were infected with a sub-lethal dose of PR8 and monitored for morbidity and mortality for two weeks. As expected, both strains of mice lost body weight on day one p.i. due to anesthesia. While B6 mice rapidly regained their pre-infection body weights, TLR7$^{-/-}$ mice maintained only 95% of their original body weight for the first week. On day 7 p.i., TLR7$^{-/-}$ mice began

showing signs of morbidity, including lethargy and ruffled fur. They also experienced more substantial weight loss which peaked with a mean maximum weight loss of 11.3% on day 8 p.i. (Figure 2-1a). These mice did not show signs of recovery until 10 days p.i. B6 mice did not show signs of morbidity at any time during the course of this study (Figure 2-1a).

Histopathological evaluation of the lung tissues shows varying degrees of inflammation in different groups of experimental animals (Figure 2-1b). No prominent inflammation was observed in the lungs of the mock infected (PBS) control mice. The inflammatory cells observed in the lungs of infected animals were mainly composed of lymphocytes, plasma cells, and macrophages in peribronchiolar areas and in alveoli. No significant differences in inflammation were seen in mice harvested on day 3 p.i. between the two groups. The degree of inflammation in the lung tissues was more intense on day 7 than on day 3 in both B6 and TLR7$^{-/-}$ mice. Furthermore, a more intense inflammation was observed in TLR7$^{-/-}$ mice compared to B6 mice on day 7 (Figure 2-1b, c).

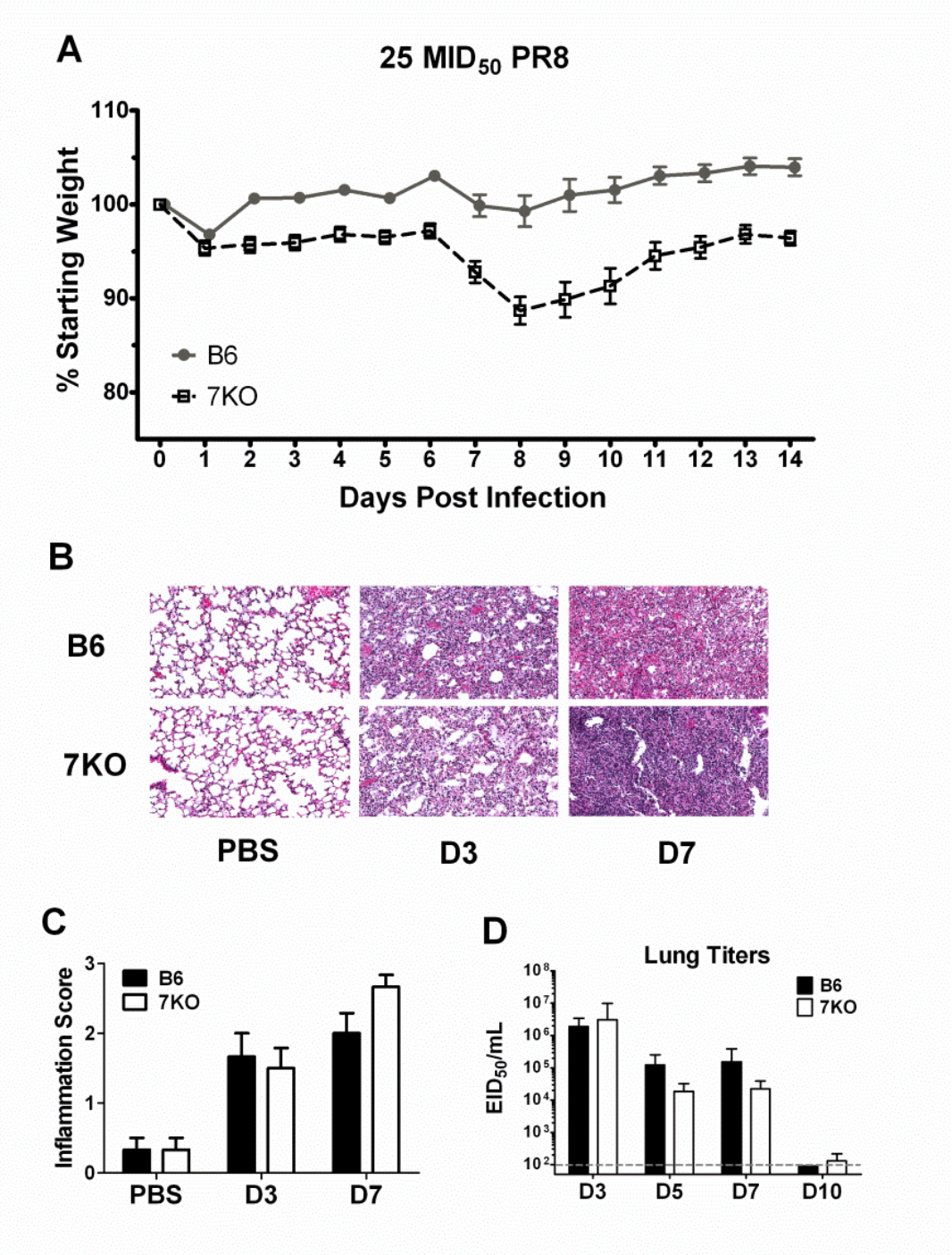
A
25 MID50 PR8
% Starting Weight
110
100
90
80
B6
7KO
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Infection
B
B6
7KO
PBS
D3
D7
C
Inflammation Score
3
2
1
0
B6
7KO
PBS
D3
D7
D
Lung Titers
EID50/mL
10^8
10^7
10^6
10^5
10^4
10^3
10^2
B6
7KO
D3
D5
D7
D10

Figure 2-1: TLR7$^{-/-}$ mice exhibit increased influenza-induced morbidity.
B6 and TLR7$^{-/-}$ mice were infected with 25mID50 of A/PR8/34 (PR8) virus and monitored daily. (A) Mice (n≥13 mice for each group) were weighed individually. Displayed is the percent of starting weight averaged for each group. Significant differences were seen between groups on days 2 & 3 ($p<0.01$), day 4 ($p<0.05$), and days 6-14 ($p<0.001$) as determined by a Two-way ANOVA. (B) Three mice per group per day per treatment were sacrificed and lung samples collected for histology days 3 and 7 p.i. Displayed is a representative H&E stained section of three individual mice with original magnification 10X. (C) An inflammation score was determined by a pathologist of each individual mouse. These scores were combined and displayed on a scale from 0-3; 0 being no inflammation, 3 being the most intense inflammation. (D) At indicated days p.i. mice (n≥5) were sacrificed, lungs were homogenized, and viral titers determined as described in the materials and methods.

Because it is possible that the increased morbidity of TLR7$^{-/-}$ mice could be due to differences in viral burden and/or kinetics of viral clearance, viral titers were quantified in the lungs on days 3, 5, 7, and 10 p.i. (Figure 2-1d). Although viral titers were modestly lower in TLR7$^{-/-}$ mice on days 5 and 7 p.i., these differences were not statistically significant. These results indicate that the increased morbidity observed in TLR7$^{-/-}$ mice is not due to increased viral replication in the lungs of the infected mice.

IAV infection in TLR7$^{-/-}$ mice does not result in hypercytokinemia

A potential cause of immunopathology during IAV infection is the excess production of inflammatory mediators, known as hypercytokinemia or a "cytokine storm", as observed in cases of highly pathogenic avian influenza (H5N1) viral infections in mice [146, 253]. To investigate this possibility that increased levels of inflammatory cytokines were present in the lungs of TLR7$^{-/-}$ mice, we harvested lungs from PR8 infected B6 and TLR7$^{-/-}$ mice on days 3, 7,

and 10 p.i. and measured the levels of cytokines produced. Levels of inflammatory cytokines and chemokines in TLR7$^{-/-}$ and B6 mice were generally similar on day 3 p.i. However, on day 7 p.i., a timepoint of increase morbidity in TLR-/- mice, these mice produced lower levels of IL-1β, IL-6, TNFα, and MCP-1 (Figure 2-2). We also observed a 60% decrease in the levels of the Th1 cytokine IFNγ in TLR7$^{-/-}$ mice compared to B6 at this time point (Figure 2-2). KC, G-CSF, and Eotaxin levels were similar between the control and TLR7$^{-/-}$ mice. These results suggest that TLR7$^{-/-}$ morbidity following IAV infection is not due to hypercytokinemia.

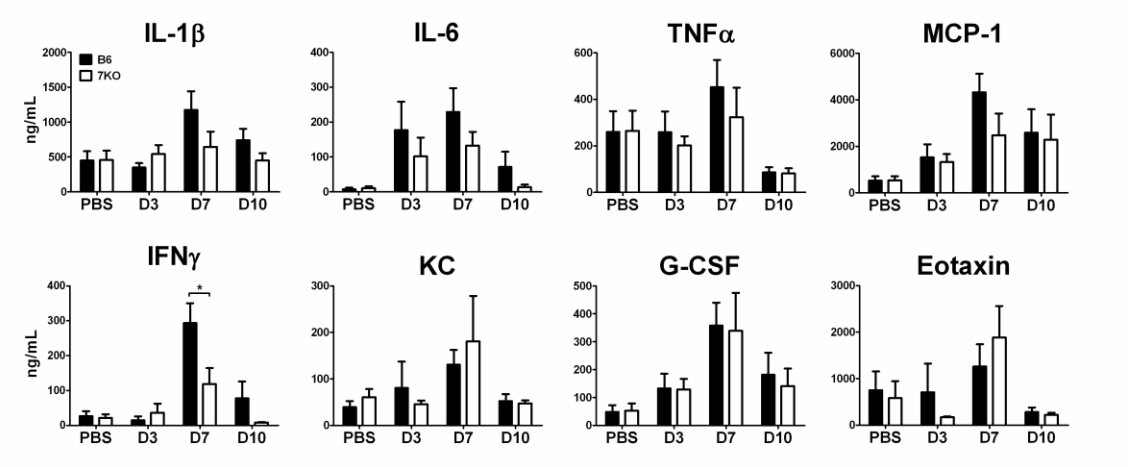

Figure 2-2: The absence of TLR7 does not significantly alter influenza-induced inflammatory cytokine profiles.

On the indicated days p.i., cytokine levels within homogenized lung samples were determined using a bead-based multiplex immunoassay as described in the materials and methods. The data represent a combination of n≥5 individual mice from two independent experiments.

TLR7$^{-/-}$ mice accumulate increased numbers of Gr1^{+}CD11b^{+} monocytes, neutrophils and dendritic cells to the lungs during IAV infection.

We next investigated whether the absence of TLR7 affected the types of cells recruited to the lungs during the first week of IAV infection. No differences were seen in the recruitment of lymphocytes between TLR7$^{-/-}$ and B6 mice on any of the days tested (Figure 2-4a). However, on day 7 p.i. the lungs of TLR7$^{-/-}$ mice had an 8-fold increase in the number of Gr1^{+}, CD11b^{+} cells (Figure 2-3a). 73% of these cells were F4/80^{+}, $SSC_{low,}$ $Gr1_{mid}$ (Figure 2-3b-c). Cells with these phenotypic markers are known to be MDSCs [167, 169, 387] or inflammatory monocytes (iMo) [391, 392]. Neutrophils and dendritic cells (DC) were also detected at significantly higher numbers in TLR7$^{-/-}$ mice (Figure 2-3c). When day 10 was examined, many of the innate cell infiltrates decreased from day 7, while T cells and B cells increased in prevalence (Figure 2-4b). No statistically significant changes were seen between the cell types, except for a relatively lower accumulation of B cells in the TLR7$^{-/-}$ mice. Our findings, consistent with those reported earlier [348, 350, 357, 386], indicate that TLR7 is not essential for the very early innate response to IAV infection. Our data suggest that TLR7 is involved in the second wave of the innate immune response, mainly through the recruitment and/or activity of the pulmonary leukocytes. Furthermore, TLR7 may play a specific role in the accumulation of MDSCs in response to IAV infection distinct from that of the other TLRs and MyD88 dependent pathways [358].

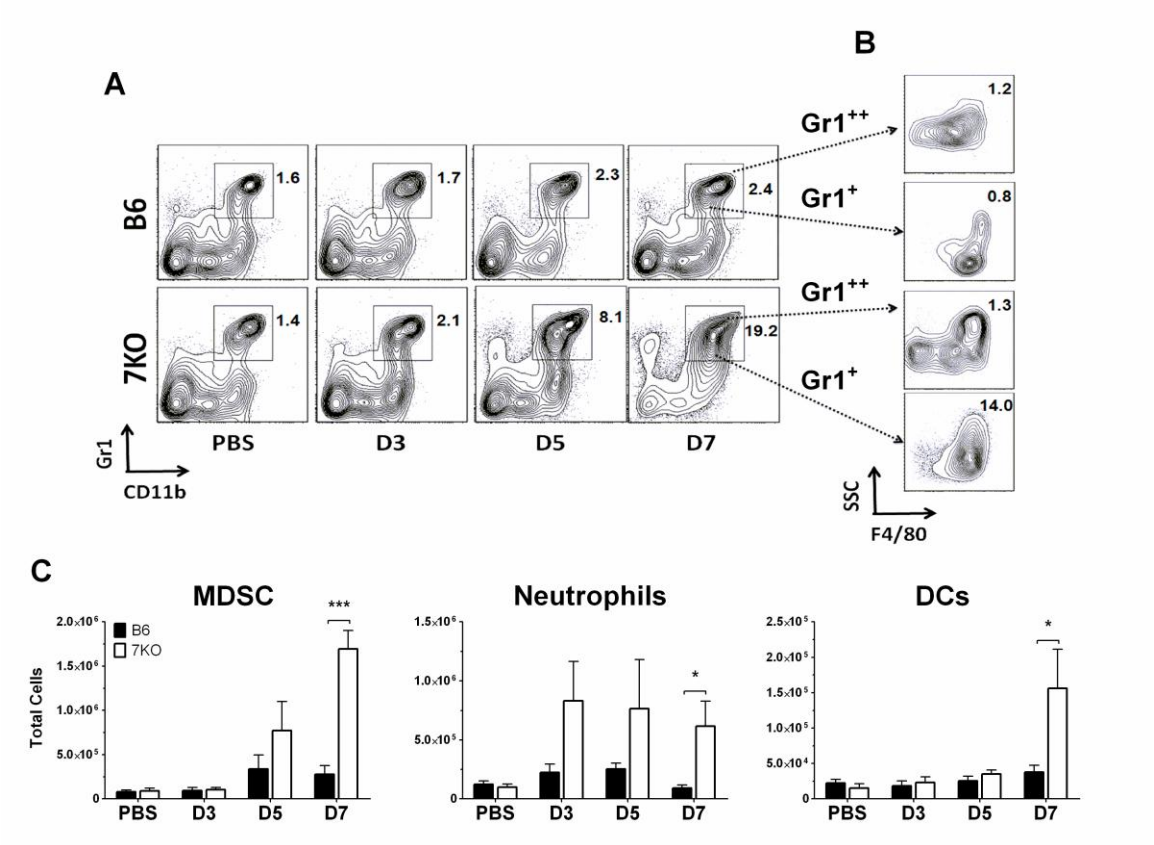

Figure 2-3: Increased recruitment of Gr1+ CD11b+ cells to the lungs of TLR7-/- mice.

On indicated days p.i., lungs from PR8 infected mice or mock infected PBS control mice (n≥5) were processed; infiltrating cells were isolated and stained for analysis by flow cytometry. (A) $CD3^{-}CD19^{-}$ cells were measured for their dual expression of Gr1 and CD11b. (B) These cells were further gated by F4/80 expression to separate neutrophils ($SSC^{+}F4/80^{-}$) from MDSCs ($SSC^{low}F4/80^{+}$). (A, B) Numbers indicated on gates of dot plots represent % total cells. (C) Total cell numbers from two independent experiments were calculated for MDSCs ($Gr1^{+}CD11b^{+}F4/80^{+}SSC^{low}$), neutrophils ($Gr1^{+}CD11b^{+}F4/80^{+}SSC^{high}$), and dendritic cells ($CD11c^{+}Gr1^{-}F4/80^{-}$).

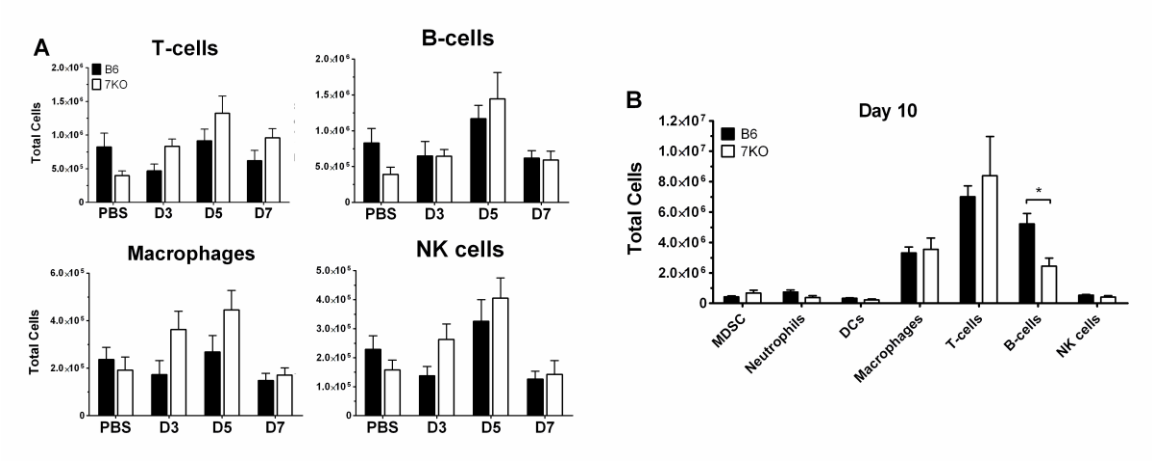

Figure 2-4: Recruitment of lymphocytes and macrophages to lungs was not significantly affected in TLR7$^{-/-}$ mice.

On indicated days p.i., lungs (n≥5 mice) were harvested, infiltrating cells were stained with appropriate fluorochrome conjugated antibodies. Total cell numbers from two independent experiments were calculated for macrophages (CD11b^{+}, Gr1-, F4/80^{+}), B cells (CD19^{+}, B220^{+}), T cells (CD3^{+}), and NK cells (CD3^{-}DX5^{+}/NK1.1^{+}). (B) A separate experiment tested the recruitment of lung infiltrates on day 10 post infection. Again, lungs (n≥4 mice) were harvested, stained for cell specific markers, and total cell numbers were calculated as described above.

Gr1^{+}CD11b^{+} monocytes from TLR7$^{-/-}$ mice are IL-10, TNFα dual producing cells that influence the cytokine production of T cells *in vitro*.

MDSCs are known to have both inhibitory and inflammatory characteristics depending on the model and the circumstance, which is why a phenotypically identical cell type has been referred to as either MDSC or iMo [161, 393]. We wanted to determine if the Gr1^{+}CD11b^{+} cells that were accumulating in the lungs of TLR7$^{-/-}$ mice during IAV infection were multifunctional, similar to those observed in other models [394]. More than 15% of the total cells in the lungs of TLR7$^{-/-}$ mice were MDSCs producing IL-10

(Figure 2-5a,b) on day 7 p.i. Not only were these MDSCs producing IL-10, but the majority of them (80%) were also co-expressing TNFα (Figure 2-5a,b). MDSCs expressing both cytokines were also observed in control B6 mice (2.1% of total lung cells), however they were lower in numbers when compared to $TLR7^{-/-}$ mice (Figure 2-5a,b).

Next, we determined the functionality of these MDSCs by assessing their influence on the activation of T cells to a novel antigen. MDSCs were purified from either B6 or $TLR7^{-/-}$ mice 7 days p.i. and co-cultured with transgenic OT-II T cells, along with OT-II peptide pulsed APCs. After 24 hours in culture, ICCS was performed. Addition of MDSCs from both B6 and $TLR7^{-/-}$ mice induced increased expression of IL-4 from $CD3^{+}CD4^{+}$ cells compared to peptide pulsed APCs alone (Figure 2-5c,d). However, IL-4 production was further increased in the wells containing $TLR7^{-/-}$ MDSCs (Figure 2-5c,d). Approximately 16% of the IL-4 producing cells in the $TLR7^{-/-}$ cultures were also activated, based on their up regulation of CD25 (Figure 2-5c). Taken together, these results suggest that TLR7 not only affects the accumulation of MDSCs at the site of infection, but can also modulate their ability to influence the subsequent T-cell response.

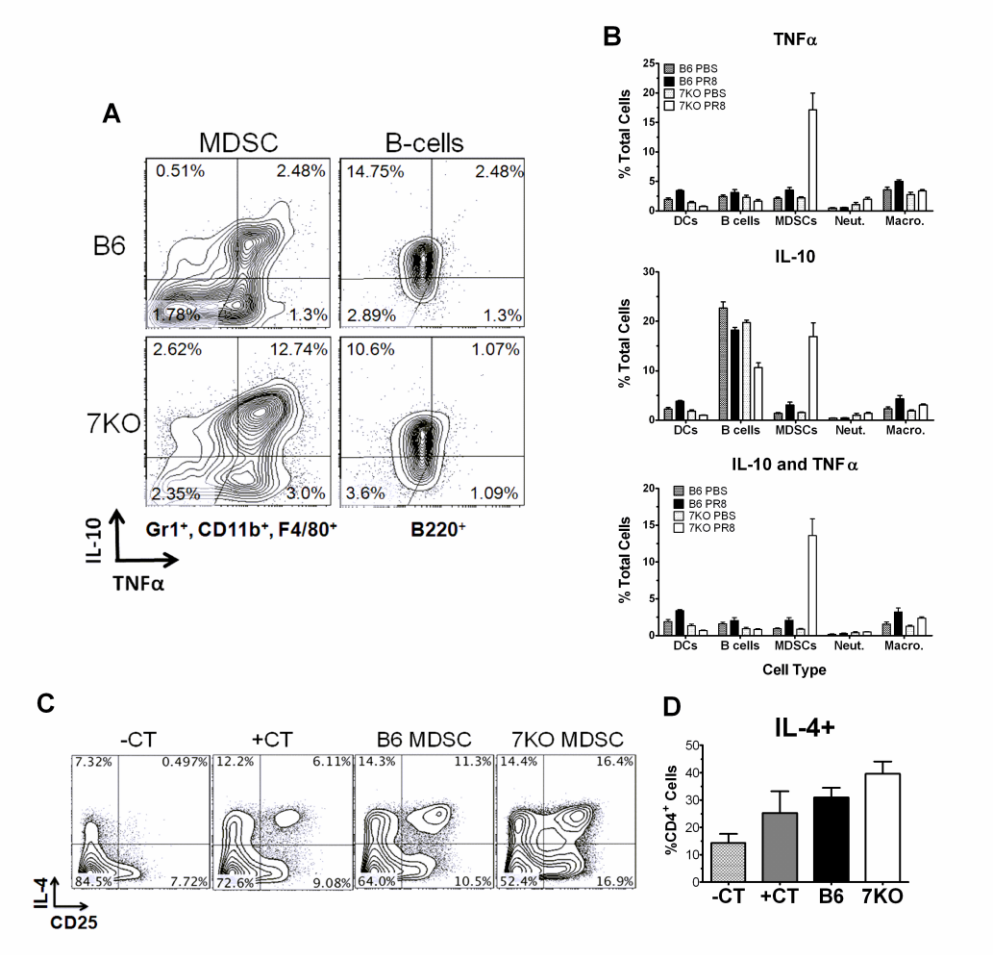

Figure 2-5: Functional analysis of lung-derived MDSCs shows greater activity in TLR7$^{-/-}$ mice.

(A,B) Lungs were harvested and processed from PR8 infected and PBS mock infected mice (n=3 for each group) 7 days p.i. Cells were stained for surface antigens and intracellular cytokines to be analyzed by flow cytometry. Individual cell types were gated and subsequently analyzed for the expression of IL-10 and TNFα. (B) Percent of total cells (n=3 lungs) expressing IL-10, TNFα, or both is shown. (C,D) MDSCs from PR8 infected B6 or TLR7$^{-/-}$ mice were co-cultured with OT-II T cells and peptide pulsed APCs. T cells were stained for intercellular cytokine expression of IL-4. Also included were T cells co-cultured with peptide pulsed APCs only (+CT) and T cells co-cultured with APCs without peptide pulsing (-CT). (C) Dot pots of a representative experiment of three independent experiments are presented. (D) Data from the combination of the three independent *in vitro* experiments was combined displaying the %CD4+ T cells expressing IL-4.

Evidence of increased Th2 polarization of T cells in both the MLNs and lungs of TLR7$^{-/-}$ mice.

Previously, it was shown that the MyD88 signaling pathway is important for the adaptive immune response to IAV [355, 357, 358], but the specific role that TLR7 plays in this response is still unclear. We next examined if there were differences in the activation of B cells in the mediastinal lymph nodes (MLN), where the B cells first encounter antigen. There was an increase in the relative number of B cells in the MLN, increasing steadily from day 3 through 7 p.i. TLR7$^{-/-}$ mice showed a greater expansion of B cells at day 7 and 10 compared to B6 mice, although these differences were not statistically significant (Figure 2-6a). Concordant with the overall increase in B cell numbers, was an increased expansion of GL7^{+} CD95^{+} germinal center B cells in TLR7$^{-/-}$ mice compared to B6 mice (Figure 2-6b,c). One explanation for this observation would be the presence of increased numbers of T-helper cells expressing the B-cell growth factor IL-4, a consequence of Th2 polarization.

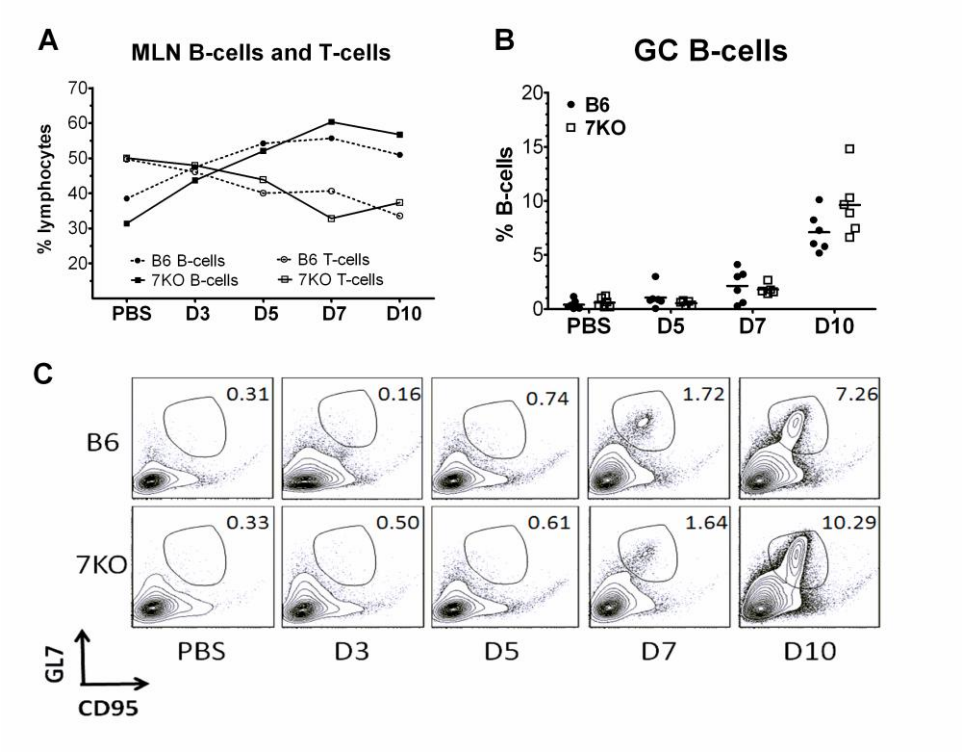

Figure 2-6: Increased expansion of germinal center B cells in TLR7$^{-/-}$ mice.

At indicated day p.i., MLN were harvested (n≥5 animals) and stained for surface antigens. (A) Changes in the relative number of B and T cells as a % of total lymphocytes are displayed over time. (B, C) B-cell germinal center activation was measured by % of B cells co-expressing GL7 and CD95. (C) A representative dot plot and (B) a combination of all experiments are displayed.

To investigate further the possibility of increased Th2 polarization, we compared T cells from the lungs of TLR7$^{-/-}$ mice with those from B6 mice on day 10 p.i. We found a 40% increase in the numbers of IL-4 producing CD4+ T cells in the lungs of TLR7$^{-/-}$ mice than in B6 mice (Figure 2-7a,b). However, we did not see significant increases in the number of IL-17, IL-10, or IFNγ producing T cells (Figure 2-7a,b). There was also no change in the production of these cytokines in spleenic T cells (Figure 2-9a). When we examined CD8^{+} T cells, we saw no differences in frequency or function of antigen specific CD8^{+} T cells (Figure

2-8a,d) as reported previously by others [355, 357, 358]. Taken together, our findings suggest that TLR7$^{-/-}$ mice have a T_h2 T cell bias in response to IAV.

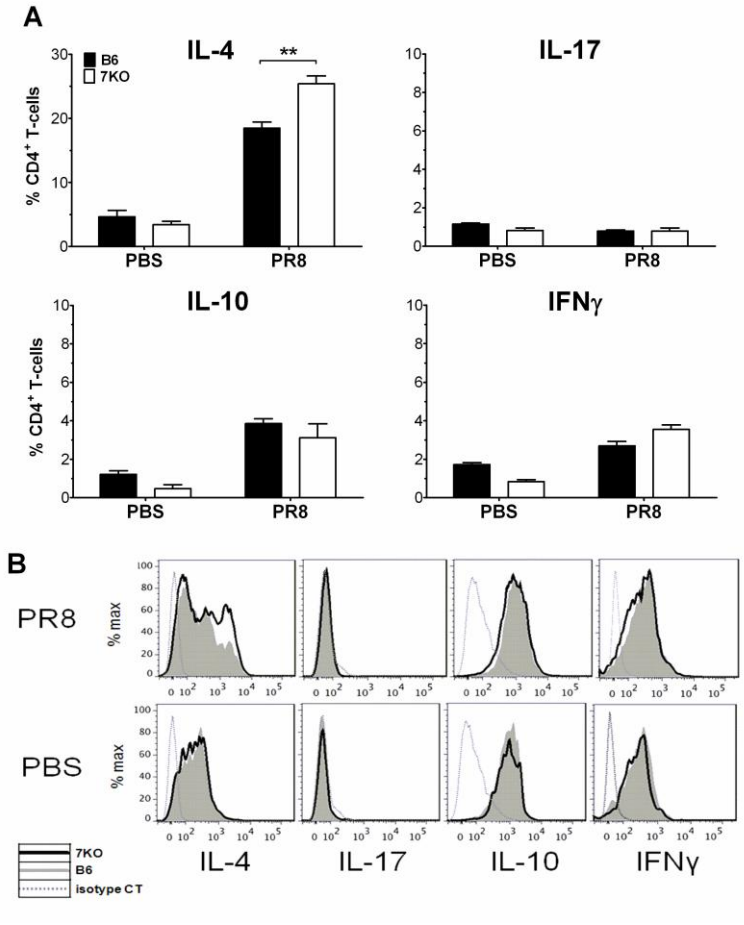

Figure 2-7: Absence of TLR7 leads to increased numbers of IL-4 producing CD4+ T cells.

10 days p.i., lungs (n≥6 animals) were harvested, infiltrating cells were stained with appropriate fluorochrome conjugated antibodies and ICCS performed as described in materials and methods. (A) Data from for each animal was combined as a percentage of CD4+ T cells expressing either IL-4, IL-17, IL-10 or IFNγ. (B) One representative histogram from each group is also shown.

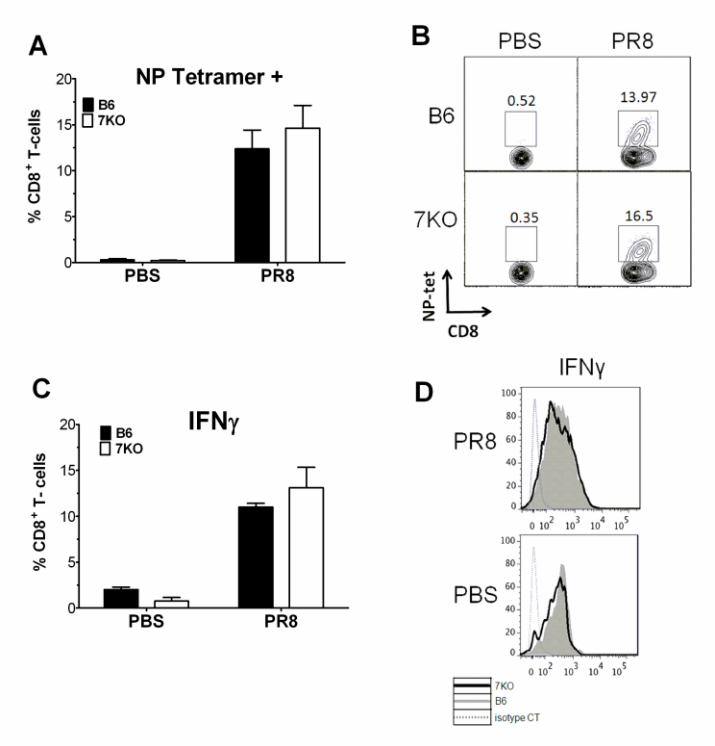

Figure 2-8: The frequency of NP-specific CD8$^+$ T cells are not affected in TLR7$^{-/-}$ mice.

10 days p.i., lungs (n≥6 animals) were harvested, infiltrating cells were stained with appropriate fluorochrome conjugated antibodies and ICCS performed as described in materials and methods. (A) Data from for each animal was combined showing the % NP-tetramer positive CD8+ T cells. (B) One representative histogram from each group is also shown. (C) Data from for each animal was combined as a percentage of CD8+ T cells expressing IFNγ. (D) One representative histogram from each group is also shown.

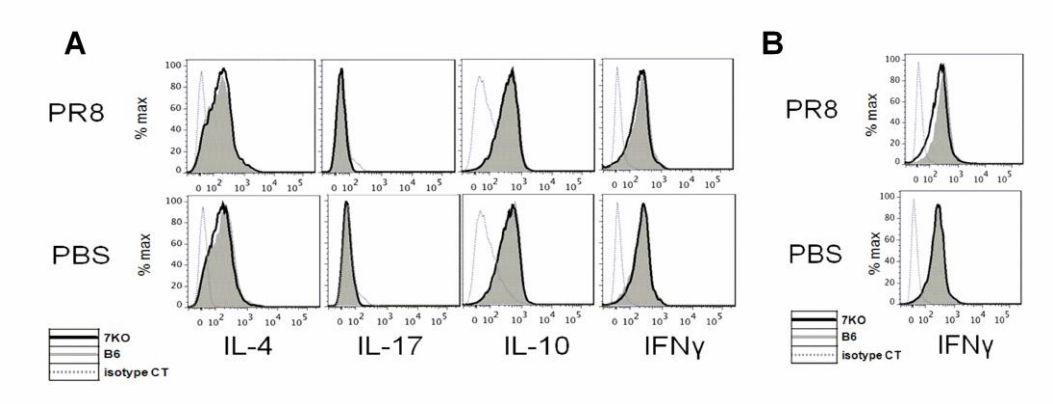

Figure 2-9: No changes in cytokine expression were observed in splenocytes on day 10 post-infection.

Splenocytes were harvested and ICCS performed as described in materials and methods. One representative histogram of three different mice showing the expression of IL-4, IL-17, IL-10 or IFNγ by (A) CD4+ cells or (B) IFNγ by CD8+ cells is shown.

B cells in TLR7$^{-/-}$ mice produce more IgG1 than IgG2a during the early adaptive immune response to IAV.

To further investigate the possible Th2 bias of the adaptive response of TLR7-/- mice, we examined the IgG subclass distribution of PR8 specific serum antibodies. A Th2 polarized response produces increased levels of IgG1, where a Th1 polarized response induces increased levels of IgG2a [202, 355, 395]. Such a Th2 bias has been previously reported in MyD88$^{-/-}$ mice [355, 357]. Although both B6 and TLR7$^{-/-}$ mice had similar levels of HI antibody titers, as well as PR8 specific IgM and IgG (Figure 2-10 a,b,d), the IgG isotypes differed on various days p.i. (Figure 2-10b). We found that TLR7$^{-/-}$ mice produced relatively higher levels of IgG1, where B6 mice produced relatively higher levels of IgG2a (Figure 2-10b,c). These results further demonstrate that the lack of TLR7 signaling leads to a Th2 polarized environment in response to IAV infection.

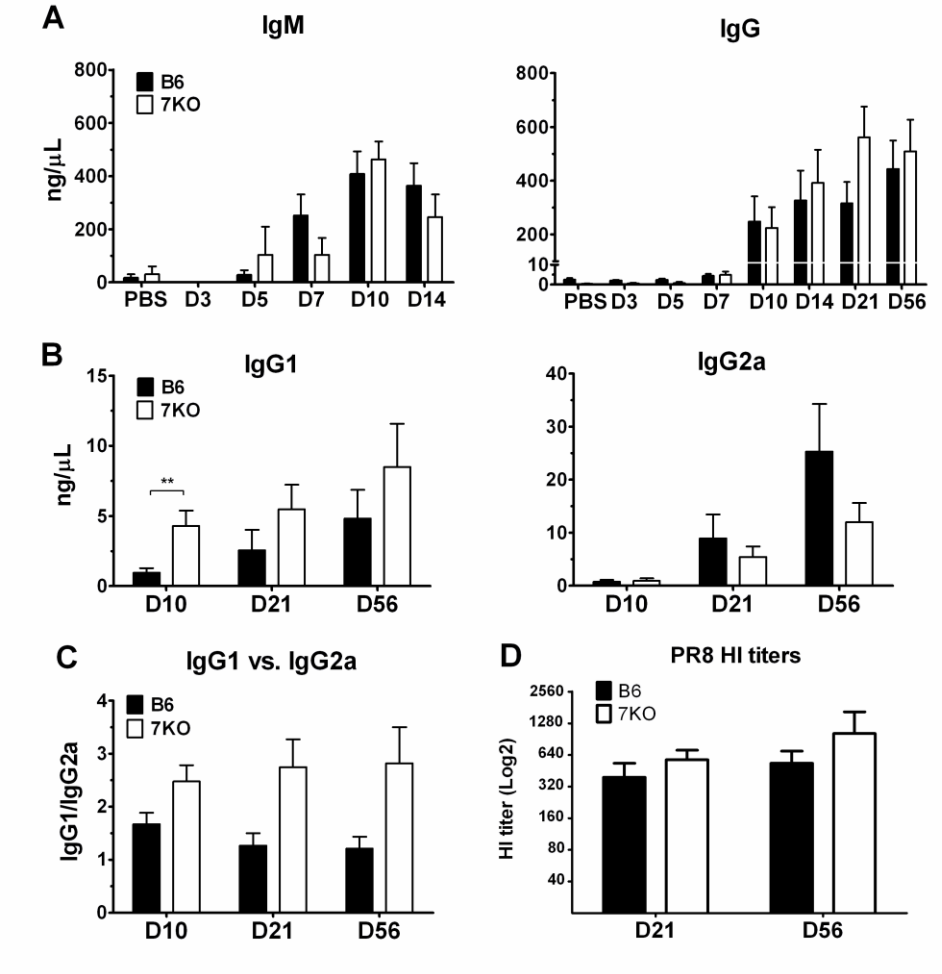

Figure 2-10: T_h2 polarization in TLR7$^{-/-}$ mice alters IgG isotype switching of influenza-specific antibodies.

Sera were collected from B6 and TLR7$^{-/-}$ infected mice (n≥6 per group) at indicated days p.i. PR8 specific (A) IgM or IgG antibodies were measured by ELISA. Sera from additional animals were collected over time (n=10 per group) and PR8-specific (B) IgG1 and IgG2a were measured. Antibody concentration was determined based on a standard curve of their respective immunoglobulin type. (C) A ratio of IgG1 versus (vs.) IgG2a prevalence was determined by dividing a sample's IgG1 A450 absorbance by its IgG2a absorbance. (E) HI titers were determined as described in the materials and methods.

Discussion

The main function of the innate immune system is not only to limit the early replication and spread of the invading pathogen, but also to initiate an adaptive response to clear the infection and establish long-lasting immunological memory. TLR7 is one of the major RNA sensing PRRs involved in the detection of IAV infection. Currently, it is understood that TLR7's downstream signaling pathway leads to the activation of proinflammatory cytokines and IFNs; other RNA sensing PRRs, such as RIG-I, activate similar responses. Previously, it was shown that MyD88 signaling, not RIG-I signaling, plays a unique role in the $CD4^+$ polarized response to IAV infection, but the mechanism of this was unknown. We demonstrate that the lack of TLR7 signaling significantly increases the accumulation of MDSCs at the site of infection. Because MDSCs have been previously shown to alter $CD4^+$ T-cell polarization, we propose that MDSC recruitment is the mechanism by which TLR7 effects the resulting T cell TH1/TH2 balance.

In a study by *Seo et al.,* MyD88 was shown to be required for protection from IAV infection, as $MyD88^{-/-}$ mice displayed increased morbidity and increased viral titers when infected with PR8 [396]. $MyD88^{-/-}$ mice were inhibited in their ability to recruit $CD11b^+$ granulocytes, produce inflammatory cytokines, and Th1 cytokine production by CD4+ T cells following IAV infection when compared to B6 mice. A study by *Koyama et al.*, conversely, showed no changes in viral titer when $MyD88^{-/-}$ mice were infected with A/New Caledonia/20/99. They demonstrated that RIG-I and MyD88 were redundant in their ability to induce the early IFNα/β response *in vivo* and *in vitro* [357].

However, they found changes in the IgG istotypes in MyD88$^{-/-}$ mice following IAV infection with increased IgG1 with a concomitant decrease in IgG2a on day D10 [357]. These studies demonstrated that MyD88 signaling is instrumental in the shaping of not only the innate but also the adaptive responses to IAV.

MyD88 is the downstream adaptor not only for many of the TLRs, but is also downstream of IL-1 receptor signaling [113]. For this reason, it is difficult to ascertain if the phenotypes observed in MyD88$^{-/-}$ mice are a result of dysfunctional IL-1 cytokine signaling or if they are due to inhibited viral recognition by a PRR. Like MyD88$^{-/-}$ mice, IL-1$^{-/-}$ mice display an increase in morbidity and lung viral titers in response to IAV [397]. Because of these similarities, it is possible that many of the effects observed in the MyD88$^{-/-}$ model could be attributed to the inhibition of IL-1 signaling more so than the inhibition of TLR signaling. To better understand the effects of PRR specific recognition of IAV, independent of cytokine signaling inhibition, we utilized TLR7$^{-/-}$ mice. We found that TLR7$^{-/-}$ mice had increased morbidity, starting on day 7 p.i. (Figure 2-1a). In many viral infections, increased morbidity is caused by increased viral titers and/or cytokine storm [146, 253]. Previously, it was shown that absence of the inflammatory cytokines TNFα and IL-1 reduces the severity of H5N1 infection [398]. In our study, viral titers did not increase in the absence of TLR7 (Figure 2-1d). Also, many of the inflammatory cytokines measured were slightly decreased rather than showing signs of a cytokine storm (Figure 2-2). The only significant change in cytokines observed in the lungs was the decrease in IFNγ.

The relative decrease in inflammatory cytokines in the lungs did not have a detrimental effect the recruitment of myeloid cells (Figure 2-3a-e). This is

contrary to what was observed in the MyD88$^{-/-}$ and IL-1R$^{-/-}$ models of IAV [396, 397]. Macrophage and neutrophil accumulation during IAV has been shown to play both a protective and pathogenic role depending on the magnitude of the cellular infiltrate. Depletion of neutrophils after a sub-lethal dose of IAV induces uncontrolled virus growth and lethality [149, 153]. In addition to hypercytokinemia, much of the pathology associated with highly pathogenic IAV infection is attributed to the recruitment of these myeloid cell types [146]. *Lin et al.* found that CCR2 dependent Gr1^{+}CD11b^{+} iMo were the largest population recruited to the lungs during PR8 infection, and were responsible for much of the pathology associated with IAV in mice, but not in the control of virus replication [391]. In our study, we also show that the relatively large recruitment of Gr1^{+}CD11b^{+} cells in TLR7$^{-/-}$ mice was likely the major cause of morbidity observed with little or no effect on lung viral titers.

Gr1^{+}CD11b^{+} cells are recruited to a site of inflammation in several models of inflammation [161, 171, 391, 394]. MDSCs have been described to be a Gr1^{+}CD11b^{+} immature myeloid cell population derived from monocytes migrating out of the blood to the site of infection [167, 399]. It has been previously shown that the presence of MDSCs skews the immune response towards a Th2 response [170, 171, 394, 400]. This is mainly attributed to their ability to induce IL-10 and reactive oxygen species [170, 400] Like other groups, the TLR7$^{-/-}$ MDSCs not only produced IL-10, but also TNFα (Figure 2-5a,b) [394]. Interestingly, TNFα has been shown to amplify the ongoing Th1 or Th2 response rather than favoring one over the other [401, 402]. The ability of TLR7$^{-/-}$ MDSCs to coproduce IL-10 and TNFα may be one mechanism that encourages the Th2 bias observed in TLR7-/-

mice during IAV infection. On day 10 p.i., the lungs of TLR7$^{-/-}$ mice had increased numbers of IL-4 producing CD4^{+} T cells present (Figure 2-7). One month following infection with IAV, we found increased levels of IgG1 and decreased amounts of IgG2a in sera of TLR7$^{-/-}$ mice (Figure 2-10), suggesting that TLR7$^{-/-}$ mice do in fact display a T_h2 bias during IAV infection. We hypothesize that this is due to the increased recruitment of MDSCs to the site of infection.

Interestingly, it has been shown in different models that MyD88 is required for the expansion of MDSCs *in vivo* [394, 396]. TLR7$^{-/-}$ MDSCs, when compared to B6, not only show increased recruitment to the site of infection and secrete increased amounts of Th1 inhibiting IL-10 *in vivo*, but also increase the amount of IL-4 production from OT-II T cells (Figure 2-3,Figure **2-5**). It is possible that while MyD88 is required for MDSC expansion, through either IL-1 signaling or another TLR becoming activated, TLR7 stimulation during IAV infection may be involved in the suppression of MDSC activity. TLR7 signaling could accomplish this through an inhibitory feedback mechanism or by inducing MDSCs to differentiate into another monocytic cell type. It has been previously shown that activation of MDSCs through either TLR9 or TLR4 can further differentiate MDSCs into a myeloid cell that no longer has tumor suppressor activity or Th2 allergy inducing polarization [169, 399]. If this is true in our model, the absence of TLR7 signaling in MDSCs may be responsible for further aggravating the Th2 bias observed due to increased MDSCs cytokine secretion and recruitment.

In the sepsis model, Gr1^{+}CD11b^{+} MDSCs have been implicated for Th2 polarization observed [394]. The accumulation of MDSCs was dependent on

MyD88 expression, as $Gr1^{+}CD11b^{+}$ cell recruitment to the spleen was inhibited during sepsis in $MyD88^{-/-}$ mice [394]. $CD11b^{+}$ cells in the spleens of wild type mice constitutively express TLR7, and TLR7's expression is highly up regulated during sepsis. When mice were pre-treated with R-848 (a TLR7 ligand) before sepsis induction, the treated mice showed an increase in their ability to control bacterial load at the site of infection. This increase in pathogen control and inflammation demonstrate that TLR7 signaling will overcome the inhibitory phenotype predominant during sepsis [403].

During IAV infection, RNA released during cell lysis serve as ligands to activate $TLR7^{+}$ myeloid cells, including macrophages, neutrophils, DCs, and MDSCs. Resident alveolar macrophages are among the first responders to infection [144]. Neutrophils and macrophages are recruited on days 3 and 5 p.i. (Figure 2-3c). On day 5 p.i., MDSCs are recruited to the site of inflammation (Figure 2-3c). They then can become activated by the presence of inflammatory mediators like IL-6 and IL-1 [387]. In mice, virus replication is present through day 7, and is cleared by day 10 (Figure 2-1d), indicating that TLR7 ligands would be present at least through day 10. Our findings suggest that the MDSCs recruited to the lungs starting on day 5 would recognize the presence of RNA through TLR7 and become hindered in their capacity to inhibit the ongoing inflammatory response similar to TLR9 ligand mediated inhibition of MDSC tumor suppression [399] (Figure 2-11a). It is possible that the sensing of RNA by TLR7 actually leads to further differentiation of MDSCs into a more classical Th1 inducing macrophage. MDSCs are known to have plasticity in their ability to further

differentiate into a different type of myeloid derived cell, as has been previously shown with activation from TLR4 activation [169, 404].

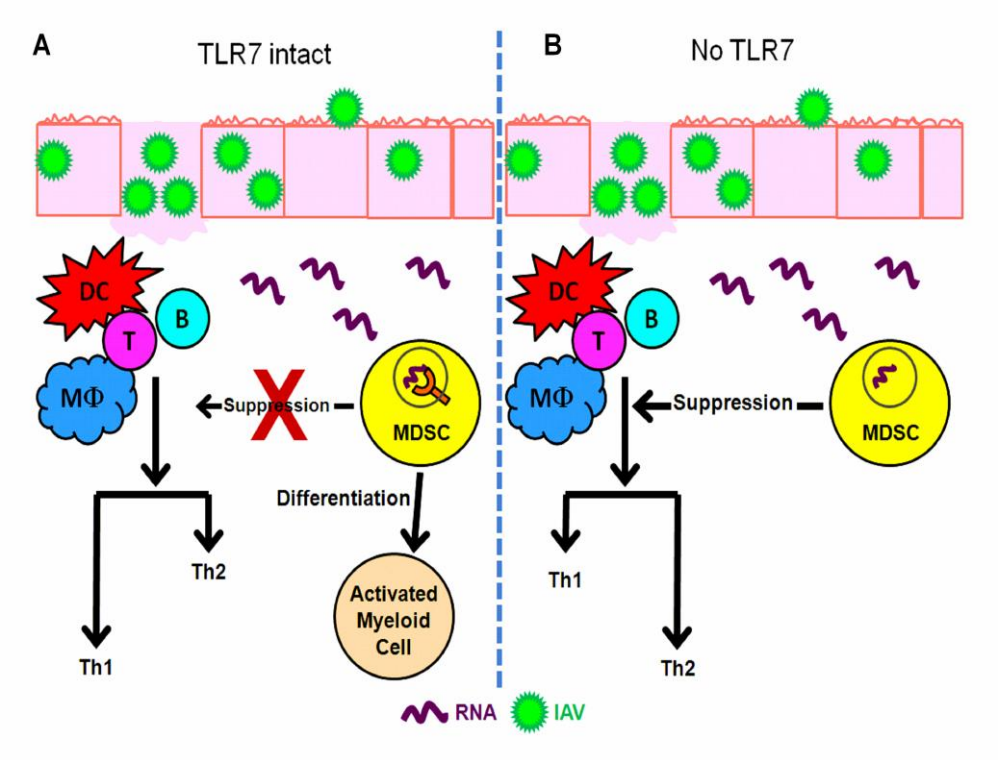

Figure 2-11: TLR7 inhibition of MDSC mediated Th1 suppression.
(A) TLR7 functions to sense the presence of RNA in the endosomes following IAV induced inflammation. TLR7 stimulation may then activate the infiltrating MDSCs to further differentiate into other activated myeloid cell types, preventing the suppression of the ongoing Th1 polarized response. (B) When TLR7 is absent, MDSCs would not recognize the presence of RNA in the context of inflammation, leading to the recruitment of additional MDSCs and suppression of the Th1 mediated response, resulting in a Th2 bias response at the site of infection.

It would not be beneficial for the host to have numerous suppressor cells present before the acute infection is cleared. After day 7 p.i., when RNA is no longer in excess, MDSC activity would no longer be inhibited and would then be allowed to suppress the remaining inflammatory response. In a situation where

TLR7 is not present, MDSCs would lose their ability to sense the presence of RNA during an ongoing infection. When the MDSCs are recruited on day 5, they would not suspend their suppressive activity in the presence of inflammation [387]. This would lead to accumulation of MDSCs and the inhibition of a Th1 polarized immune response, further aggravating inflammation in an unbalanced manner (Figure 2-11b).

Together, our findings suggest that TLR7 signaling inhibits the suppressive activity of MDSCs during IAV infection in mice, resulting in a predominant Th1 response. It has yet to be determined what the long term effects of a Th2 polarized response observed in these TLR7$^{-/-}$ mice are on the memory response. It would be interesting to see if this Th2 polarization increases or decreases the recall response to subsequent infections, and what the consequences of this would be on vaccine design. Because of TLR7 ability to promote a Th1 biased response, it is possible that the inclusion of TLR7 ligands may enhance the immunogenicity of influenza vaccines [383, 405].

Chapter 3 : "TLR7 recognition is dispensable for influenza virus A infection, but important for the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice."

In submission: The Journal of Virology

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Contributions: VJS harvested all samples, contributed data for figures 3.1-3.6, and 3.8-3.10, and wrote the paper. JHK assisted with ELISpot assays and edited the paper. WGD assisted with sample preparation and sequenced viral RNA from the influenza split vaccine. WC contributed data for figure 3.7. JMK and SS edited the paper.

Abstract

Recognition of pathogen associated molecular patterns by pattern recognition receptors of the innate immune system is crucial for the initiation of innate and adaptive responses and for immunological memory. We investigated the role of TLR7 in the induction of adaptive immunity and long-term memory following influenza virus infection and vaccination. We identified a differential role of TLR7 dependent on the context of viral antigen exposure. During infection with influenza A/PR8/34 virus, the absence of either TLR7 or MyD88 leads to reduced serum IgM and fewer IgM secreting B cells in their secondary lymphoid organs, particularly in bone marrow. In spite of this, the absence of TLR7/MyD88 signaling did not impair the production of protective antibodies. However, following immunization with the 2009 pandemic split vaccine, $TLR7^{-/-}$ mice had significantly lower levels of germinal center formation, antibody secreting cells and circulating influenza-specific antibodies. Consequently, $TLR7^{-/-}$ mice failed to develop protective immunological memory upon challenge. Furthermore, the immunogenicity of the split vaccine was likely due to TLR7 recognition of virion RNA, as its removal from the split vaccine significantly reduced levels of influenza-specific antibodies, and compromised the vaccine protective efficacy in C57Bl/6 animals. Taken together, our data demonstrate that TLR7 plays an important role in vaccine-induced humoral immune responses to influenza through the interaction with viral RNA present in the split vaccine.

Introduction

Influenza viruses continue to be a considerable public health burden. Each year, influenza viruses infect 3-5 million people worldwide resulting in 250 to 500 thousand deaths [206]. In addition, influenza A viruses (IAV) from animal reservoirs remain a pandemic threat which is undiminished by the 2009 H1N1 pandemic. Currently, vaccination remains the most cost-effective public health counter-measure to prevent seasonal and pandemic influenza. However, renewed efforts are needed to improve influenza vaccine efficacy in immunocompromised populations, older adults and young children [317, 319, 406]. Therefore, understanding the immune response to infection and vaccination with IAV, and especially how the interplay of host and viral components shape the immune response, is critical for designing influenza vaccines with improved immunogenicity and effectiveness.

The immune response to IAV culminates in the production of protective neutralizing antibodies against the major surface protein, the hemagglutinin (HA). Influenza infection can lead to production of neutralizing antibodies that provide life-long protection from infection with antigenically closely related viruses. This was exemplified by the recent spread of the 2009 pandemic influenza A/H1N1 virus [A(H1N1)pdm09], which caused an estimated 86 million cases and up to 17,620 deaths in the U.S. by April 2010 [407]. Compared with seasonal influenza outbreaks, the overall impact of the 2009 H1N1 pandemic was lower in adults ≥65 years of age, possibly due to the presence of protective cross-reactive antibodies developed through childhood exposure to early 20th century H1N1 viruses which shared antigenic similarity with the A(H1N1)pdm09 virus

[245]. The immune response to influenza infection is initiated through the engagement of the innate immune system. The IAV genome consists of negative sense, single-stranded ribonucleic acid (RNA) that is recognized by host pattern recognition receptors (PRRs). Many PRR ligands have previously been shown to improve the magnitude, duration, as well as breadth of neutralizing antibody responses [318, 383, 408].

Upon infection of host cells by IAV, viral RNAs (vRNA) are sensed by PRRs, such as toll-like receptor 7 (TLR7), retinoid acid inducible gene-I (RIG-I), and nucleotide-binding domain and leucine-rich-repeat-containing protein 3 (NLRP3), which forms multimolecular complexes termed 'inflammasomes' [54]. Activation of these pathways leads to downstream signaling through myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), or caspase 1 respectively. The subsequent cascade signal induces type I interferons (IFNα/β) and production of inflammatory ctytokines. Of these PRRs, TLR7 is not only important for the activation of the innate anti-viral response, but also for the induction of adaptive immunity [55, 355, 357, 409-411]. A study by Heer *et al.* showed that TLR7 signaling is critical for antibody isotype class-switching [355]. This could be due to B-cell intrinsic TLR7 signaling or indirect B-cell stimulation by extrinsic TLR7-dependent production of IFNα/β. Recently, we have shown that TLR7 signaling is involved in the recruitment of myeloid derived suppressor cells (MDSCs) and for the shaping of humoral immunity in response to IAV infection [409]. Data by Boeglin *et al.* later showed that a combination of B-cell receptor, CD40 and TLR7 stimulation on B cells augments antibody secreting cell (ASC) differentiation

[410]. Collectively, these data suggest that TLR7 signaling is important in adaptive immunity, particularly in the enhancement of B-cell responses.

In this study, we investigated the role of TLR7 in the long-term memory responses to IAV infection and vaccination. In the case of infection, we found that serum IgM levels and the frequency of IgM^+ ASCs in secondary lymphoid organs were reduced in the absence of TLR7. However, TLR7 signaling played a minimal role in the production of HA-specific antibodies. Conversely, TLR7 was critical during immunization with an inactivated split influenza vaccine. The absence of TLR7 signaling led to reduced germinal center (GC) formation, expression of B7 on B cells, IAV-specific ASCs, and serum antibody responses. When challenged, $TLR7^{-/-}$ mice had significantly higher lung viral titers compared to wild-type mice. The immunogenicity of the split vaccine was likely due to the presence of vRNA and its potential interaction with TLR7. Overall, we demonstrate that TLR7 plays an important role in the induction of immune responses to an IAV monovalent split vaccine.

Methods

Mice

C57Bl/6 (B6) mice were purchased from Charles River Laboratories (Wilmington, MA). $TLR7^{-/-}$ mice on B6 background were a gift from Akiko Iwasaki (Yale University, New Haven, CT) [363] and Regeneron Pharmaceuticals, Inc (Tarrytown, NY). $MyD88^{-/-}$ mice were also on the B6 background and a gift from Bruce Beutler (Scripps Research Institute, LaJolla, CA) [412]. Both strains were bred at Charles River Laboratories. Animals were age matched and housed

under pathogen-free conditions. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Influenza Viruses and vaccines

The mouse-adapted A/Puerto Rico/8/34 virus (PR8) and A(H1N1)pdm09 A/Mexico/4108/2009 (Mex4108), and A/California/08/2009 (Cal/08) viruses were propagated by allantoic inoculation of 10-day old embryonated chicken eggs. The 50% egg infectious dose (EID_{50}), 50% mouse infectious dose (MID_{50}), and 50% mouse lethal dose (MLD_{50}) were determined using methods described previously [388]. For PR8, 1 MID_{50} was equivalent to 10 EID_{50} and 0.01 MLD_{50}. For Mex4108, 1 plaque forming units (PFU) were equivalent to 28 EID_{50}. B6 or $TLR7^{-/-}$ mice were vaccinated intramuscularly (i.m.) in the hind leg with 10μg of A/California/07/2009 X-179A (Cal/07) monovalent split vaccine (Sanofi Pasteur, Swiftwater, PA) in a volume of 100μL. Viral genomic RNA components in the concentrated monovalent vaccine were inactivated by treatment with 20μg/mL of Ribonuclease A (RNase) (Quiagen, Valencia, CA) at 37°C for 30 min. The HA activity of the vaccines pre and post RNase treatment was verified using a hemagglutination assay and found HA activity to be equivalent with and without RNAse treatment. RNase-treated vaccine (RTV) and heated untreated vaccine were run on an 1.2% ethidium bromide e-gel (Life Technologies, Grand Island, NY) to check for the presence of RNA. RTV was then diluted to the equivalent concentration used for the non-inactivated vaccine inoculations, and administered as described above.

Infections of mice and harvesting tissues for flow cytometric analysis and viral titer quantification

Infection experiments were carried out as described previously [409]. Briefly, mice were infected intranasally (i.n.) with 25 MID_{50} of PR8 under anesthesia in a volume of 50 μL or mock infected with 50 μL of phosphate buffered saline (PBS). On the indicated days post-infection (p.i.), animals were sacrificed to harvest lung, spleen, bone marrow (BM) and sera. Single cell suspensions were stained with fluorochrome-conjugated antibodies for flow cytometric analysis, using an LSR-II (BD Biosciences, San Jose, CA). In some experiments, mice were challenged with 100 MLD_{50} of PR8 for recall responses or with $9.3x10^4$ EID_{50} Mex4108 as a challenge for vaccine studies. To determine viral titers, lungs were homogenized in 1mL of cold PBS. Clarified homogenates were titrated in 10–11 day old eggs to determine the EID_{50} viral titers in the lungs as previously described [389]. Numbers were converted to a log 10 scale to determine statistical significance.

IAV-specific antibody secreting cell ELISpot

Allantoic fluid clarified virus was inactivated using 5000 units of ultraviolet (UV) light. PVDF multiscreen 96-well plates (Millipore, Billerica, MA) were coated with either 5 μg/mL anti-mouse IgG or IgM antibodies (Southern Biotech, Birmingham, AL) or 1000 HA units (HAU) of UV-inactivated PR8 or Cal/08 virus overnight at 4°C and washed 3 times with PBS. The plates were then blocked for 1hr at 37°C with 200 μL/well of RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, and 0.01M Hepes buffer (cRPMI). Spleen, BM, and lung were harvested and a single cell

suspension was prepared in cRPMI after red blood cell lysis. Cells (10-15x10^6 cells/mL) were added onto IAV-coated plates and incubated for overnight at 37°C in a humidified atmosphere with 5% CO_2. The plates were washed 4 times with 0.5% Tween 20 in PBS (PBST) and incubated with biotinylated anti-mouse IgG or IgM antibodies (Southern Biotech, Birmingham, AL) for 1hr at room temperature. Plates were washed 4 times with PBST, and alkaline phosphatase-conjugated streptavidin (Vector, Burlingame, CA) in PBST was added for 1hr at room temperature. Plates were washed 4 times and spot development was achieved by adding 100 µL of Vector Blue alkaline phosphatase substrate kit III (Vector, Burlingame, CA) to each well. Spot forming units were counted using ImmunoSpot® (Cellular Technology Ltd., Cleveland, OH) and expressed as spots per 10^6 cells.

Hemagglutination Inhibition (HI)

Hemagglutination Inhibition (HI) assay serum samples were treated with receptor-destroying enzyme (RDE) (Denka Seiken Co., Tokyo) overnight at 37°C, followed by heat inactivation (56°C for 30 min). Serially diluted sera in V-bottom 96-well plates were tested in duplicate for their ability to inhibit the agglutination of 0.5% turkey red blood cells by 4 HAU of PR8 or Cal/08 virus in a standard HI assay as described previously [413]. Numbers were converted to a log 2 scale to determine statistical significance.

Antibody isotype determination by ELISA

An antibody ELISA assay was performed as described previously [409]. Briefly, 96 well, flat bottom plates were coated with 100 HAU/well of PR8, washed with PBST and then blocked with 5% bovine serum albumin (BSA). Mouse sera were

diluted 1:10. Absorbance was read at 450 nm. A standard curve of purified mouse IgM, IgG, IgG1, or IgG2c was used to convert absorbance readings to immunoglobulin concentration. Numbers were converted to a log 10 scale to determine statistical significance.

Antibodies for surface staining for flow cytomertic analysis

Single cell suspensions were stained with Fluorochrome-conjugated anti-mouse antibodies to measure B-cell markers, as well as antigen specific $CD8^+$ T cells. The following antibodies were used for B cells: GL7 (FITC), CD19 (APC-Cy7) purchased from BD Bioscience (San Jose, CA); CD80 (PE) purchased from BioLegend (San Diego, CA); and CD38 (Alexa Fluor 700), IgD (Pacific Blue) purchased from eBiosciences (San Diego, CA). The following antibodies were used for T cells: CD8 (Alexa Fluor 700), CD3 (APC) purchased from BD Bioscience, and PR8 nucleoprotein (NP)-tetramer (H-2Db ASNENMETM) (PE) was purchased from ProImmune (Sarasota, FL).

Intracellular cytokine staining

Splenocytes were seeded at 1 x 10^6 in 100µL of plain RPMI-1640 in 96-well round-bottomed plates and Cal/08 virus was added in a volume of 50 µL at 0.1 multiplicity of infection (MOI) in each well. After 1hr incubation with the virus, 50 µL RPMI-1640 containing 40% FBS and 400 U/mL Penicillin and 400 µg/mL Streptomycin solution was added to wells and plates were incubated for 5 days at 37°C in a humidified atmosphere with 5% CO_2. Cells were then collected and restimulated for 6hr with plate-bound anti-CD3 (10 µg/mL; clone 1452C11from eBioscience, San Diego, CA) and anti-CD28 (2 µg/ml, clone 37.51, eBioscience) in the presence of GolgiStop (BD Bioscience, San Jose, CA). Cells were surface

stained with CD4 (PE-Cy7) and CD8 (Alexa Fluor 700), purchased from eBioscience, for 15 min at 4°C. Cells were then made permeable with Cytofix/Cytoperm (BD Biosciences) followed by intracellular staining with IFNγ (PerCP-Cy5.5) purchased from BioLegend (San Diego, CA); TNFα (eFluor 450) purchased from eBioscience; IL-17 (Alexa 488), IL-4 (PE) and IL-10 (APC) purchased from BD Bioscience in perm/wash solution (BD Bioscience) for 30 min at 4°C. Cells were washed twice with perm/wash and resuspended in PBS/10%FBS. The samples were analyzed using LSRII Flowcytometer (BD Biosciences, Sam Jose, CA) and the cytometry data were analyzed using FlowJo software (Treestar, Ashland, OR).

RNA purification/sequencing

Viral ribonucleic acid (RNA) was purified from vaccine using the RNeasy Plus mini kit (QIAGEN, Valencia, CA). RNA was reverse transcribed and amplified with the following primers (F: 5'-AGC AAA AAG CAG GGT GAC AAG ACA-3' R: 5'-AGT AGA AAC AAG GGT GTT TTT TAT) using the Superscript One Step RT-PCR System with Platinum Taq (Life Technologies Corp., Carlsbad, CA.). Prior to sequencing, the PCR product was cleaned using Exosap-IT (Affymatrix, Santa Clara, CA.). A sequencing reaction was performed using a Big Dye Terminator v3.1 Cycle Seqencing kit and run on a 3730xl Sequencer (Life Technologies). Genetic analysis was performed using the program BioEdit Sequence Alignment Editor [414].

Statistical analysis

Statistical significance was determined by a two tailed, student's t-test measured against control B6 mice. Significant p-values were indicated. Error bars represent the standard error of the mean (SEM) unless otherwise mentioned.

Results

TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had reduced GC reactions and IAV-specific IgM^{+} ASCs following IAV infection.

We and others have previously shown that TLR7 is important in the induction of the adaptive immune response to IAV infection [355, 357, 409]. In this study, we investigated the long-term consequences of memory responses to IAV infection in the absence signaling by TLR7 or TLR7's downstream adaptor MyD88. B6, TLR7$^{-/-}$, MyD88$^{-/-}$ mice were infected with 25 MID_{50} PR8 or were mock infected with PBS. Primary B- and T-cell responses were then assessed at both 1 and 6 months p.i. Spleen, lung and BM samples were harvested at each time point. For primary B-cell responses, the frequency of GC B cells ($CD19^{+}GL7^{+}CD38^{-}$) and PR8-specific ASCs were measured in both spleen and lung. Both TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had at least a 2 fold reduction in the frequency of GC B cells in response to IAV infection as compared to control B6 mice, most strikingly in the spleen at 6 months p.i. with a greater than 3 fold reduction (Figure 3-1a).

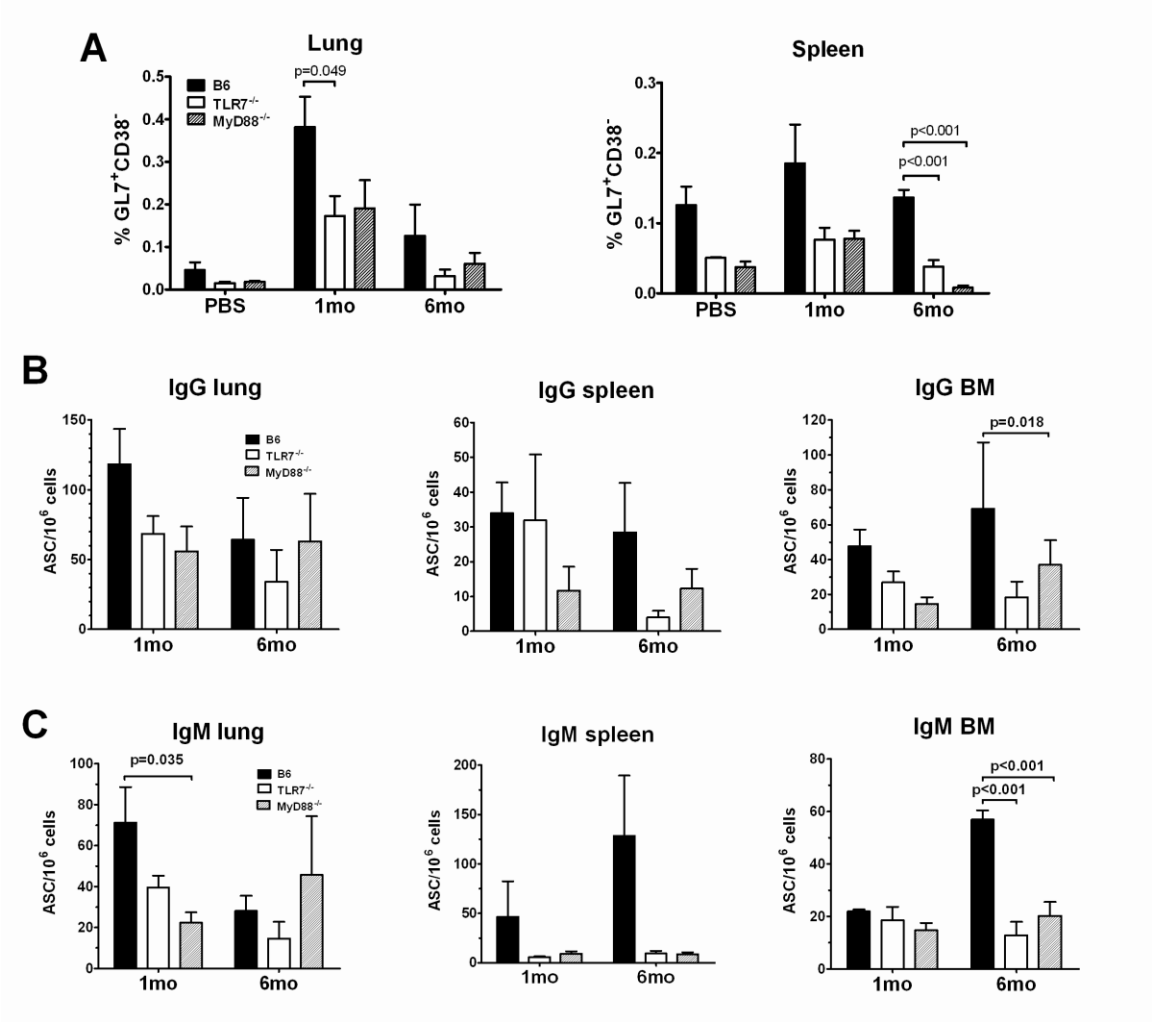

Figure 3-1: TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had fewer GC-B cells and PR8-specific IgM^{+} ASCs following primary infection.

Mice (n≥4) were infected with 25 MID_{50} of PR8 virus and spleen, lung and BM were harvested at the time points indicated. (A) B cells entering GC reactions were identified as CD19^{+}IgD^{-}GL7^{+}CD38^{-} in both lung and spleen. Data are shown as %GC-B cells out of total CD19^{+} cells gated. (B-C) PR8-specific IgG+ ASCs (B) or IgM^{+} ASCs (C) from lung, spleen and BM were measured by ELISpot assay against whole, UV-inactivated PR8 virus. Data are shown as number of PR8-specific spots per million cells plated.

Despite a reduced frequency of GC B cells in these tissues, the numbers of class-switched IgG^{+} PR8-specific ASCs in the lung and spleen were not

significantly decreased in either TLR7$^{-/-}$ or MyD88$^{-/-}$ mice (Figure 3-1b). However, there was a general reduction in the frequency of IgM^{+}, PR8-specific ASCs in these mice at the site of infection (the lung) as well as in memory lymphoid tissues (Figure 3-1c). A lack of TLR7/MyD88 signaling did not impact the memory CD8^{+} T cell response, as both TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had comparable frequencies of NP-specific CD8^{+} T cells in both lungs and spleen following infection (Figure 3-2). Collectively, these data indicate that a deficiency in TLR7 signaling results in a minor reduction in primary B-cell responses, but not in memory CD8^{+}T-cell responses following infection.

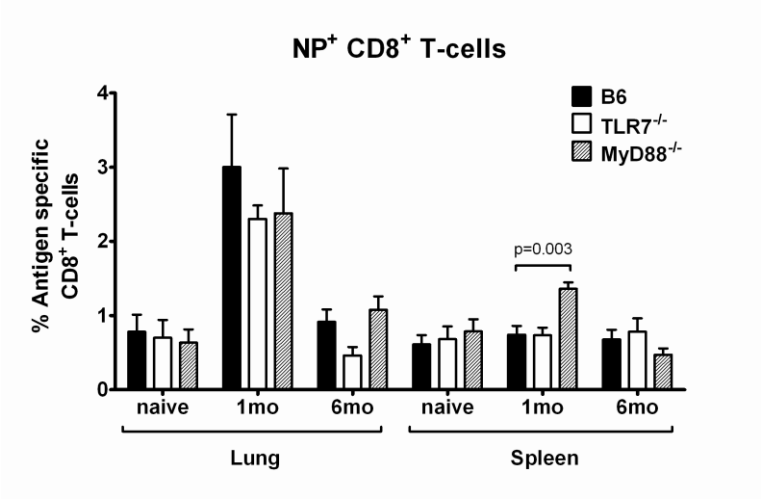

Figure 3-2: TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had comparable levels of PR8-specific CD8 T cells following IAV infection.

Mice (n≥4) were infected with 25 MID_{50} of PR8 virus and lungs and spleens were harvested at the time points indicated. NP tetramer-binding CD8^{+} T cells were analyzed by flow cytometry and shown as %PR8-specific CD8 T cells out of total CD8 T cells.

TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had reduced GC reactions and lower levels of IgM^{+} BM PR8-specific ASCs following a lethal challenge.

Our data suggest that following influenza virus infection, loss of innate recognition of IAV through TLR7 and its adaptor MyD88 only modestly affects the development of B-cell adaptive immune memory. Nonetheless, it remains possible that these molecules regulate the quality of long-term protective memory responses, thereby impairing the ability of a host to respond to future challenge. To test this idea, previously infected mice were challenged with a lethal dose (100 MLD_{50}) of homologous PR8 virus one or six months after the primary infection, and subsequently monitored for B- and T-cell recall responses 5 days following challenge. PR8 virus challenge resulted in a 2-fold increase in GC reactions in the lungs of B6 mice one month post-challenge and a 6-fold increase in the spleen at 6 months p.i., relative to the primary response. In contrast, no such recall response was observed in $TLR7^{-/-}$ or $MyD88^{-/-}$ mice at either time point (Figure 3-3a-f). Nonetheless, the frequencies of PR8-specific IgG^+ or IgM^+ ASCs from $TLR7^{-/-}$ mice were generally comparable to those from B6 mice in these tissues, except for a 5-fold decrease in IgG^+ ASCs in the spleen at 6 months post-challenge (Figure 3-3g-h). However, the frequency of PR8-specific ASCs, and particularly IgM^+ ASCs, was significantly reduced in the BM of both knockout mice; $TLR7^{-/-}$ and $MyD88^{-/-}$ mice exhibited a 7- and 11- fold reduction by 6 months compared to B6, respectively (Figure 3-3g-h). This result suggests reduced recruitment of memory B cells to the BM from either the spleen or lung due to impaired GC reactions in the knockout animals (Figure 3-3a-f). Consistent with the primary infection, the frequency of recall PR8-specific $CD8^+$ T cells and the levels of cytokines secreted by $CD4^+$ and $CD8^+$ T cells were comparable in lung and spleen either in the absence of presence of TLR7 or MyD88 signaling

(Figure 3-4). Collectively, these findings suggest that TLR7 signaling has a minimal role in the establishment of memory cells during IAV infection.

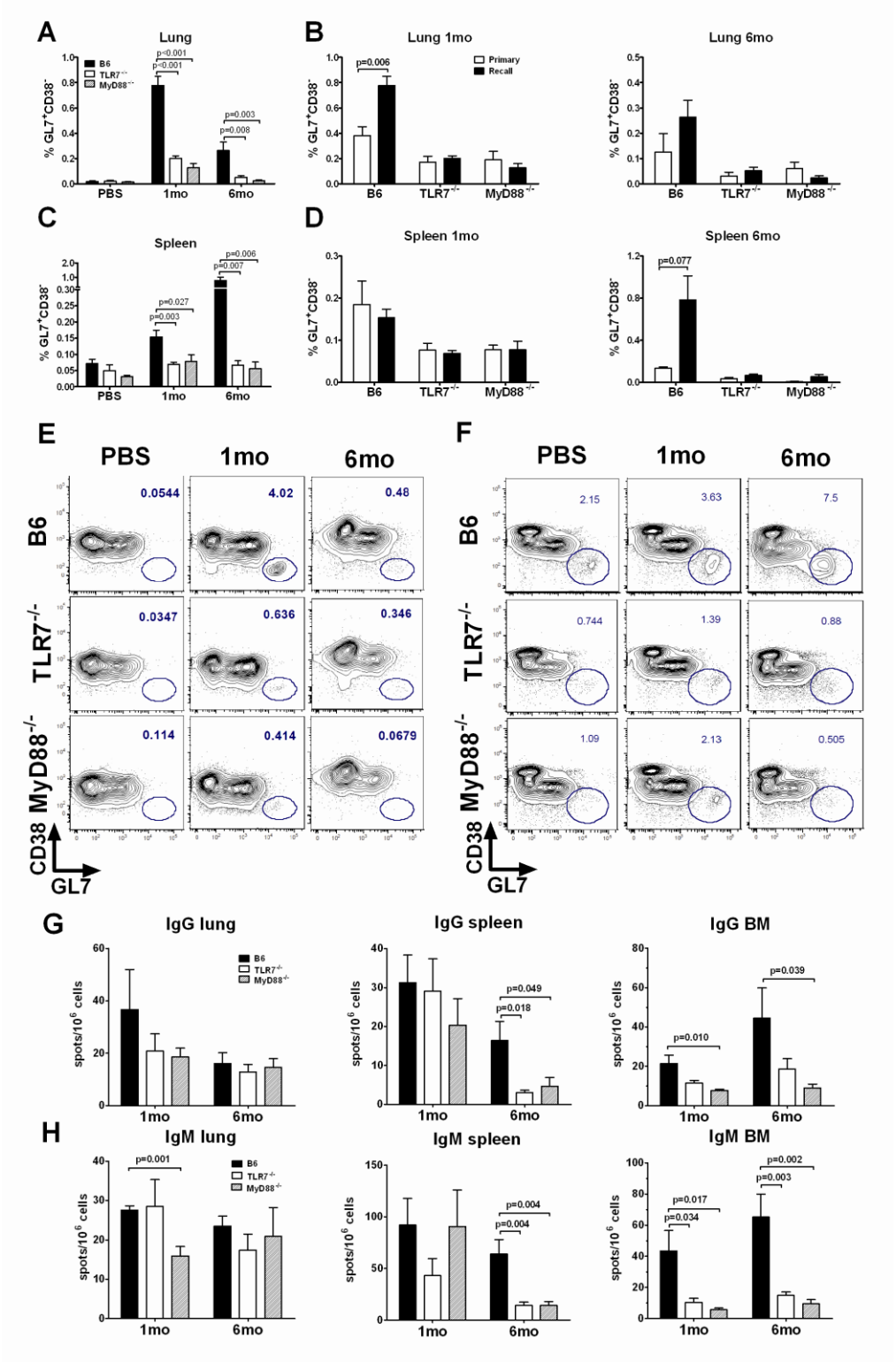

Figure 3-3: TLR7$^{-/-}$ mice had reduced GC reaction and lower frequencies of IgM^{+} BM PR8-specific ASCs following a lethal IAV challenge.

Mice (n≥6) were infected with 25 MID_{50} of PR8 then challenged at either 1 or 6 month following primary infection with 100 MLD_{50} of PR8 virus. Lung, spleen, and BM were harvested on day 5 post-challenge. (A-F) GC B cells ($CD19^{+}IgD^{-}GL7^{+}CD38^{-}$) in either lung or spleen were measured by flow cytometry. (B, D) GC-B cells from primary infection and post-challenge were shown at each time point. Data are shown as %GC B cells out of total $CD19^{+}$ cells gated. Representative dot plots of the recall responses of $CD19^{+}IgD^{-}$ cells are shown for both lung (E) and spleen (F). Numbers represent % of $CD19^{+}IgD^{-}$ cells. (G-H) PR8-specific IgG^{+} ASCs (G) or IgM^{+} ASCs (H) from lung, spleen and BM were measured by ELISpot assay against whole, UV-inactivated PR8 virus. Data are shown as number of PR8-specific spots per million cells plated.

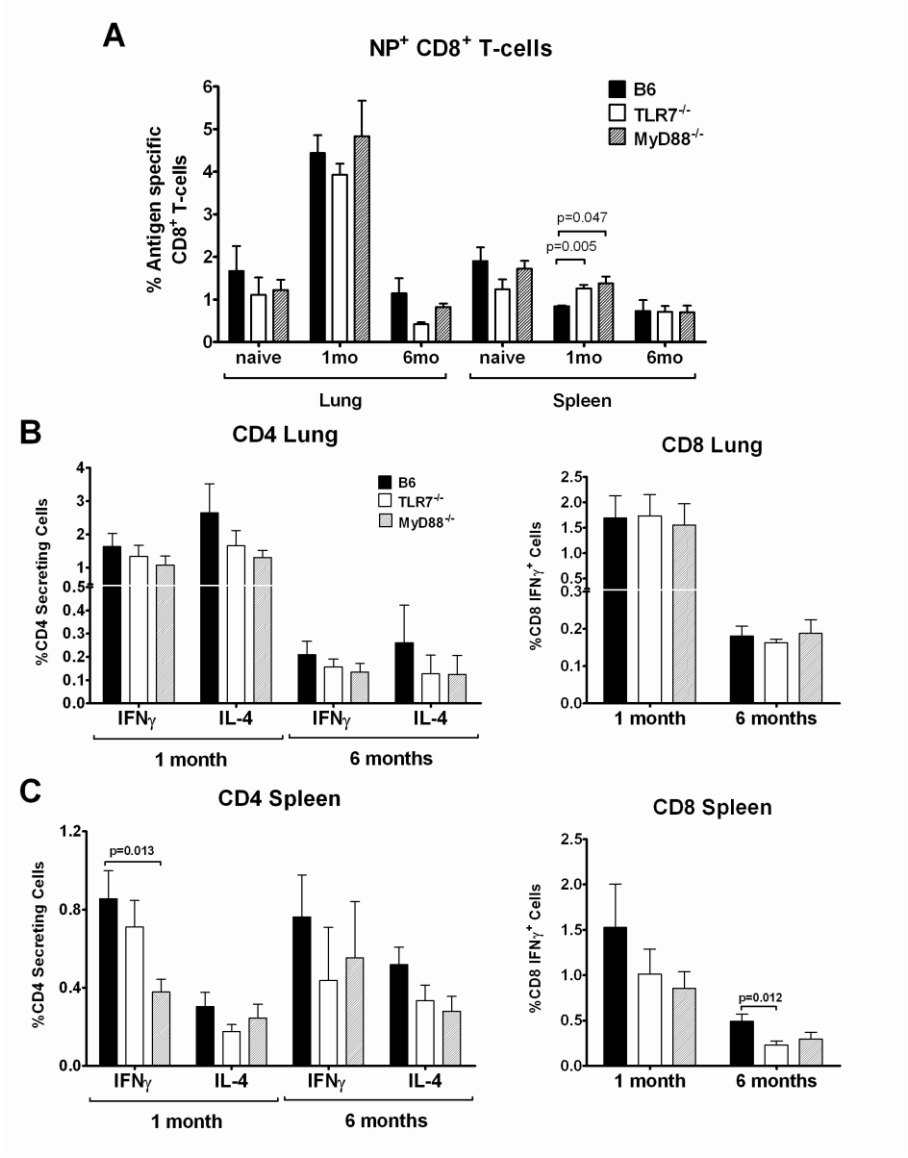

Figure 3-4: TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had comparable levels of PR8-specific and cytokine secreting T cells following challenge.

Mice (n≥4) were infected with 25 MID_{50} of PR8 virus and then challenged at either 1 or 6 months following primary infection with 100 MLD_{50} of PR8 virus. Lungs and spleens were harvested 5 days following challenge. (A) NP tetramer-binding CD8$^+$ T cells were analyzed by flow cytometry and shown as % PR8-specific CD8$^+$ T cells out of total CD8$^+$ T cells. (B-C) Cells were stained ex-vivo for cytokine production in both lung (B) and spleen (C). Data is shown as % CD4$^+$ or CD8$^+$ T cell secreting the cytokine indicated.

TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had lower levels of serum PR8-specific IgM, yet comparable IgG and HI titers following IAV infection.

HA-specific serum antibodies are the major immune correlate of protection against IAV infection. Since both TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had a generally reduced memory B-cell response (Figure 3-1,Figure 3-3), we wanted to determine if the virus-specific circulating antibodies were also reduced following infection in these mice. Sera were collected from the three groups of mice at 1, 3, and 6 months following primary infection and tested for the presence of PR8-specific antibodies by ELISA and HI assay (Figure 3-5). Consistent with the observed differences in frequency of ASCs between control B6 and knockout mice (Figure 3-1,Figure 3-3), the levels of PR8-specific IgM antibodies were higher in B6 mice than those in either TLR7$^{-/-}$ or MyD88$^{-/-}$ mice, with IgM responses in TLR7$^{-/-}$ mice being significantly reduced at 3 and 6 months p.i. (Figure 3-5a), In contrast, IgG antibodies were comparable in the early time points, with a modest reduction by 6 months p.i. (Figure 3-5b). When IgG subclasses were measured, both knockout mice had elevated levels of PR8-specific IgG1 with reduced levels of IgG2c (Figure 3-5c,d), indicating that the antibody responses in these mice was polarized towards a T_h2 response as demonstrated previously [355, 357, 409].

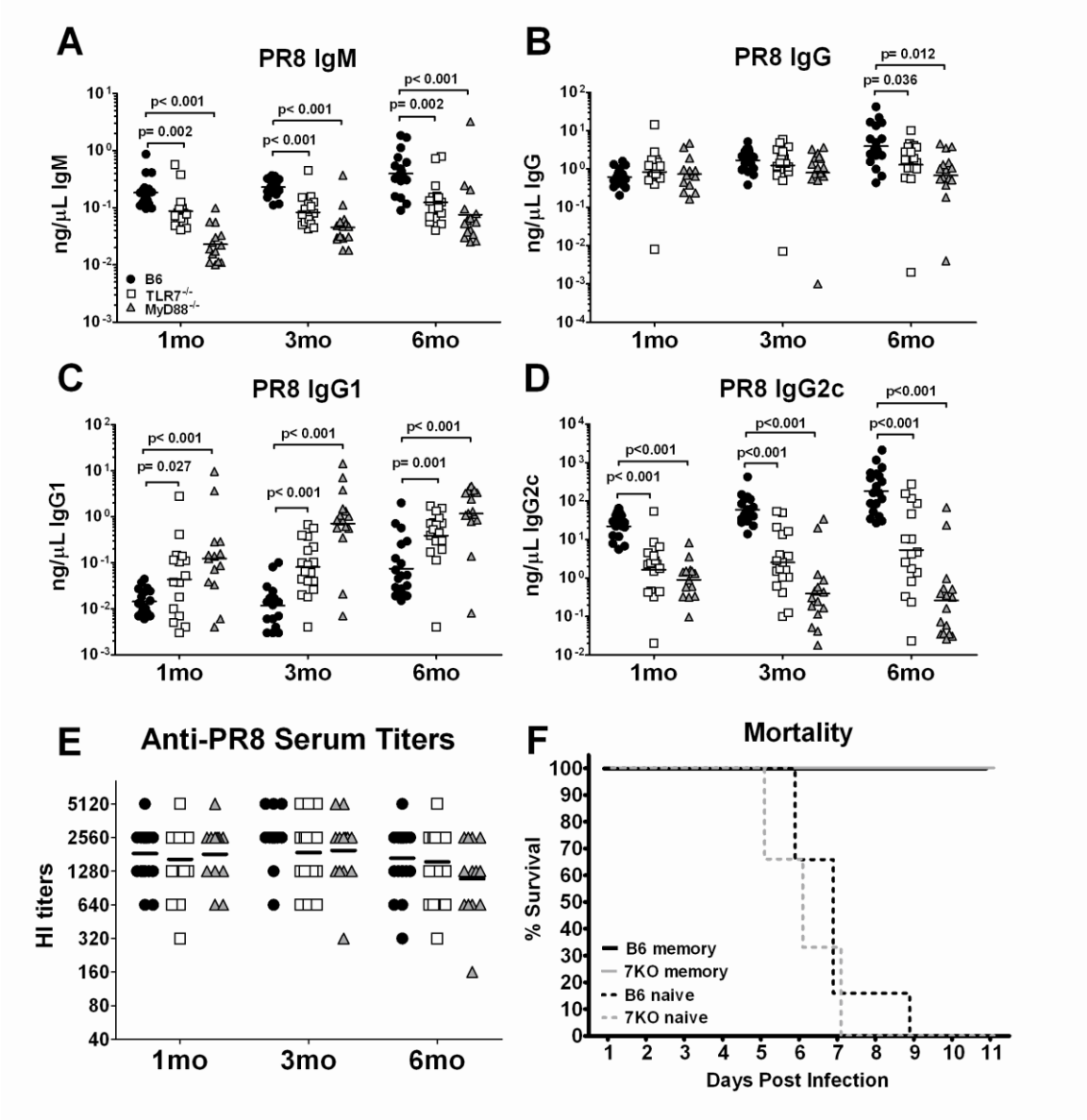
A
PR8 IgM
ng/μL IgM
B6
TLR7-/-
MyD88-/-
p< 0.001
p= 0.002
p< 0.001
p< 0.001
p= 0.002
p< 0.001
1mo
3mo
6mo
B
PR8 IgG
ng/μL IgG
p= 0.012
p= 0.036
1mo
3mo
6mo
C
PR8 IgG1
ng/μL IgG1
p< 0.001
p= 0.027
p< 0.001
p< 0.001
p= 0.001
p< 0.001
1mo
3mo
6mo
D
PR8 IgG2c
ng/μL IgG2c
p<0.001
p< 0.001
p<0.001
p<0.001
p<0.001
p<0.001
1mo
3mo
6mo
E
Anti-PR8 Serum Titers
HI titers
5120
2560
1280
640
320
160
80
40
1mo
3mo
6mo
F
Mortality
% Survival
B6 memory
7KO memory
B6 naive
7KO naive
Days Post Infection

Figure 3-5: TLR7$^{-/-}$ and MyD88$^{-/-}$ mice had lower levels of circulating IAV-specific IgM, yet comparable levels of IAV-specific IgG and HI titers following IAV infection.

Mice (n≥15) were infected with 25 MID_{50} of PR8 virus and sera were collected at 1, 3, and 6 months following infection. (A-D) The levels of circulating PR8-specfic IgM, IgG, IgG1 and IgG2c were measured by ELISA using whole, UV-inactivated PR8 virus. Each mouse represents one value, and average is the geometric mean. (E) Sera were treated with RDE and HI titers were measured by HI assay. Shown is each individual mouse with the geometric mean. (F) Six months post primary infection or PBS control B6 and TLR7$^{-/-}$ mice (n=10) were challenged with 500 mLD50 PR8 and monitored for mortality for 14 days. Mice were euthanized after losing 25% of their original body weight.

Despite the reduction of PR8-specific IgM levels and evidence for T_h2 skewed responses, HI titers in TLR7$^{-/-}$ and MyD88$^{-/-}$ mice were comparable to those from control B6 mice at all time-points measured following infection (Figure 3-5e). Upon lethal challenge, all groups of mice were protected, showing no lethality or weight loss; whereas control mice succumbed to infection between 6 and 9 days post-infection (Figure 3-5f). This demonstrates that the signaling pathway through TLR7/MyD88 is dispensable for protective antibody responses following primary IAV infection, although the composition of this antibody response is different from control B6 mice. Collectively, these data suggest that although TLR7/MyD88 signaling regulates certain aspects of the memory B-cell response, it plays a dispensable role in the protective humoral response against IAV infection.

The B-cell response to pandemic split vaccine was compromised in TLR7$^{-/-}$ mice.

Since TLR7 signaling was not critical for protective immunity following infection (Figure 3-1Figure 3-3Figure 3-5), we next investigated whether TLR7 signaling affects the outcome of immunization with influenza vaccine. B6 and $TLR7^{-/-}$ mice were immunized i.m. with 10 μg of monvalent A(H1N1)pdm09 Cal/07 split vaccine. Sera were collected 15 days following vaccination to measure early HI titers. B6 mice developed measurable HI titers (~80) by day 15; however, $TLR7^{-/-}$ mice had undetectable levels of HI antibody (Figure 3-6a). Also decreased were the levels of B cells entering GC reactions; $TLR7^{-/-}$ mice had fewer GC B cells than B6 mice on day 10 post-immunization (Figure 3-6b). $TLR7^{-/-}$ B cells also exhibited a modest reduction in expression of the costimulatory molecule CD80 on their surface compared with B6 B cells, both in B cells that were actively part of a GC ($GL7^{+}CD38^{-}$) as well as those B cells outside of the GC ($CD38^{+}$) (Figure 3-6c). There was no significant difference in the frequencies of cytokine producing $CD4^{+}$T cells and only a modest reduction of $CD8^{+}$ TNFα-producing T cells ($p=0.048$) in in the absence of TLR7 signaling (Figure 3-7). Therefore, these results suggest that TLR7 plays a key role in early B-cell activation and antibody production in the context of immunization with split vaccine.

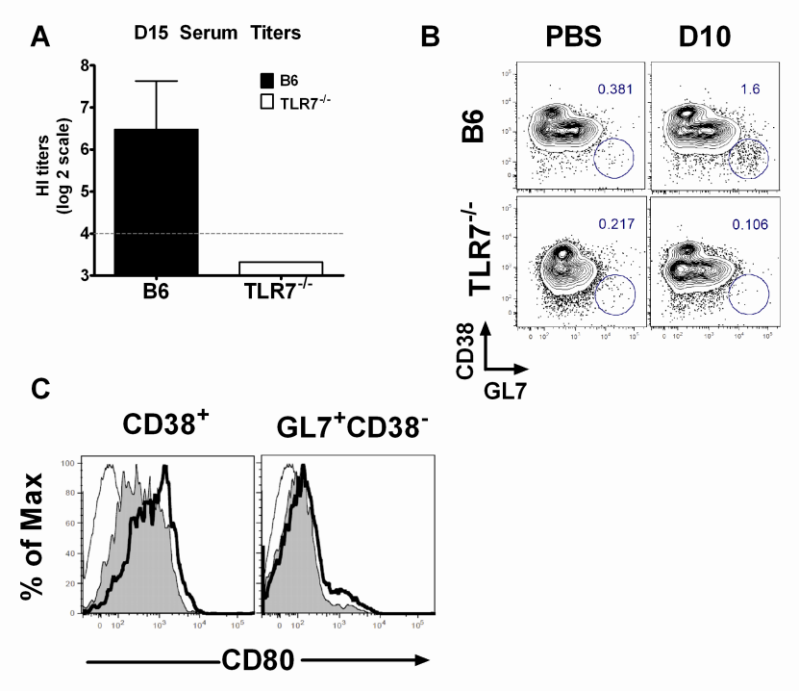

Figure 3-6: The B-cell response to pandemic split vaccine was compromised in TLR7$^{-/-}$ mice.

Mice (n=5) were vaccinated i.m. with 10µg pandemic 2009 Cal/07 split vaccine. (A) Sera were collected on day 15 following vaccination, RDE treated, and measured for HI titers against Cal/08 virus. Dashed line represents lower limit of assay detection. Shown is the geometric mean with a 95% confidence interval. (B) A representative dot plot of CD19^{+}IgD^{-} GC-B cells (GL7^{+}CD38^{-}) from each group of mice is shown. (C) A representative histogram of CD80 expression pattern from GC-B cells (GL7^{+}CD38^{-}) or non GC-B cells (CD38^{+}) is shown. The Isotype control is the thin line; TLR7$^{-/-}$ response is the grey filled line; B6 response is the thick black line.

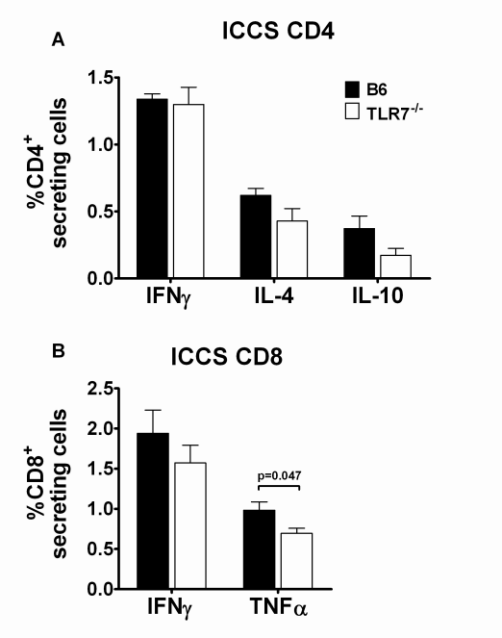

Figure 3-7: The T-cell response in vaccinated mice was comparable following vaccination in the presence or absence of TLR7 signaling.
Mice (n=5) were vaccinated i.m. with pandemic 2009 Cal/07 split vaccine. Spleens were harvested 10 days following vaccination. Collected splenocytes were infected for 1hr with Cal/08 virus at 0.1 MOI and incubated for 5 days. Intracellular cytokine production from $CD4^+$ T cells (A) or $CD8^+$ T cells (B) was measured by flow cytometry.

The vRNA component of the split vaccine contributes to the vaccine's immunogenicity.

Our findings that the primary B-cell response was reduced in $TLR7^{-/-}$ mice indicate that TLR7 signaling is important for an optimal response to inactivated split influenza vaccine. It also suggests that the pandemic split vaccine may contain TLR7 ligand(s) that potentiate the immunogenicity of this vaccine. To determine if the pandemic split vaccine contained RNA, the Cal/07 vaccine was treated with RNase or left untreated and then purified RNAs were run on an ethidium bromide gel. Indeed, untreated Cal/07 split vaccine contained

detectable amounts of RNA, whereas RNase-treated vaccine was devoid of RNA (Figure 3-8). Sequencing of the vRNA isolated from untreated vaccine confirmed the origin of the RNA to be from PR8 virus, the parental high growth donor used to produce influenza vaccine reassortant viruses.

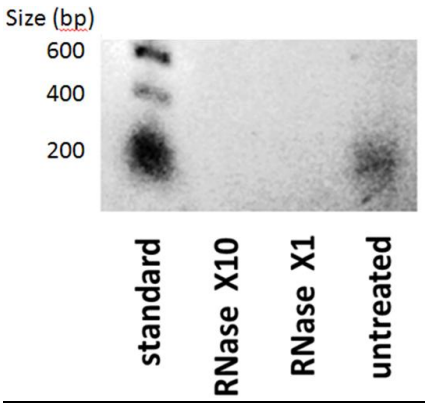

Figure 3-8: The A(H1N1)pdm09 Split vaccine contains viral genomic RNA.
Vaccine was treated with various concentrations of RNase (10x = 100µg, 1x = 10µg) or left untreated under the same conditions. RNA was then purified and concentrated from vaccine using a QIAGEN kit. Samples were then run on an Ethidium Bromide gel.

Next, we asked whether this RNA was responsible for the immunogenicity of the Cal/07 split vaccine. The B-cell response in B6 and TLR7$^{-/-}$ mice immunized i.m. with Cal/07 vaccine were compared with those of B6 mice immunized with RNase-treated vaccine (RTV). Spleen and BM were harvested at 1 month following immunization and the frequency of Cal/08-specific ASCs was determined (Figure 3-9). TLR7$^{-/-}$ mice had reduced numbers of Cal/08-specific, IgG^{+} and IgM^{+} ASCs in both spleen and BM (Figure 3-9a,b) with the most significant reduction being IgM^{+} ASCs in the BM. In the case of RTV immunized mice, the BM, but not the spleen, exhibited reduced numbers of ACSs similar to those detected in TLR7$^{-/-}$ mice immunized with non-RNAse treated vaccine (Figure 3-9a,b). These results suggest that the RNA component of the split vaccine is responsible for the immunogenicity of the vaccine and the host memory B cell response to vaccine.

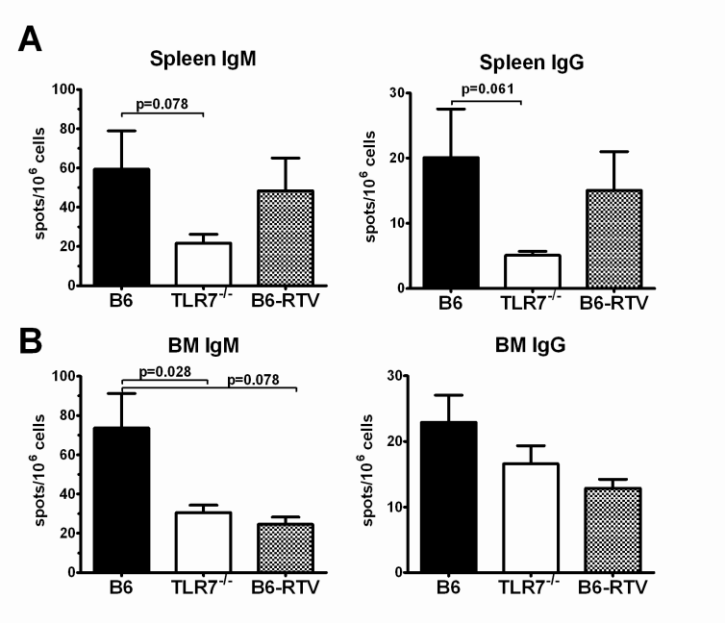

Figure 3-9: TLR7 recognition contributed to the B cell response to split influenza vaccine.

B6 and TLR7$^{-/-}$ mice (n=10) were immunized i.m. with 10μg Cal/07 split vaccine as well as a control group of B6 mice immunized with RNase-treated Cal/07 vaccine (RTV) (n=5). Spleen (A) and BM (B) were harvested at 1 month post-vaccination and Cal/08-specific IgG+ or IgM^{+} ASCs were measured by ELISpot assay against whole, UV-inactivated Cal/08 virus. Data are shown as number of spots from Cal/08 specific ASCs per million cells plated.

TLR7$^{-/-}$ mice failed to develop protective immunity following immunization.

Since an optimal B-cell response to split vaccine was dependent on TLR7 signaling, we next tested whether TLR7 was critical for the development of protective humoral immunity. B6 and TLR7$^{-/-}$ mice were immunized with the Cal/07 split vaccine and compared with B6 mice immunized with RTV. Sera were collected 1 month later and HI titers were measured (Figure 3-10a). Consistent with the reduced frequency of Cal/08-specific ASCs (Figure 3-9), the HI titers

were significantly reduced in TLR7$^{-/-}$ mice immunized with untreated Cal/07 vaccine and undetectable in B6 mice immunized with RTV (Figure 3-10a). Consequently, upon challenge with 2x10^5 MID_{50} of Mex4108 A(H1N1)pdm09, TLR7$^{-/-}$ mice had 30-fold higher lung viral titers at day 4 following challenge compared with those in B6 mice (Figure 3-10b). RTV immunized mice were equally unprotected from challenge. These data indicate that TLR7 signaling regulates the development of humoral immunity following split vaccine immunization. Additionally, the presence of a TLR7 ligand in the vaccine is critical for protective vaccine efficacy

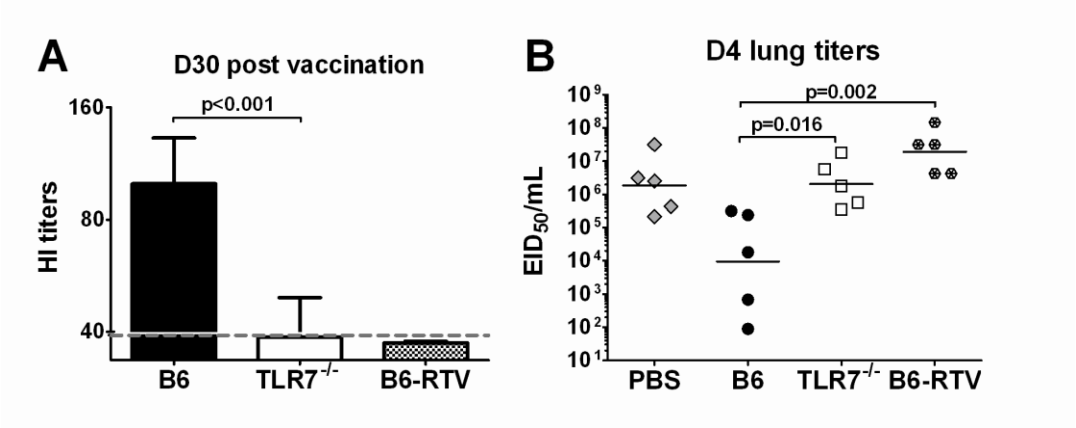

Figure 3-10: TLR7$^{-/-}$ mice failed to develop protective immunity following immunization.

B6 and TLR7$^{-/-}$ mice were immunized i.m. with 10μg Cal/07 split vaccine as well as a control group of B6 mice immunized with RNase-treated Cal/07 vaccine (RTV). (A) Sera were collected at 1 month post-vaccination for each group tested (n=25), RDE treated, and measured for HI titers against Cal/08 virus. Shown are the geometric means of the titers collected with a 95% confidence interval. The grey dashed line represents lower limit of assay detection. (B) After 1 month following immunization, mice (n=5) from all groups vaccinated were challenged with $9.3x10^4$ EID_{50} Mex4108. A control group of B6 mice received PBS only and then challenged. Lungs were harvested at day 4 post-challenge and lung viral titers were measured in eggs. Shown is each individual titer with the geometric means of the titers collected.

Discussion

Engaging innate PRRs is an important step in eliciting an effective adaptive response against IAV. Since TLR7 recognizes IAV genomic RNA, delineating the impact of TLR7 signaling on the innate and adaptive response is key to our understanding of the overall immune response to influenza infection and vaccination. Recently, we reported that deficient TLR7 signaling increased MDSC recruitment to the lungs and polarized the acute adaptive response towards T_h2 following IAV infection [409]. In the present study, we hypothesized that TLR7 signaling would enhance the long-term memory response to IAV. We tested this hypothesis both in the case of infection and immunization. In our infection model, we found that a deficiency in TLR7 or MyD88 signaling reduced certain aspects of B-cell memory (Figure 3-1Figure 3-3Figure 3-5). However, HI titers following primary infection were comparable and mice were equally well protected from a lethal challenge (Figure 3-5e). These data indicate that TLR7

signaling is dispensable for the production of protective antibodies following IAV infection. In contrast, TLR7 signaling during immunization contributed to the development of a robust HI antibody response to the A(H1N1)pdm09 split vaccine and optimal clearance of challenge virus which appeared to be dependent on the presence of RNA in the vaccine (Figure 3-10). Together, our data demonstrate that TLR7 signaling is dispensable for the establishment of memory to IAV infection, but is important for humoral responses following vaccination with split vaccine.

The differential role of TLR7 during infection vs. immunization indicates that the context by which vRNA is recognized by host PRRs may be different in the two circumstances. In the context of infection, upon entry to host cell through endocytosis, vRNA is released from the late endosome following fusion of viral and host membranes [415]. Throughout IAV's lifecycle, vRNA can be recognized by at least 3 different PRRs: TLR7 in late endosomes, as well as RIG-I and NOD-like receptors (NLRs) in the cytosol [55]. These innate immune receptors initiate antigen-specific adaptive immune response by the induction of IFNα/β and pro-inflammatory cytokines. Thus, it is possible that in the absence of TLR7, the collective signaling through other PRRs is sufficient to facilitate the induction of adaptive immunity during virus infection [357, 358, 386, 416]. In the context of immunization with split inactivated influenza vaccine, the vaccine components are confined to the MHC class II endocytic pathway. Therefore, endosomal TLR7 is the only PRR that can activate innate signals during vaccination.

There are three types of influenza vaccines licensed in the U.S. today, the live attenuated influenza vaccine (LAIV) and the split and subunit trivalent

inactivated vaccines (TIVs) [292-294]. TIV remains the most widely administered influenza vaccine, but its immunogenicity is reduced in young children and older adults [318, 319, 406]. Formulation of TIV with adjuvants is one strategy to improve vaccine immunogenicity, but adjuvanted influenza vaccines are not licensed in the U.S. Since vRNA is a natural ligand for TLR7, having these innate signals in TIVs may improve their immunogenicity. Indeed the antibody responses of B6 mice immunized with the A(H1N1)pdm09 split vaccine was dependent on RNA (Figure 3-8), as RNase treatment abrogated the immunogenicity of this vaccine (Figure 3-10), suggesting that the presence of vRNA is important for the immunogenicity of the split vaccine.

Our findings highlight the important role of TLR7 signaling in vaccine design. In support of this idea, other studies have shown that TLR7 is important for the induction of a protective memory response against influenza whole-inactivated vaccine [357, 405, 411], while split vaccines lacking intact RNAs are defective in inducing protective antibody response [411]. In addition, a study by Co *et al.* showed that a seasonal split vaccine activated human T cells more efficiently than a subunit vaccine, which contains only highly purified surface antigens HA and neuraminidase (NA) [417]. In a separate study, Kasturi *et al.* showed that purified HA alone did not elicit virus-neutralizing antibody titers, whereas the addition of a TLR7 ligand to HA increased antibody production in mice [383]. The additions of synthetic TLR7 agonist to subunit, purified protein, or mRNA-based vaccines were shown to enhance the immunogenicity of vaccines against a variety of targets [418-422]. Collectively, these studies show that the

presence of vRNA that engage TLR7 signaling contributes to the immunogenicity of commercially available TIVs.

Induction of protective, durable antibody responses is a desirable outcome of vaccination. Protective antibodies are generally considered to be the products of class-switched, highly specific IgG producing ASCs and memory B cells generated from GCs. However, growing evidence suggests that IgM producing memory B cells and ASCs also actively participate in adaptive immune responses [423, 424]. After infection with IAV, we found that TLR7$^{-/-}$ mice had reduced levels of circulating serum IgM (Figure 3-5a) and IgM^{+} ASCs, particularly in the long-term memory compartment of the BM (Figure 3-1Figure 3-3c). This IgM^{+} ASC deficiency was even more pronounced following immunization with split influenza vaccine (Figure 3-9). In addition, the average spot size of a TLR7$^{-/-}$ IgM^{+} secreting ASCs was significantly reduced (data not shown). Other groups similarly have reported that IgM production is reduced in the absence of TLR7/MyD88 signaling [92, 194, 425, 426]. A study by Fink *et al.* showed that TLR7$^{-/-}$ mice produced lower levels of IgM, but not IgG, neutralizing antibodies against vesicular stomatitis virus (VSV) [92]. They also found that TLR7$^{-/-}$ VSV-specific B cells did not expand nor differentiate as well as the controls. Additionally, Kang *et al.* demonstrated that MyD88$^{-/-}$ mice produced lower levels of circulating IgM and fewer IgM^{+} ASCs in the spleen and BM following influenza virus like particle vaccination [359]. The association between TLR7 signaling and IgM production is also apparent in human B-cell responses [370, 374, 427]. The precise long-term consequence of reduced IgM production as a result of TLR7 deficiency remains unclear. A recent study by Pape *et al.* showed that IgM^{+}

memory B cells survive longer than class-switched memory B cells [424]. Thus, it is possible that TLR7-mediated IgM memory cell formation/maintenance is critical after the humoral memory provided by IgG wanes. Alternatively, since IgM antibodies are considered as an early adaptive defense due to their polyreactivity, TLR7 signaling in IgM production may be important in limiting viral spread at an early phase of infection [92, 428-430].

It remains unclear if the impaired B cell response of TLR7$^{-/-}$ mice in response to vaccination is due to B-cell intrinsic or extrinsic effects. However, growing evidences suggest that TLR7 signaling directly on B cells contributes to enhanced antibody response. A study by Kasturi *et al.* showed that TLR7 expression by B cells is required for an effective antibody response against an adjuvanted ovalbumin antigen [383]. In addition, Hou *et al.* showed that intrinsic TLR7 signaling on B cells, and less so in DCs, is important for antiviral antibody responses [197]. Later, Browne, using conditional knock-out mice demonstrated that intrinsic MyD88 expression by B cells controlled GC formation as well as an animal's ability to control a retroviral infection [375]. MyD88 expression by DCs was also important for viral control, but not to the extent of its expression by B cells. Thus, intrinsic TLR signaling on B cells may augment the signaling through B-cell receptor and T-cell help to stimulate B-cell proliferation and differentiation as previously suggested [7, 431].

Our data demonstrate that TLR7 signaling is particularly important for the immunogenicity of some currently available TIVs. We propose that engaging TLR7 in the context of vaccination is one strategy to improve immunogenicity of

influenza vaccines, and that this could be especially beneficial in populations for which the immunogenicity of influenza vaccine is reduced.

Acknowledgements:

We would like to thank Kortney Gustin and Steve Lindstrom for the use of their reagents for this study. We also greatly appreciate Sanofi Pasteur for allowing us the use of their A/California/07/2009 X-179A (H1N1) monovalent split vaccine. VJS received a post-graduate stipend form the Oak Ridge Institute for Science and Education.

Chapter 4 : Discussion

Influenza and its complications remains a major public health burden. Currently, annual vaccination is the most effective strategy for mitigating seasonal and pandemic influenza related disease and death. Unfortunately, populations that suffer the most severe consequences of influenza infection are also those that respond poorly to influenza vaccines. Therefore, development of improved seasonal influenza vaccines strategies are needed to optimally protect individuals of all ages and risk groups. Although the world recently experienced the 2009 H1N1 pandemic, the threat of another pandemic virus arising from animal sources has not diminished. Recently, a new zoonotic reassortant virus capable of transmitting in the ferret model has emerged from swine. The H3N2 variant (H3N2v) virus has acquired the M gene from the A(H1N1)pdm09 virus and has caused human disease in 13 individuals (12 of which were children) in the U.S. since July 2011 [325, 432-434]. Highly pathogenic H5N1 viruses are endemic in several Asian and African countries, although these viruses lack the ability to transmit efficiently among people [435]; however, Imai *et al.* have recently generated a reassortant H5N1 virus capable of efficient transmission in the ferret model [436]. These findings highlight the pandemic potential of influenza viruses and the need to develop immunogenic, dose-sparing vaccine against these public health threats. The goal of my work was to better understand the role of TLR7 in the development of innate and adaptive immune responses to influenza virus infection in order to improve vaccines for both seasonal and pandemic influenza.

My specific aims sought to identify the consequences of TLR7 signaling for IAV infection and vaccination. Our data confirmed earlier findings that TLR7 signaling was involved in the Th1/Th2 polarization of the response to primary IAV infection (Figure 2-7Figure 2-10) [355, 357, 359, 409]. TLR7$^{-/-}$ mice also had large numbers of MDSCs recruited to the site of infection 7 days p.i. (Figure 2-3). The overabundance of MDSCs secreting T_h2 polarizing factors at the site of infection may be affecting the polarization of the DCs bringing antigen to the draining LNs [437]. Such an environment could induce the Th2 bias of the resulting adaptive immune response (Figure 2-7Figure 2-10). The recruitment of MDSCs to sites of malignancy has been well studied in mice and humans and is known to be responsible for Th2 polarization [166, 170, 171, 400, 438-443]. When MDSCs are removed from an animal, existing tumors are eliminated and anti-tumor vaccine efficacy is improved [166, 444-446], although this phenomenon is not restricted to malignancy. A deleterious role for MDSCs has been associated with exacerbating complications from sepsis and in the maintenance of chronic viral infection in both mice and humans [162, 169, 394, 447-449]. However, the involvement of MDSCs in TLR7 regulation of IAV infection had not been demonstrated prior to our work. In the context of IAV infection, we demonstrated that increased numbers of MDSCs at the site of infection corresponds with the increased morbidity seen in virus infected TLR7$^{-/-}$ mice (Figure 2-1**Figure** 2-3). Inflammation is initiated during an IAV infection and MDSCs are recruited. According to our model, MDSCs cannot sense viral RNA at the site of infection without TLR7 signaling and cannot recognize inflammation that is occurring in the context of an ongoing infection, leading to

increased recruitment of MDSCs to suppress the perceived inappropriate inflammation (Figure 2-11). Hence, it is important for the host to control the accumulation of MDSCs during infection, as an excess of these cells can lead to an unbalanced immune response with exacerbated disease.

In our study, all cells of the TLR7$^{-/-}$ mice lacked the ability to utilize TLR7. We could not identify the specific cell type that induces MDSC recruitment to the site of infection in our model. Bone marrow (BM) chimeras and adoptive transfer experiments could be employed to identify whether lung epithelial or hemopoietic cells are responsible for MDSC accumulation. Initially, one could lethally irradiate and reconstitute a WT mouse with BM from a TLR7$^{-/-}$ mouse to identify if TLR7 signaling is required by the hematopoietic or the non-hematopoietic cells. To further identify the specific cell type responsible, one could adaptively transfer CFSE-labeled TLR7$^{-/-}$ CD11b^{+} cells into a WT mouse. If relatively larger numbers of transferred TLR7$^{-/-}$ myeloid cells were recruited to the lungs of WT mice, one could conclude that TLR7 signaling intrinsic to the MDSC was essential for their accumulation.

The second goal of the study was to investigate the potential consequences of T_h1/T_h2 polarization for the memory response to IAV. In our initial study, we observed a T_h2 biased humoral immune response to primary IAV infection; evidence indicates that simply altering the T_h1/T_h2 balance in response to antigen determines the quality of the memory response induced [450]. Certain groups, including pregnant women, asthmatics and obese individuals, had increased complications during the 2009 pandemic from infection with A(H1N1)pdm09 [197, 451-453]. A common trait within each of these risk groups was an increased

T_h2-tropic environment inherent to their medical condition [454-458]. In this context, we hypothesized that programming a memory response with a T_h2 bias during the primary infection would be deleterious to the host during a secondary IAV challenge.

To better understand the contribution of TLR7 induced T_h1/T_h2 polarization, we monitored WT and TLR7$^{-/-}$ mice for 6 months p.i. to identify variations in the long-term memory response to IAV infection. We determined that the humoral Th2 bias in TLR7$^{-/-}$ mice was maintained during the duration of the study with no significant difference in the memory T cells between the two groups (Figure 3-2Figure 3-4). Despite certain aspects of B-cell memory being different in TLR7$^{-/-}$ compared to WT mice (Figure 3-1Figure 3-3Figure 3-5), this polarization did not translate into reduced long-term protective humoral immune response to homologous challenge 6 months p.i. (Figure 3-5f). A similar observation was made by Seo *et al.* in the MyD88$^{-/-}$ model, although they only monitored their mice up to 4 weeks post-primary infection [358]. My data suggest that the levels of IgG memory B cells in the KO mice were sufficient to produce the antibodies necessary for protection from a homologous viral challenge; hence TLR7 is dispensable for the establishment of an effective long-term memory response against IAV infection.

The major difference we observed between the immunological memory post-IAV infection was that TLR7$^{-/-}$ mice had fewer IgM memory B cells and fewer germinal center (GC) reactions compared with B6 mice (Figure 3-1Figure 3-3Figure 3-5). Two independent studies have provided evidence that murine IgM memory cells are the source of B cells re-entering GC reactions after a

secondary challenge to antigen, whereas IgG memory B cells can only act as effector antibody secreting cells (ASC) [424, 459]. It is possible that the inability to induce GC recall responses by TLR7$^{-/-}$ mice is a result of their low levels of IgM memory B cells. A study by Kopf *et al.* demonstrated that IgM$^{-/-}$ mice have increased viral titers post-primary IAV infection compared to WT mice [423]. Their work suggests natural IgM provides a level of protection to naïve mice from IAV and implies IgM is more broadly reactive than IgG. In addition, the presence of long-lived IgM memory B cells has been identified, and could be the source of long-term memory B-cell maintenance [191, 424, 459]. Taking these studies into consideration, it is possible that TLR7$^{-/-}$ mice infected with PR8 virus would be more susceptible to a secondary infection with a drift variant IAV because of their IgM memory B-cell deficiency. For future studies, it would be important to investigate the role of TLR7-mediated IgM memory in response to a secondary challenge from a drift variant virus, as these are the type of viruses responsible for annual influenza epidemics.

Our work also demonstrated the importance of TLR7 signaling in mice for the establishment of an effective immune response to a commercially available influenza SV (Figure 3-6Figure 3-10). TLR7/MyD88 signaling was also shown to be important in the establishment of immunity against WIV influenza vaccines [405, 411]. Recently, Koyama *et al.* demonstrated that TLR7$^{-/-}$ mice could not produce either influenza-specific IgA or IgG in response to an intranasally administered WIV vaccine and were not protected from a subsequent lethal challenge. However, the immune response to WIV was not dependent on RIG-I/IPS1 signaling, as IPS1$^{-/-}$ mice could produce virus-specific antibodies and were

protected from a lethal challenge. Similarly, a study by Geeraedts *et al.* demonstrated that an H5N1 WIV vaccine was not immunogenic in TLR7$^{-/-}$ mice [405]. Additionally, these two studies examined the importance of TLR7 signaling against a lab-produced, inactivated influenza SV and found them to be non-immunogenic in naïve WT mice [405, 411]. Both groups suggested that their results were due to the degradation of RNA in their SV preparations. Interestingly, we detected viral RNA within the commercially available monovalent 2009 pandemic SV (Figure 3-8). Similar to what was shown in the WIV model [411], treating the SV with RNase significantly reduced the HA-specific response to SV in WT mice (Figure 3-10a). As a result, mice immunized with RNase treated vaccine (RTV) were more susceptible to viral challenge (Figure 3-10b). The phenomenon of vaccine dependence on RNA has also been observed in other systems [143, 460]. For example, Sander *et al.* compared live-attenuated to whole-killed bacterial vaccines and found a substantial difference in their immunogenicity [460]. Based on their data, they hypothesized there was a bacterial component that distinguished live from dead bacteria called viability-associated PAMPs (vita-PAMP) [461]. Upon further investigation, they identified RNA as the essential vita-PAMP for the immunogenicity of these vaccines [460]. The cumulative data suggest RNA is an important determinant of vaccine immunogenicity in naïve animals.

A limitation of current influenza vaccines is that individuals who respond poorly to immunization are also often the groups at the highest risk for complications due to influenza infection [250, 294, 315-320]. Furthermore, vaccines are less immunogenic in unprimed populations. This is problematic for

the development of effective vaccines against avian subtypes H5 and H7 with pandemic potential [327, 330, 337, 340], as these HAs have been proven to be poorly immunogenic [337-340]. The addition of oil-in-water emulsion adjuvants to the seasonal TIV and pandemic influenza vaccines has already proven to increase immunogenicity in clinical trials [318, 346, 347, 462-466]. However, vaccines formulated with adjuvants are not currently approved for use in the U.S. The development of new adjuvants targeting innate immune receptors that manipulate the adaptive immune response is an active area of research; TLR ligands are good candidates for this purpose [383, 467, 468]. Clinical studies have already been conducted using TLR ligands as adjuvants in the formulation of vaccines for Epstein-Barr virus, Hepatitis B, Polio, influenza and human papillomavirus with promising results [469-474]. In addition, several pre-clinical studies have been conducted using ligands for TLRs, showing them to enhance immunogenicity of influenza vaccines [383, 475-482]. Unfortunately, TLR ligands which are bacterial in origin may have high levels of reactogenicity [483]. Because viral RNA is one of the major PAMPs within IAV and would likely have low reactogenicity, the use of RNA as a vaccine adjuvant for influenza vaccines should be evaluated. Any adjuvanted influenza vaccine must have an acceptable safety profile since currently licensed influenza vaccines are extremely safe [484-486].

TLR7 recognizes single-stranded RNA [118, 120, 363], but the exact nature of the RNA it binds during a viral infection is not known. IAV ssRNA segments are coated in NP and are part of the vRNP [213, 227]. More research is required to determine whether TLR7 binds naked ssRNA or NP-coated ssRNA. One

method to determine the nature of RNA bound to TLR7 would be to immunoprecipitate TLR7 from IAV-infected cells and subsequently gel-purify the components to determine any viral proteins complexed with the RNA. Identifying the natural ligand of TLR7 would best replicate the immune response to viral infection, making it an ideal adjuvant for influenza vaccines.

It has been previously demonstrated that the addition of synthetic TLR7 ligands [383, 420-422, 482, 487] and mRNA [418, 419] to vaccines can improve the immunogenicity of vaccines. For example, mRNA vaccines are dually effective because of their ability to transcribe anti-tumor/pathogen proteins while simultaneously having the ability to stimulate TLR7 [418, 419, 488]. In addition, imiquimod, a TLR7 ligand, is currently being used clinically for treatment against cancer and warts [489-491]. Three independent studies have examined the use of synthetic TLR7 ligand in influenza vaccines as an adjuvant and have shown increased immunogenicity in animal models [383, 482, 487]. It would be important to examine the effects of adding synthetic TLR7 ligands on the immunogenicity of influenza vaccines for at-risk populations. Since TLR expression and function declines with age, it is possible TLR7 ligands may not have adjuvant activity in all age groups [151, 317, 492, 493].

The demonstration of a role for TLR7 signaling in the immunogenicity of inactivated influenza vaccines but not infection suggests TLR7 is required for situations where IFNα/β production and T-cell help are limited [7, 355, 357, 396, 405, 411, 494] (Figure 4-1c). Several studies have demonstrated that in addition to BCR stimulation (signal 1) and T-cell help through CD40/CD40L interaction (signal 2), naïve B cells require additional stimulation for their activation (signal

3) [194, 368, 410, 431] (Figure 4-1). The third signal for B-cell activation can come from either TLR stimulation [7, 194, 368, 410, 431, 495] or cytokines provided by innate cells [377, 431] (Figure 4-1c,d). It would be important to identify which cell type requires TLR7 signaling during influenza SV immunization with and without a strong adjuvant. This could be evaluated by immunizing conditional B cell or DC TLR7$^{-/-}$ mice to determine which cells are dependent on TLR7 signaling. Based on previous studies [197, 375], vaccines formulated with strong adjuvants may not depend on TLR7 signaling by B cells, whereas the immunogenicity of unadjuvanted SV could require TLR7 recognition of RNA by B cells. Support for this hypothesis would demonstrate RNA-dependent TLR7 signaling by B cells is only required in situations where inflammatory responses are minimal.

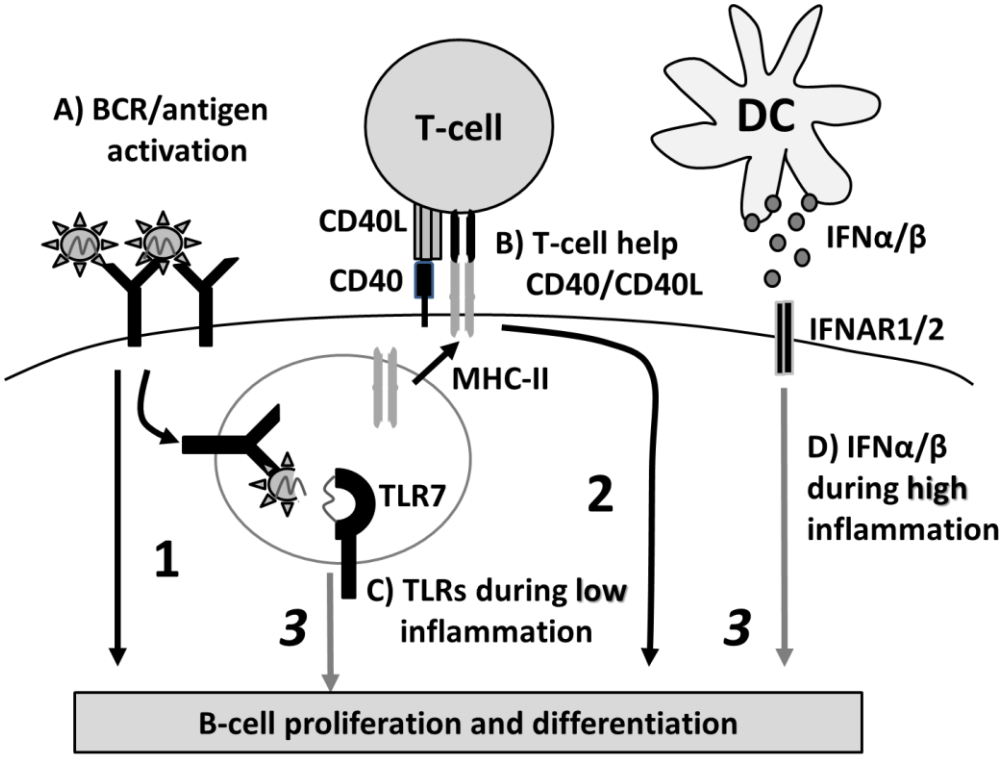

Figure 4-1: B cell Signal 3 Hypothesis

Naïve B cells require (a) signal 1 from the BCR and (b) signal 2 from T-cell help to respond to T-cell dependent antigens. To become fully activated, B cells may also require signal 3. This third signal can come from (c) TLR stimulation in the context of low inflammation or (d) from cytokines produced by DCs or other sources. Figure modified from Lanzavecchia 2007 [7].

A thorough understanding of the innate immune system and the role of pathogen sensing mechanisms will provide a greater opportunity for the development of improved, more immunogenic vaccines. TLR7 is a prime target for investigation, as adding viral RNA or synthetic TLR7 ligands to vaccines could greatly enhance the immunogenicity of influenza vaccines against seasonal and pandemic influenza. The current study has highlighted the fact that non-HA components of influenza vaccines may contribute to vaccine immunogenicity and that PRR signaling offers a target for modulation of vaccine responses. Additional research is needed to aid in the formulation of improved pandemic influenza vaccines due to the ongoing potential of novel influenza viruses emerging from birds and other animals.

Chapter 5 : References

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