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The Role of Innate Immune Recognition in Chronic Viral Infection

by

Jonathan M. Clingan

DISSERTATION

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Contributions of Co-Authors

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1. Division of Rheumatology, Department of Medicine, University of California, San Francisco

2. Rosalind Russell Medical Research Center for Arthritis, University of California, San Francisco

3. Howard Hughes Medical Institute, University of Texas Southwestern Medical Center

4. Department of Molecular Biology, University of Texas Southwestern Medical Center

5. Department of Microbiology and Immunology and the Cancer Research Institute, University of California, San Francisco

The Role of Innate Immune Recognition in Chronic Viral Infection

Jonathan M. Clingan

Abstract

The role of innate immune recognition and the importance of these responses for control of acute virus infections are well documented. However, how these pathways contribute to control of chronic virus infections, and to what extent persistent stimulation of innate immunity is necessary, are less well understood. In the studies described herein, we utilized the lymphocytic choriomeningitis virus (LCMV) infection model, where infection with various strains can lead to either acute or persistent infection in mice.

First, we used mice deficient in signaling through either the cytosolic RIG-I-like receptor (RLR) pathway, or the endosomal Toll-like receptor (TLR) nucleic-acid sensing pathways to demonstrate that the RLR pathway was required for normal clearance of acute LCMV infection, whereas the TLR pathway was necessary for clearance of chronic infection. The RLR pathway was the primary means of type I IFN production, which was necessary for early virus control and activation of $CD8^+$ T cell responses. In contrast, the TLR pathway was required for optimal antibody responses, particularly during chronic infection.

Upon investigation of the mechanism by which TLR signaling was required for antibody responses, we used bone marrow chimeric mice to demonstrate a B cell intrinsic role for TLR7 signaling in antibody, germinal center (GC) B cell and plasma cell responses. GC B cells in TLR7-deficient mice exhibited decreased proliferation and an altered GC response, leading to defective plasma cell formation.

We further examined a role for prolonged pDC activation in the control of LCMV infection. Using IFN-β reporter mice, we found that pDCs underwent rapid activation-dependent cell death following virus infection, pDCs were not activated during chronic infection, and were refractory to further stimulus following acute infection. This state was not dependent on prior cell-intrinsic activation or presence of pDCs during the early immune response.

These data together imply that different innate immune recognition pathways impact adaptive immune responses during chronic infection through various means. It is likely that the importance of these pathways for viral clearance is determined by the nature of infection and cell types involved.

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Chapter 1

Introduction

Innate Immune Recognition of Viruses

In the broadest sense, the innate immune system serves to initiate an immune response by 'sensing' molecules that differ from that of the host organism, or by the recognition of molecules that are mislocalized relative to their usual location (1). In regards to immunity to infectious diseases, as opposed to adaptive immunity, which is marked by exquisitely specific responses to individual pathogens, the innate immune system instead recognizes broad classes of pathogens. Initial recognition occurs when pathogen-associated molecular patterns (PAMPs) engage pattern-recognition receptors (PRRs) initiating signaling cascades, which induce expression of cytokines and type I interferons (IFNs) that serve to limit virus replication or promote activation of the adaptive immune response.

Innate immune recognition of pathogens occurs via a variety of germline-encoded receptors including the membrane-associated C-type lectin receptors (CLRs) and Toll-like receptors (TLRs), and the cytosolic NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and sensors of cytosolic DNA (2). CLRs are capable of binding carbohydrate ligands on the surface of microbial pathogens, including those expressed on the surfaces of some viruses (3). However, in contrast to TLRs and cytosolic receptors, few CLRs are, on their own, capable of initiating downstream signaling cascades that induce innate immune activation, and generally serve a more modulatory function. Therefore, the rest of this introduction will focus on the role of TLRs and cytosolic receptors in the recognition of viral ligands.

The importance of TLRs for the initiation of innate immune responses was first recognized in Drosophila when flies mutant for components in the Toll pathway were

susceptible to fungal infection and showed impaired expression of the antifungal peptide drosomycin (4). TLRs (10 in humans, 12 in mice) are comprised of an extracellular leucine-rich repeat (LRR) domain which mediates ligand binding, and an intracellular Toll/IL-1R (TIR) domain that is required for downstream signaling through association with TRIF (TLRs 3 and 4) or MyD88 (all but TLR3) (2). TLRs can be divided into those that are cell membrane-associated receptors, which generally recognize ligands present on microbial surfaces, and endosome-localized TLRs, which recognize nucleic acids (5). As viruses replicate by pirating host cellular pathways, and can be highly mutative, changing amino acid sequences, innate recognition of viruses using protein determinants is of limited value, and is generally restricted to those viral surface proteins that are conserved due to high selection pressure (6). For example, TLR2, originally shown to recognize Gram-positive lipoteichoic acids (7), and which heterodimerizes with TLRs 1 or 6 to recognize lipoproteins (2), can also initiate innate immune responses to viruses. These include the activation of TLR2 by measles virus hemagglutinin protein (8), and TLR2-dependent responses to mouse cytomegalovirus (MCMV) and vaccinia virus (VV), among others (6, 9). Likewise, TLR4, which functions primarily in the recognition of the Gram-negative bacterial cell wall component lipopolysaccharide (LPS) (10), also has limited roles in antiviral immunity through the recognition of the respiratory syncytial virus fusion protein (11) and mouse mammary tumor virus, during which TLR4 engagement is actual beneficial to the virus by promoting an immunosuppressive IL-10-mediated response (12).

An alternative to the recognition of viral surface proteins is that of nucleic acid sensing, a strategy employed by both endosomal TLRs and cytosolic receptors of nucleic

acids, and is the primary mechanism by which mammalian hosts sense viral infection. Although particular biochemical properties can vary among classes of nucleic acid ligands, these are generally divided into three classes: double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA (5). Self/non-self discrimination is dependent primarily on nucleic acid modifications not present on host nucleic acids, or through specific localization of receptors to subcellular compartments where they are unlikely to come into contact with host nucleic acids (1).

Nucleic Acid-Sensing Toll-like Receptors

The family of TLRs involved in nucleic acid sensing includes: TLR3, which recognizes ssRNA (13), TLR7 and 8, which recognize the imidazoquinoline class of anti-viral compounds along with ssRNA (14-17), and TLR9, which binds to DNA (18). Whereas human TLR8 was able to confer responsiveness to guanosine (G)- and uridine (U)-rich ssRNA derived from HIV-1 (15), whether *Tlr8* is functional in mice is unclear, as rodent species lack a five amino acid motif in the undefined region of *Tlr8*, which is conserved in non-rodent species and confers responsiveness to TLR8 ligands (19).

Host specificity is conferred in the case of TLR3 because mammals largely lack dsRNA of sufficient immunostimulatory capacity, whereas dsRNA is a common replication intermediate of RNA, as well as DNA viruses (6). Discrimination of DNA is largely due to a preference for nonmethylated CpG motifs found in prokaryotes, whereas mammalian DNA is usually methylated. Specific motifs for TLR7 are unclear, but discrimination is likely due to endosomal localization as extracellular host ssRNAs would

likely be quickly degraded, but viral RNA is protected within the virion until uncoating in the endosome.

Proper localization of nucleic acid-sensing TLRs to the endosome is critical for both activation under normal conditions, but also to prevent autoimmunity. In a forward genetic screen seeking to identify genes important for responsiveness to TLR3, 7, and 9 ligands, a missense mutation in *Unc93b1* was identified and termed '3d' as it conferred unresponsiveness to ligands for these three TLRs (20). It was later shown that UNC93B1 is necessary for exit of these TLRs from the endoplasmic reticulum and their trafficking to the endolysosome (21). Indeed, 3d mice were more susceptible to MCMV, consistent with roles for both TLR3 and TLR9 during MCMV infection (20, 22). Endosomal localization is additionally crucial for activation of nucleic acid-sensing TLRs and prevention of autoimmunity. Cleavage of TLR7 and TLR9 occurs in the endolysosome and is necessary for activation (23-25). However, if TLR9 is mutated in the transmembrane domain, such that it is no longer enzymatically cleaved, TLR9 is now able to traffic to the cell surface and respond to DNA ligands independently of receptor processing (26). Mice reconstituted with bone marrow expressing the mutant TLR9 receptor died of lethal autoinflammatory disease, underscoring the importance of proper localization in self/non-self discrimination and demonstrating that although PRRs such as TLRs preferentially respond to forms of nucleic acid different from self, such as unmethylated CpG DNA motifs, this does not exclude the recognition of host nucleic acids under certain conditions.

Cytosolic Nucleic Acid Recognition

As previously mentioned, in addition to TLRs, a variety of cytosolic PRRs are capable of recognizing nucleic acids in the cytosol. The best characterized of these are the RNA-sensing RLRs, which consist of retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (27). Though TLRs sensing nucleic acids were already known, a cytosolic pathway for sensing of dsRNA was hypothesized based on a TLR3-independent type I IFN response, induced when dsRNA was introduced into the cytosol of bone marrow-derived dendritic cells (DCs) (28). RIG-I was the first RLR to be discovered in a screen for molecules capable of enhancing activation of type I IFN when cells were transfected with polyriboinosinic:polyribocytidylic acid (polyI:C), a synthetic form of dsRNA (29). Subsequently, MDA5, first identified as an IFN-inducible gene that promoted apoptosis in melanoma cells (30), was shown to also enhance type I IFN responses to intracellular dsRNA (31). LGP2 was the last RLR to be identified based on sequence homology to MDA5 and RIG-I (32). However, unlike RIG-I and MDA5, which are able to transmit downstream activating signals through interaction with the adaptor mitochondrial antiviral signaling (MAVS), also known as VISA/Cardif/IPS-1, via caspase activation and recruitment domains (CARDs) (33-36), LGP2, although it can bind dsRNA was shown to lack a CARD, and knockdown of LGP2 *in vitro* enhanced type I IFN reporter expression (32). In contrast to this initial report, more recent studies with *Lgp2*$^{-/-}$ mice indicate that LGP2 may, in fact, facilitate recognition of viruses by RIG-I, and in particular, MDA5, though there are some discrepancies between these

studies, and the exact mechanism by which LGP2 performs these functions are unknown (37, 38).

Although originally identified based on their ability to recognize cytosolic dsRNA in the form of polyI:C, RIG-I and MDA5 do differ in their ability to recognize various RNA species. This was first demonstrated by the generation of gene-targeted mice, which showed that RIG-I and MDA5 recognize distinct families of viruses and that while MDA5 seemed to be the primary sensor of polyI:C, RIG-I mediated recognition of *in vitro* transcribed RNA (39, 40). However, it was later shown that RIG-I too responds to polyI:C, and that differential recognition by MDA5 or RIG-I depended on the length of the polyI:C with MDA5 preferentially binding longer and RIG-I shorter species (41). Additionally, RIG-I has been shown to recognize RNA containing 5' triphosphates and polyuridine motifs (42-44). This is important for self/non-self discrimination of RNAs containing primarily ssRNA, as mammalian RNAs present in the cytosol generally contain a 5' cap not present on many viral and synthetic RNA species (5).

In addition to RLRs, NLRs have been shown to have some role in the recognition of cytosolic RNA. Nod2 was shown to induce type I IFN in response to cytosolic viral ssRNA in a MAVS-dependent manner, and Nod2 deficiency resulted in increased susceptibility to various RNA virus infections *in vivo* (45). The Nalp3 inflammasome likewise is an NLR that is capable of being activated by cytosolic RNA, however, in contrast to Nod2, appears to not induce type I IFNs, but promotes the maturation of IL-1β and IL-18 via caspase-1-dependent mechanisms (46-48).

Similarly to RNA, introduction of DNA into the cytosol is also capable of inducing pro-inflammatory cytokines including type I IFN (49, 50); however, much less

is known about the upstream receptors required for the initial recognition of DNA and initiation of downstream signaling events. The first presumptive receptor hypothesized to play a role in cytosolic DNA sensing was DNA-dependent activator of IFN-regulatory factors (DAI), which was shown to bind to cytosolic DNA and was necessary for type I IFN production induced by DNA (51). However, when DAI knockout mice were generated, the cytosolic DNA response was not impaired in mouse embryonic fibroblasts (MEFs) or bone marrow-derived DCs transfected with DNA (52).

An important advance was the identification of stimulator of interferon genes (STING), which was shown to be necessary for induction of type I IFNs in response to cytosolic DNA both *in vitro* and *in vivo*, and conferred protection to infection with the DNA virus, herpes simplex virus-1 (53, 54). Interestingly, these studies showed that STING might also contribute to the recognition of RNA viruses detected by RIG-I, but not MDA5. More recently, the AIM2-like receptors (ALR) IFI16 and DDX41 were shown to bind cytosolic DNA and induce STING-dependent type I IFN and inflammatory cytokine responses (55, 56). Together with the observation that cytosolic DNA can also induce activation of the inflammasome via AIM2 (57-60), a recent study examined all known human and mouse ALRs for their ability to induce type I IFN and caspase-1-dependent IL-1β secretion and identified several new ALRs capable of conferring such activity (61). Importantly, the ability to induce IL-1β or type I IFNs was largely segregated. Furthermore, knockdown of any individual ALR did not significantly affect type I IFN induction by cytosolic DNA, thereby implicating redundancy among this class of receptors.

In addition to AIM2, inflammasome-dependent IL-1β production can also be mediated in response to cytosolic DNA by the Nalp3 inflammasome (62). A STING-independent mechanism also exists for the induction of type I IFN by cytosolic DNA. In this scenario, cytosolic DNA containing poly(dA:dT) motifs induces type I IFN in a RIG-I dependent manner (63, 64). The mechanism of RIG-I dependency was elucidated by the discovery of a dsRNA intermediate transcribed by RNA polymerase III from poly(dA:dT), recognition of which by RIG-I was dependent on the presence of a 5' triphosphate moiety.

Innate Signaling Activates Type I Interferon and NF-κB Pathways

Despite the plethora of innate PRRs that detect viruses, the signaling cascades downstream of the various sensing pathways are relatively conserved. With the exception of PRRs that activate the inflammasome and induce caspase-1-dependent release of IL-1β, TLRs and cytoplasmic nucleic acid-sensing PRRs initiate an antiviral program largely through the transcriptional induction of type I interferons and inflammatory cytokines by the transcription factors IFN regulatory factors (IRFs) 3 and 7 and NF-κB (2, 5). Whereas induction of type I IFNs requires activation of IRF3 and IRF7 (65), inflammatory cytokine responses require NF-κB.

Induction of inflammatory cytokines is a common feature to PAMP recognition by all TLR, RLR, and DNA-sensing pathways (2, 5, 49, 50). However, PRRs differ in their ability to activate IRF3 and IRF7, and thus, the expression of type I IFNs. Although the cytosolic RLRs and putative DNA-sensor(s) can all induce the expression of type I IFNs, TLRs differ in their ability to activate IRF3 and IRF7 largely due to differing usage

of adaptor proteins and subcellular localization (2, 66). Much of the insight into different adaptor usage was provided from studies of TLR4, which recognizes LPS (10).

It was observed that *Myd88*$^{-/-}$ mice largely mirrored the response of TLR4 mutant mice in that they were resistant to LPS-induced shock (67). However, unlike the response to most other TLR ligands, cells from these mice still observed delayed activation of NF-κB, indicating a MyD88-independent TLR4-signaling mechanism. This MyD88-independent pathway was shown to activate IRF3, whereas TLR2 could not, indicating that TLR4 was unique in this respect (68, 69). Likewise, activation of TLR3, which is activated in response to dsRNA, was also shown to involve a MyD88-independent signaling pathway (13). TIR domain-containing adapter inducing IFN-β (TRIF) was then identified as a TIR domain-containing protein that associated with TLR3 and was capable of activating IRF3, and therefore type I IFN production (70). TRIF was shown to be crucial to activation of IRF3 by TLR3 or TLR4 ligands, but did not participate in the response to other TLR stimuli (71, 72). TRIF-related adaptor molecule (TRAM) was also identified as a component of the MyD88-independent response, but only in regard to TLR4, and not TLR3, ligands (73). Another adaptor, TIRAP, in contrast, was shown to be important for MyD88-dependent cytokine production downstream of TLR 1, 2, 4, and 6, but not MyD88-independent responses (74, 75).

Interestingly, TLR7 and TLR9 are capable of inducing type I IFNs in a MyD88-dependent manner, suggesting that receptor access to the adaptor TRIF is required for TLRs to engage in induction of type I IFNs (14-18). These TLRs use a unique mechanism in which IRF7 directly associates in a complex with MyD88, and upon activation by ligand is phosphorylated and moves to the nucleus in a MyD88-dependent

manner (76). Type I IFN production via this pathway is regulated, in part, by subcellular localization. Prolonged containment of nucleic acid ligands in endosomes was shown to be important for strong induction of type I IFNs (77), and may further require transit to a lysosome related organelle, whereas inflammatory cytokine production through this pathway occurs earlier (78). The endosomal localization of TLR 3, 7, and 9, and the discovery that TRAM-dependent TLR4 internalization and relocalization to the endosome upon LPS ligation is necessary for TLR4-dependent type I IFN production via TRIF, supports a model in which subcellular localization of TLRs may be critical for access to IFN-inducing signaling components (66, 79).

In contrast to TLRs, which use a variety of adaptors to promote downstream signaling, RLRs rely on the adaptor MAVS to initiate downstream signaling (33-36). Similarly, activation of downstream signaling by cytosolic DNA depends on the adaptor STING (53, 54). As mentioned previously, there may be some interplay between these pathways as RIG-I-mediated recognition of some RNA viruses is suboptimal in the absence of STING, and MAVS and STING may directly interact, although the details are unclear (5).

In the 'classic' model of IFN induction, which occurs downstream of cytosolic PRRs and has been traditionally studied in fibroblasts, transcription of type I IFN is dependent on both IRF3 and IRF7. IRF3 is expressed constitutively and upon virus infection is phosphorylated and translocates to the nucleus to induce the expression of type I IFNs and other IFN-responsive genes (80-83). In contrast, IRF7 is expressed at low levels, induced by type I IFN signaling, and is required for signal transducer and activator of transcription 1 (STAT1)-dependent induction of IFN-α genes downstream of type I

IFN receptor (IFNAR) signaling (84). However, despite low levels of IRF7 in uninfected cells, IFN-β induction is also critically dependent upon IRF7, as mouse embryonic fibroblasts (MEFs) lacking IRF7 are severely defective in their induction of IFN-β, and only in MEFs lacking both IRF3 and IRF7 is the type I IFN response completely abrogated (85, 86). In contrast, type I IFN production downstream of TLR7 and TLR9 is solely dependent on IRF7.

Plasmacytoid Dendritic Cells and Cell-Specific Expression of PRRs

Whereas RLRs are expressed in most cells, across many different tissues and cell types including non-immune cells, TLRs capable of inducing type I IFN have a more restricted expression to mostly those cells of the myeloid lineage (2, 27). Cell-type specific expression of various TLRs is a critical determinant of their responsiveness to various ligands. Macrophages and conventional DCs (cDCs) express TLR3 and TLR4 and are capable of inducing type I IFN via these pathways, but although they can also express TLR7 and 9, they are not capable of making type I IFN in response to TLR7 or 9 ligands, and instead make only inflammatory cytokines (2, 85). This is presumably because these ligands do not reside in the endosomal pathway long enough to form the signaling apparatus required to induce type I IFN (77). In contrast, plasmacytoid DCs (pDCs) do not express TLR3 and are uniquely capable of inducing type I IFN in response to TLR7 and 9 ligands (2, 5). Plasmacytoid DCs have the further distinction of being able to produce approximately 100 to 1000-fold more type I IFN per cell compared to other blood cells, and have been shown to contribute to control of infections such as MCMV and vesicular stomatitis virus (VSV) (87, 88).

Plasmacytoid dendritic cells were first described as cells with a lymphoid morphology, containing large amounts of rough endoplasmic reticulum, and capable of rapidly secreting large amounts of IFN-α following virus exposure (89, 90). Early studies identified mouse pDCs as Gr-1^{+}, and anti-Gr-1 antibody-mediated depletion of these cells demonstrated their requirement for type I IFN production in response to MCMV infection *in vivo* (91, 92). However, depletion of these cells, despite their property of high IFN production, did not affect type I IFN responses to all viruses, as those to LCMV were unaffected (92).

Although depletion studies with anti-Gr-1 gave insight into the role of pDCs in antiviral responses, anti-Gr-1 recognizes both Ly6C and Ly6G and treatment can deplete other cell types such as neutrophils and monocytes. In order to more specifically deplete pDCs, bone marrow stromal cell antigen 2 (BST2) was identified as a largely pDC-specific marker in naïve mice that when targeted with various antibodies, could deplete pDCs *in vivo* (93, 94). Although an improvement over anti-Gr-1 treatment, this strategy still suffered from the fact that although fairly specific in naïve mice, BST2 is upregulated in response to type I IFN on other cells such as B cells and cDCs.

More recently, genetic manipulation in mice has provided for somewhat more specific depletion of pDCs to study their roles *in vivo*. The transcription factor E2-2 was shown to be essential for pDC development, and when E2-2 was ablated pDC development was blocked at an early stage, converting these cells to a cDC-like phenotype with diminished responses to CpG (95, 96). Sialic acid binding Ig-like lectin (Siglec)-H is specifically expressed on pDCs (97), and another group introduced the human diptheria toxin receptor (DTR) into the 3' untranslated region of *Siglech* in order

to specifically deplete pDCs using diptheria toxin (DT) (98). Mice in which pDCs were ablated with DT were more susceptible to infection with herpes simplex virus-1, and showed reduced $CD8^+$ T cell responses to this virus. Another model of pDC ablation *in vivo*, utilizes BDCA-2, an antigen specifically expressed by human pDCs (99). In this approach, transgenic (Tg) mice were made which express the human DTR under the control of the BDCA-2 promoter (88). Consistent with earlier reports, when pDCs were depleted in these mice, immune responses to MCMV and VSV were impaired, particularly very early following infection.

Type I Interferon Functions in Innate and Adaptive Immunity

The induction of type I IFNs by PRR engagement is one of the primary means by which these receptors exert antiviral effects. The type I interferons consist of one IFN-β and multiple IFN-α genes (13 in humans and 14 in mice), as well as other lesser-defined subtypes (65, 100). Following induction, type I IFNs act in both autocrine and paracrine manners through a single IFNAR to restrict viral replication in both infected and neighboring cells, and in addition, promote other immune responses. Type I IFNs are critically important for protection from viral pathogens as evidenced by severe pathogenesis in mice lacking one of the two components of the IFNAR (101, 102).

It is through the induction of interferon-stimulated genes (ISGs) whereby type I IFNs confer an 'antiviral state.' Most of the well-characterized ISGs known to date do so by interfering with cellular processes required for viral replication. For example, protein kinase R inhibits protein translation and RNase L degrades RNA (103). Two recent studies, which sought to identify novel ISGs and ascertain their antiviral effects, have

demonstrated that ISGs are a diverse set of genes some of which can inhibit replication by a broad range of viruses, some acting preferentially against RNA or DNA viruses, and some in fact capable of enhancing viral replication (104, 105). Additionally, ISG inhibition of viral replication was additive, suggesting multiple strategies by which ISGs exert antiviral function.

In addition to the expression of ISGs, type I IFNs also have pleiotropic effects on other cells of both the innate and adaptive immunity important in the control of viral infections. Natural killer cells are an 'innate' lymphocyte important for elimination of virally infected cells through both direct cytotoxic effects and the production of the type II IFN, IFN-γ (106). Type I IFNs both increased cytotoxicity of NK cells and increased proliferation of NK cells in response to mouse cytomegalovirus (MCMV) infection, through the induction of IL-15 (107).

Type I IFNs further link innate and adaptive immune responses through the activation of cDCs, which are particularly capable of promoting T and B cell responses through their antigen presentation function. These include activation of $CD4^+$ T cell subsets, which drive a diverse array of immune responses, $CD8^+$ T cells, which are cytotoxic lymphocytes capable of eliminating virus infected cells, and B cells, which differentiate into antibody-secreting plasma cells. Type I IFN potently stimulates cDCs to increase expression of co-stimulatory molecules such as CD80 and CD86, along with major histocompatibility complexes (MHC) to augment their T cell stimulatory capacity (108). The direct action of type I IFNs on cDCs has been shown to facilitate a diverse range of adaptive immune responses. When only cDCs were capable of responding to type I IFN, use of IFN-α/β as an adjuvant, was sufficient for increased antibody

responses and isotype class switching (109). Antibody responses are also augmented by pDC-derived type I IFN (110). In this case, IFN-α/β from pDCs synergized with IL-6 and CD40 signaling (via $CD4^+$ T cells) to promote the differentiation of B cells into plasmablasts and plasma cells. Cell-intrinsic requirements for type I IFNs in cDCs were also shown to be critical in promoting $CD4^+$ T cell responses in a vaccine model (111), and for cross-presentation of soluble antigen to $CD8^+$ T cells (112).

In addition to effects on B and T cell responses mediated by type I IFN action on cDCs, IFNs are also capable of influencing the responses of these cells directly through engagement of the IFNAR. Antibody production in response to influenza virus infection was decreased when B cells lacked the IFNAR (113). Likewise, IFNAR signaling in $CD4^+$ T cells has been shown to be important for clonal expansion of lymphocytic choriomeningitis virus (LCMV)-specific T cells (114, 115), and for survival and granzyme B expression in $CD8^+$ LCMV-specific T cells (115). Therefore, type I IFN shapes the adaptive immune response both through modulation of cDC functions, but also by acting directly on B and T cells to influence their differentiation.

Other Roles for PRRs in Shaping Adaptive Immune Responses

As mentioned previously, PRRs, and in particular TLRs, are highly expressed on immune cells involved in antigen presentation to T cells, such as cDCs. Activation of APCs via PRRs is a highly effective method by which APCs become competent to activate T cells as it leads to the up-regulation of MHC molecules, the co-stimulatory receptors CD80 and CD86, as well as the chemokine receptor CCR7, which enables migration to T cell zones of secondary lymphoid organs where APCs can interact with T

cells (116). Increased expression of MHC and co-stimulatory molecules can be downstream of both PRR engagement, or via exposure of APCs to inflammatory factors. Interestingly, when cDCs were 'matured' with or without TLR engagement, CD80, CD86 and MHC II were expressed at similar levels and the ability to promote $CD4^+$ T cell proliferation was equivalent (117). However, only those DCs that underwent TLR activation were able to make IL-12 and promote $CD4^+$ T cell IFN-γ production and helper cell differentiation. These data indicate that signals from PRRs are crucial for making qualitatively more effective APCs, particularly with regards to the expression of particular cytokines, such as IL-12 or IFN-α/β, which can act as a 'signal 3' for T cell differentiation in addition to signals through the T cell receptor (TCR) and co-stimulatory molecules. In addition to $CD4^+$ T cell responses, TLR activation on DCs can also promote cross-priming and activation of $CD8^+$ T cells (118). RLRs can also contribute to T cell activation through activation of cDCs to increase expression of co-stimulatory molecules and inflammatory cytokines (119, 120). Hence, PAMPs can activate DCs through multiple pathways to enhance their antigen presentation functions to stimulate T cell responses.

PRRs expressed on DCs can also influence antibody production through both type I IFNs, as previously described, and activation of cDCs, which elicit $CD4^+$ T cell responses capable of initiating B cell activation and differentiation into antibody-secreting plasma cells (121). Recently, evidence has accumulated for a novel $CD4^+$ T cell subset, known as T follicular helper (Tfh) cells, important for germinal center (GC) responses that drive B cell differentiation processes such as somatic hypermutation (SHM), class switch recombination (CSR), and memory B cell and plasma cell

differentiation (122). Tfh cell development is dependent on the transcription factor Bcl6, and when $CD4^+$ T cells lack Bcl6, GC B cell responses were abrogated (123-125). Bcl6 is first expressed in Tfh cells following interaction with antigen presented on DCs, and is further stabilized by interactions with cognate B cells (126-130). As both TLR signaling in DCs and B cells has been shown to affect GC responses and antibody production (131, 132), it is conceivable that this may in turn impact the generation of Tfh cells, which would further impact antibody responses. Consistent with a role for PAMP recognition in DCs influencing Tfh cell development, polyI:C was shown to augment Tfh cell development, presumably because IFN-α/β induced by polyI:C caused DCs to make IL-6, which promotes Tfh cell differentation (133).

To what extent TLR signaling within B cells augments antibody responses via Tfh cell maintenance is unclear. However, when B cells alone lacked the ability to respond to IL-21, a cytokine produced largely by Tfh cells and critical to GC B cell responses (134-136), administration of virus-like particles (VLPs) containing RNA, but not RNA-free VLPs were able to rescue much of the defective antibody production (137). Since this response to VLPs is B cell-intrinsic (132), it is likely that TLR engagement on B cells is able to drive GC B cell responses independently of Tfh cells. Furthermore, a recent study of patients with deficiency in DOCK8, an adaptor downstream of TLR9 signaling, revealed that these patients had impaired antibody and memory B cell responses to vaccines, and impaired B cell proliferation and immunoglobulin (Ig) production in response to CpG (138). In contrast, other TLR9-dependent pathways, such as the production of IFN-α by pDCs were unaffected, suggesting a B cell-specific defect in TLR9 signaling. These results are consistent with other studies in which B cells deficient

in MyD88 or various TLRs demonstrate defective B cell responses (139). Therefore, PAMPs can promote antibody through a variety of means incorporating DCs, Tfh, and B cells.

The LCMV Model of Virus Infection

As described above, the engagement of PAMPs by PRRs in a variety of cell types can influence both innate and adaptive immune responses, which are ultimately required for control of infections. Virus infection is likely to generate multiple PAMPs, which can be recognized by different PRRs. Furthermore, some PAMPs, such as dsRNA, can be recognized by multiple PRRs (MDA5, RIG-I, and TLR3). Therefore, the entanglement of the roles different PRRs play, and in what cell types, during the course of a natural virus infection can help to elucidate the relevant biological functions for these PRRs. One model of virus infection that has been immensely informative to immunologists, and which our lab primarily uses, is that of infection of mice with LCMV.

LCMV is a bi-segmented, single-stranded RNA virus of the Arenavirus family (140). Whereas infection of wild-type (WT) mice with the LCMV Armstrong (Arm) strain resulted in clearance within 8-10 d postinfection (p.i.), infection with the variant clone 13, which was isolated from the spleen of an LCMV Arm-infected carrier mouse, resulted in chronic infection that was maintained for two or more months (141). Upon sequencing, five nucleotide differences were identified between clone 13 and the parental Arm strains, two of which resulted in non-conserved amino acid mutations (142-145). One amino acid mutation resulted in a F to L change at amino acid residue 260 in the viral glycoprotein (GP)-1 encoded on the viral short (S) segment. The other amino acid

mutation, which occurred in the viral polymerase on the long (L) segment, resulted in a change from K to Q at amino acid residue 1079. Both mutations are required for persistence *in vivo* as reassortant viruses containing isolated L or S segment mutations had intermediate clearance (142, 143, 145, 146).

Following the identification of α-dystroglycan (DG) as the cellular receptor for LCMV it was shown that the clone 13 F→L GP-1 mutation confers 2- to 3-log increased binding affinity to α-DG relative to LCMV Arm (147, 148). This receptor seems to be preferentially expressed on DCs and on fibroblastic reticular cells (FRCs) (148, 149). This confers clone 13 with the ability to infect the white pulp of the spleen. The destruction of FRCs, in particular, by $CD8^+$ T cells results in prolonged disorganization of lymphoid architecture contributing to the persistent phenotype (150, 151). In addition to the F260L mutation in GP1 that was originally described, more recently, another non-conserved amino acid mutation in some clone 13 stocks was discovered that results in a N→D mutation at residue 176 (152). However, this mutation does not to contribute to the chronic phenotype. Thus far, the only evidence regarding a mechanistic role for the polymerase K1079Q mutation in viral pathogenesis has been the observation that clone 13 replicates to higher titers in macrophages early during infection, and that this phenotype maps to the K→Q mutation (143, 153). Therefore, subversion of innate immune responses by clone 13 likely contributes to its ability to establish persistent infection.

Using infection with LCMV Arm and clone 13 as a model has generated a great understanding of the roles various immune responses play in the clearance of acute or chronic viral infections, and in the formation of immune memory following clearance.

Normal control of acute and chronic LCMV infections is dependent on type I and II IFNs (102, 154, 155). Mice deficient in IFNAR demonstrate delayed clearance of acute or chronic LCMV, and in the absence of both IFNAR and IFNγR, fail to eliminate virus. $CD8^+$ T cells are also required for clearance of both acute and chronic LCMV infections, as mice that lack $CD8^+$ T cells fail to clear either strain (156). In contrast, although $CD4^+$ T cells are not required for clearance of acute infection with LCMV, even transient depletion of $CD4^+$ T cells is enough to establish lifelong persistence in mice infected with clone 13 (156, 157). Likewise, B cells are not required for control of acute LCMV infection, but mice lacking B cells fail to control various chronic strains of LCMV (158-160).

The mechanisms by which these various cell populations contribute to effective elimination of virus are complex and multifaceted. $CD8^+$ T cells eliminate infected cells through perforin-dependent mechanisms that are required for LCMV clearance (161, 162). $CD8^+$ T cells also secrete multiple effector cytokines, including IL-2, IFN-γ, and TNF-α, but over the course of chronic infection become 'exhausted,' gradually losing the ability to make these cytokines (157, 163, 164). In addition, as $CD8^+$ T cells become more exhausted, they increase expression of multiple inhibitory receptors, the blockade of which has been shown to increase effector function and reduce viral titers (164-167). Similarly, blockade of IL-10, which is increased during chronic infection, also augments $CD8^+$ T cell function and decreases viral loads (168, 169). Thus, the maintenance of $CD8^+$ T cell function is critical to eventual control of chronic LCMV infection.

$CD4^+$ T cells are central to maintaining $CD8^+$ T cell function during chronic viral infection (156, 157, 170). In the absence of $CD4^+$ T cells, mice fail to clear clone 13, but

with $CD4^+$ T cells, mice are eventually able to control virus infection with serum titers becoming undetectable at about 2 months postinfection. The precise nature of the help provided by $CD4^+$ T cells is unclear, but recent studies indicate that IL-21 produced by LCMV-specific $CD4^+$ T cells acts directly on $CD8^+$ T cells to maintain their function during chronic infection (171-173). Among $CD4^+$ T cells, primarily Tfh cells produce IL-21, and chronic infection drives the differentiation of $CD4^+$ T cells to become Tfh cells (171-173).

$CD4^+$ T cells are also important for B cell responses, which contribute to control of chronic LCMV infection (159, 160, 174). Consistent with a role for $CD4^+$ T cells in maintaining antibody responses, $CD4^+$ T cell depletion or an inability for Tfh cells to provide help to GC B cells resulted in decreased antibody titers (175). Not only do $CD4^+$ T cells provide help to B cells, but B cells also reciprocate by providing help to $CD4^+$ T cells, which makes it difficult to differentiate between the antibody-producing function of B cells and their helper function (160, 176). However, studies using mice that have B cells, but are unable to class switch or secrete antibody have demonstrated a role for antibodies in control of LCMV infection (159).

In contrast to the role for T and B cells in control of LCMV infection, the role of the innate immune system is less well defined. As previously mentioned, IFNAR-deficient mice have delayed clearance of both acute and chronic LCMV infections (102, 154, 155). In addition to any direct antiviral effects, type I IFN is also important for differentiation, expansion and survival of LCMV-specific $CD4^+$ and $CD8^+$ T cells, demonstrating the requirement for innate immunity for optimal adaptive immune responses (114, 115). Despite the important role played by type I IFNs, conflicting

reports make it difficult to discern which PRRs, and in what cell types, the induction of type I IFNs and other innate responses to LCMV occur. Originally, using *Ifna6* reporter mice, the Akira group identified pDCs as the primary producers of type I IFN in response to LCMV infection (177). This response was MyD88-dependent, and *Myd88*$^{-/-}$ mice demonstrated ineffective clearance of LCMV. However, the contribution of pDCs to virus clearance was brought into question by a study demonstrating that MyD88 is also required intrinsically within CD8^{+} T cells for proper expansion, and thus defective CD8^{+} T cells in the absence of MyD88 is the most likely culprit for high viral loads in *Myd88*$^{-/-}$ mice, presumably via the IL-33 receptor (178, 179). A more recent study indicated a role for MDA5, but not TLR pathways in early type I IFN production to acute LCMV infection, but effects on viral titers or adaptive immune responses were not reported (180). Furthermore, prior to my studies, the PRRs engaged during chronic LCMV infection had yet to be reported. Therefore, throughout the following studies, I have sought to address the role for various PRRs in different cell types in the control of acute versus chronic LCMV infection in the hope that it can shed light on the functions of these receptors in the course of chronic viral disease.

Chapter 2

Differential roles for RIG-I-like receptors and nucleic acid-sensing TLR pathways in controlling a chronic viral infection

Abstract

The necessity for pathogen recognition of viral infection by the innate immune system in initiating early innate and adaptive host defenses is well documented. However, little is known about the role these receptors play in the maintenance of adaptive immune responses and their contribution to resolution of persistent viral infections. Here, we demonstrate a non-redundant functional requirement for both nucleic acid-sensing Toll-like receptors (TLR) and RIG-I-like receptors (RLR) in the control of a mouse model of chronic viral infection. Whereas the RLR pathway was important for production of type I interferons and optimal $CD8^+$ T cell responses, nucleic acid-sensing TLRs were largely dispensable. In contrast, optimal anti-viral antibody responses required intact signaling through nucleic acid-sensing TLRs, and the absence of this pathway correlated with less virus-specific antibody and deficient long-term virus control of a chronic infection. Surprisingly, absence of the TLR pathway had only modest effects on antibody production in an acute infection with a closely related virus strain, suggesting that persistent TLR stimulation may be necessary for optimal antibody responses in a chronic infection. These results indicate that innate virus recognition pathways may play critical roles in the outcome of chronic viral infections through distinct mechanisms.

Introduction

Chronic viral infections such as those caused by human immunodeficiency virus (HIV) and hepatitis B and C viruses are a major global health problem infecting millions worldwide (181). The mechanisms of immune-mediated control of chronic viral infections in humans or mouse models have been well studied and are multifaceted. Virus-specific $CD8^+$ T cells are important for elimination of virally infected cells and gradually lose effector function throughout the course of infection (157, 163, 164). In addition, exhausted $CD8^+$ T cells express multiple inhibitory receptors, which further limit their function (165-167, 182, 183). In addition to $CD8^+$ T cells, $CD4^+$ T cells also play an important role through both help to $CD8^+$ T cells and to B cells for antibody responses, which contribute to virus control (156, 157, 159, 171-173, 175). Despite this depth of understanding regarding adaptive immune mechanisms of virus control, little is known regarding the role of innate immune recognition of virus during the control of chronic viral infections and how innate immune activation may influence pathogenesis.

The innate immune system senses virus infection primarily, but not exclusively, through the recognition of viral nucleic acid. The main pattern recognition receptors involved in sensing of viral nucleic acid include RIG-I-like receptors (RLRs), expressed by most cells, and Toll-like receptors (TLRs), which are expressed primarily by macrophages, dendritic cells (DCs) and also B cells (2, 27, 184). The RLRs RIG-I and MDA5 detect cytosolic viral RNA replication intermediates and signal through the adaptor MAVS (IPS-1/VISA/Cardif) to initiate the production of type I interferons (IFN) and other inflammatory cytokines, whereas the nucleic acid-sensing TLRs, TLR3 (dsRNA), TLR7 (ssRNA) and TLR9 (DNA) rely on the adaptors MyD88 and TRIF and

localize to endosomes in an UNC93B-dependent manner. Signaling is abrogated in cells with a missense mutation in Unc93b1, termed '3d' because it affects TLRs 3, 7, and 9 (20, 21). Recognition of virus by pattern recognition receptors is known to be important for both restricting early virus replication through the induction of antiviral genes, but also by initiating adaptive immune responses.

In order to study the role of these pathways in controlling chronic viral infection, we used a mouse model of infection with lymphocytic choriomeningitis virus (LCMV), comparing differences between acute and chronic infections. Type I IFNs (IFN-α/β) have previously been shown to be important for control of both acute and chronic LCMV infections (102, 154, 155), and for optimal $CD8^+$ T cell responses (115). However, the role of innate immune nucleic-acid recognition pathways in the induction of type I IFN by LCMV, a single-stranded RNA virus, has been controversial, as either MAVS or MyD88-dependent pathways have been implicated during response to an acute infection (177, 180). In addition, because MyD88 is needed in a $CD8^+$ T cell-intrinsic manner for proper LCMV-specific $CD8^+$ T cell expansion, the role of the TLR pathway in controlling LCMV infection has been difficult to differentiate from its effects on other MyD88-dependent signaling pathways (179). Furthermore, whether these pathways are engaged differently during chronic infection, and the relative importance of RLR and TLR pathways for adaptive immune responses and virus control during both acute and chronic infections are not known. In order to address these important questions, and circumvent the intrinsic $CD8^+$ T cell defect in *Myd88*$^{-/-}$ mice, we used *Mavs*$^{-/-}$ and *Unc93b1*$^{3d/3d}$ (3d) mice to examine the role of RLRs and nucleic acid-sensing TLRs, respectively, for: early and long-term virus control; production of type I IFNs; and the

generation and maintenance of $CD8^+$ T cell and antibody responses in both acute and chronic LCMV infections. We find that in *Mavs*$^{-/-}$ mice, type I IFN production, inhibition of early virus replication, and $CD8^+$ T cell responses were severely affected, leading to delayed but eventual clearance of both acute and chronic LCMV infections. In contrast, in 3d mice, type I IFN production, early virus control and $CD8^+$ T cell responses were not greatly affected and these mice were able to clear acute LCMV infection normally. Surprisingly, despite the ability of these mice to clear acute infection, control of chronic infection was severely impaired correlating with a defect in antibody responses and progressive $CD8^+$ T cell exhaustion. These results demonstrate critical roles for innate immune recognition of virus in maintaining adaptive immune responses during chronic viral infection.

Materials and Methods

Mice

C57BL/6 (CD45.2$^+$) mice were purchased from the Jackson Laboratory or the National Cancer Institute. CD45.1$^+$ P14 mice bearing the D^{b}GP33-specific TCR were maintained in our animal colony. Mob (IFN-β) reporter mice were generously provided by R. Locksley (University of California, San Francisco, CA) (185). *Mavs*$^{-/-}$ mice were generously provided by Z. Chen (University of Texas, Southwestern Medical Center, Dallas, TX) (186) and crossed to C57BL/6 mice for at least eight generations. *Unc93b1* "3d" mice on a C57BL/6 background were purchased from the Mutant Mouse Regional Resource Center (University of California, Davis, CA) (20). *Myd88*$^{-/-}$ mice on a C57BL/6 background were obtained from A. DeFranco (UCSF) (187). All experiments were done in accordance with University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

Viral infections and virus titration

LCMV Armstrong and clone 13 were propagated on BHK cells and titered on Vero cells as previously described (141). For acute infection, mice were infected by intravenous (i.v.) injection with 2×10^6 PFU LCMV Armstrong (Arm). For chronic infection, mice were infected i.v. with either 2×10^5 or 2×10^6 PFU LCMV clone 13 where indicated.

Cell Sorting

Plasmacytoid DCs were sorted from uninfected and LCMV-infected spleens at day 1 post-infection. Plasmacytoid DCs were enriched by negative selection using a biotinylated antibody mixture (CD4, CD19, and Thy1.2), streptavidin microbeads, and an autoMACS instrument (Miltenyi Biotec). Enriched cells were then sorted using

antibodies to B220 and Siglec-H with DAPI to exclude dead cells on a FACSAria (BD Biosciences). For CFSE-labeling, P14 cells were enriched from spleens of transgenic mice using a biotinylated antibody mixture (CD4, B220, I-A^b, NK1.1, CD11b, CD11c, and Ly6G), streptavidin microbeads, and an autoMACS instrument. The purity of P14 cells enriched by autoMACS was ~90% and the purity of FACS-sorted pDCs was ~98%.

Adoptive transfers and CFSE labeling

For non-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled P14 adoptive transfers, 2×10^4 (acute) or 1×10^3 (chronic) $CD45.1^+$ P14 cells were transferred into naïve $CD45.2^+$ recipients one day prior to infection and analyzed day 5 or 8 post-infection. For transfer of CFSE-labeled P14 cells, P14 cells were first enriched using an autoMACS instrument as described above. Enriched P14 cells were then labeled in PBS containing 7 μM CFSE (Invitrogen) for 7 min at room temperature. CFSE labeling was quenched with FBS and cells were washed with RPMI-1640 medium. 1×10^6 labeled P14 cells were transferred one day prior to infection and analyzed 48 hours post-infection.

Flow cytometry, antibodies, and intracellular staining

MHC class I tetramers were a generous gift from R. Ahmed (Emory University School of Medicine, Atlanta, GA). All antibodies were purchased from BioLegend except for anti-human granzyme B (Invitrogen); anti-mouse Ly6C and CD62L (BD Pharmingen); anti-mouse CD4, CD16+CD32, B220, CD11b, CD19, and Ly6G (University of California, San Francisco Antibody Core Facility); and anti-mouse PDCA-1 (eBioscience). Intracellular cytokine staining was performed as previously described (163). Spleen cells were cultured 5 h in the presence of 1 μg/ml GolgiPlug (BD Biosciences) and 0.2 μg/ml

of the indicated peptide prior to staining for surface markers and intracellular cytokines. Flow cytometry was performed on LSRFortessa or FACSCalibur (BD Biosciences) cytometers and analyzed with FlowJo 9.0.2 software (TreeStar).

Quantitative RT-PCR

RNA was isolated from snap-frozen tissues using TRIzol Reagent (Invitrogen). Following DNase treatment with a Turbo DNA-free kit (Ambion), cDNA was generated from total RNA using an M-MLV reverse transcriptase kit (Invitrogen). Gene expression was determined by quantitative PCR using FastStart Universal SYBR Green Master (ROX; Roche) on an ABI 7300 Real Time PCR System (Applied Biosystems). Expression of type I IFN RNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR primer pairs are as follows: GAPDH Fwd: 5'-GGTCTACATGTTCCAGTATGACTCCAC-3'; GAPDH Rev: 5'-GGGTCTCGCTCCTGGAAGAT-3'; IFN-β Fwd: 5'-CGCTGCGTTCCTGCTGTGCTT-3'; IFN-β Rev: 5'-AGGTGAGGTTGATCTTTCCATTCAG-3'; IFN-α Fwd: 5'-CTCCTAGACTCAKTCTGCA-3'; IFN-α Rev: 5'-ACACAGTGATSCTGTGGAA-3' (188).

ELISA

LCMV-specific IgG in serum was detected by solid-phase ELISA as previously described (141). Briefly, 96-well flat-bottom plates were coated with LCMV-infected BHK lysate, and then blocked with PBS containing 0.5% Tween 20 (Bio-Rad) and 10% FBS. After blocking, serially diluted serum was added and anti-mouse IgG conjugated to horseradish peroxidase was used as a secondary antibody (SouthernBiotech). 3, 3′, 5 ,5′ tetramethylbenzidine was used as substrate. Antibody titers were determined as the

reciprocal of the dilution that gave an optical density value (450 nm) reading more than two-fold above naïve control sera. For each graph, sera samples were analyzed by ELISA concurrently.

Statistical analysis

All statistical analysis was performed using a two-tailed unpaired Student's t-test with Prism software (GraphPad Software, Inc.).

Results

Differential contributions of innate virus-sensing pathways to the control of an acute or chronic viral infection

To determine how innate virus-sensing pathways contributed to the control of a chronic viral infection**,** we used the LCMV model in which infection of wild-type (WT) mice with the Armstrong (Arm) strain results in acute infection and viral clearance within 8-10 days, whereas infection with a closely related variant, clone 13, results in chronic infection with viral persistence for several months (141). Whereas WT mice efficiently controlled virus by 8 days following infection with LCMV Arm, *Mavs*$^{-/-}$ mice had elevated virus titers in serum, spleen, and liver (Fig. 1A). In contrast, 3d mice had similar levels of virus control as in WT mice. Although *Mavs*$^{-/-}$ mice had high levels of virus at day 8 post-infection (p.i.), these mice eventually cleared virus from serum and tissues by day 15 or shortly thereafter. Furthermore, we did not detect any infectious virus in serum and multiple tissues, including brain and kidney, at day 30 and later post-infection (data not shown). These data suggest that although the RLR pathway is primarily important in controlling acute infection with LCMV, deficiency in one pathway alone is not sufficient for establishment of a chronic infection with LCMV Arm.

Since the necessity for virus-sensing pathways may differ in acute and chronic infections, we investigated the role of RLR and nucleic acid-sensing TLR pathways in a chronic viral infection with clone 13. Clone 13 has been well established to replicate and spread more rapidly within the first few days of infection when compared to Arm (143). Since the dose of clone 13 used in most studies, 2 million PFU per mouse, already seems to overcome effects of the innate response and rapidly lead to $CD8^+$ T cell exhaustion in a

WT host, for our initial studies, we infected WT, *Mavs*$^{-/-}$, and 3d mice with a ten-fold lower dose of this strain and monitored virus clearance. Whereas WT mice eliminated virus in serum, spleen, and liver by day 44 p.i., similarly to acute infection with LCMV, *Mavs*$^{-/-}$ mice exhibited defective clearance of clone 13 at this time point, with higher levels of viremia at days 15 and 30 p.i., and detectable virus present in serum and tissues at day 44 following infection (Fig. 1B and C). Surprisingly, although nucleic acid-sensing TLRs were dispensable for clearance of acute LCMV infection, 3d mice failed to control infection with clone 13. Levels of virus in WT and 3d mice were relatively equivalent at day 15 p.i. (Fig. 1B), but 3d mice maintained elevated virus levels in serum and tissues throughout the course of observation. Consistent with these results, when infected with 2 × 10^6 PFU clone 13, 3d mice failed to clear virus at times as late as d160 p.i. (Fig. 1D). In contrast, with the higher dose of clone 13, kinetics of clearance in *Mavs*$^{-/-}$ mice were similar as that in WT mice indicating that the higher dose of virus likely overcomes MAVS-dependent effects on virus control (Fig. 1D). These data demonstrate that both the RLR and nucleic acid-sensing TLR pathways are necessary for control of LCMV infection. Importantly, though these receptors have the potential to activate similar downstream pathways, they act in seemingly non-redundant ways due to the differing necessity for these pathways in control of acute or chronic LCMV infections.

Impaired CD8$^+$ T cell function resulting from deficiency in innate virus-sensing pathways during acute and chronic infection

To understand the mechanism(s) by which the RLR and nucleic acid-sensing TLR pathways may affect the clearance of LCMV, we analyzed virus-specific CD8$^+$ T cell

responses, since $CD8^+$ T cells are required for control of acute infection (161, 162), and chronic infection has been shown to lead to the loss of $CD8^+$ T cell effector functions, which contributes to virus persistence (157, 163). At the peak of the $CD8^+$ T cell response to acute LCMV infection, whereas ~25% and ~18% of $CD8^+$ T cells in WT and 3d mice made IFN-γ in response to the GP33-41 and NP396-404 LCMV epitopes, respectively, only ~5% of $CD8^+$ T cells in *Mavs*$^{-/-}$ mice responded to stimulation with these peptides (Fig. 2A). Furthermore, only ~1% or less of $CD8^+$ T cells in *Mavs*$^{-/-}$ mice were able to make both IFN-γ and TNF-α to all epitopes, indicating a functional state of exhaustion (Fig. 2A and C). In addition to defective cytokine production, $CD8^+$ T cells in *Mavs*$^{-/-}$ mice exhibited severely impaired production of granzyme B, in contrast to WT and 3d mice, which expressed equivalent levels (Fig. 2A). We did observe some minor differences between WT and 3d mice in the frequency of $CD8^+$ T cells that made both IFN-γ and TNF-α in response to one epitope, GP276-86, but not to GP33-41 or NP396-404 (Fig. 2A and C). However, the response to this epitope in 3d mice was still greater than that in *Mavs*$^{-/-}$ mice, and due to the relatively low frequency of $CD8^+$ T cells that respond to this peptide when compared to NP396 and GP33, it is likely that the dominant effect of these epitopes is enough to control the infection (Fig. 1A).

In addition to qualitative deficiencies, $CD8^+$ T cells in *Mavs*$^{-/-}$ mice also exhibited quantitative differences in the number of LCMV-specific $CD8^+$ T cells, as determined by tetramer staining at the peak of response (Fig. 2B). *Mavs*$^{-/-}$ mice had approximately four-fold fewer D^bGP33-specific and three-fold fewer D^bGP276-specific $CD8^+$ T cells compared to WT mice. In contrast, 3d mice had roughly equivalent numbers of D^bGP33-

specific $CD8^+$ T cells as WT mice, and although they had fewer D^bGP276-specific $CD8^+$ T cells, this difference was less than two-fold and was not statistically significant.

Functional exhaustion in $CD8^+$ T cells is associated with altered expression of activation markers, and upregulation of multiple inhibitory receptors (164, 166). Consistent with previous reports describing higher expression of T cell immunoglobulin and mucin domain–containing molecule 3 (Tim-3) and programmed death 1 (PD-1) on virus-specific $CD8^+$ T cells during chronic viral infection (165, 167, 182, 183), D^bGP33-specific (Fig. 2D) and D^bGP276-specific (Fig. 3A) $CD8^+$ T cells in *Mavs*$^{-/-}$ mice expressed higher levels of these inhibitory receptors when compared to WT and 3d mice. In addition, LCMV-specific $CD8^+$ T cells in *Mavs*$^{-/-}$, but not 3d mice, had decreased expression of Ly6C, which decreases in expression on virus-specific T cells in chronically infected mice (164). These results demonstrate that the RLR pathway, but not nucleic acid-sensing TLRs, is critical for optimal $CD8^+$ T cell responses and virus clearance during acute LCMV infection.

Next, we examined the role for the RLR and nucleic acid-sensing TLR pathways in maintaining $CD8^+$ T cell function during chronic infection with LCMV. Similarly to acute infection, virus-specific $CD8^+$ T cells in *Mavs*$^{-/-}$ mice were severely impaired in their ability to make IFN-γ and TNF-α at days 8, 15, and 44 p.i. in response to GP33 peptide (Fig. 2E and G). However, in contrast to the dispensability of nucleic acid-sensing TLRs for acute infection, absence of these TLRs led to a gradual loss of $CD8^+$ T cell function during chronic infection. At days 8 and 15 following infection with clone 13, the frequency of $CD8^+$ T cells making IFN-γ in response to GP33 (Fig. 2E) were roughly equivalent in WT and 3d mice, although the frequency of $CD8^+$ T cells

producing both IFN-γ and TNF-α varied slightly (Fig. 2G). Approximately 3% fewer $CD8^+$ T cells in 3d mice expressed both IFN-γ and TNF-α in response to GP33 peptide at day 8, but not day 15 following infection when compared to WT (Fig. 2G). Though these differences in T cell function at days 8 and 15 were minimal, by day 44 p.i., LCMV-specific $CD8^+$ T cells in 3d mice were severely exhausted, corresponding to their inability to control viral replication (Fig. 1B). At day 44, LCMV-specific $CD8^+$ T cells in 3d mice had impaired production of both IFN-γ and TNF-α to levels comparable to those in *Mavs*$^{-/-}$ mice (Fig. 2E and G). Consistent with increased levels of viremia (Fig. 1B) and the altered functional status of virus-specific $CD8^+$ T cells in *Mavs*$^{-/-}$ and 3d mice at day 44 p.i., these cells also expressed higher levels of the inhibitory receptors Tim-3 and PD-1 when compared to WT mice (Fig. 2H). Interestingly, Ly6C expression at day 44 was significantly decreased in *Mavs*$^{-/-}$ but not 3d mice and may indicate different rates of exhaustion since its expression has been shown to gradually decrease throughout the course of chronic infection in WT mice (164). Similar results were observed in response to GP276 peptide (Fig. 3B, D-E).

Numbers of virus-specific $CD8^+$ T cells in spleens were also measured by tetramer staining at days 8, 15, and 44 following clone 13 infection (Fig. 2F). Although numbers of D^bGP33-specific cells in *Mavs*$^{-/-}$ and 3d mice were slightly lower when compared to WT at day 8 following infection, these differences were less than two-fold in magnitude (Fig. 2F). However, by day 44 following infection, 3d mice had a more than three-fold decrease in numbers of D^bGP33-specific $CD8^+$ T cells. Similar results were seen for D^bGP276-specific $CD8^+$ T cells (Fig. 3C). *Mavs*$^{-/-}$ mice had consistently lower frequencies of tetramer-positive cells within the $CD8^+$ T cell population, but when total

numbers of antigen-specific cells were evaluated, these deficiencies were largely offset by larger spleen sizes. This suggests that early $CD8^+$ T cell exhaustion in *Mavs*$^{-/-}$ mice may prevent immune-mediated immunopathology. This is consistent with the observation that mice depleted of $CD8^+$ T cells show reduced disruption of splenic architecture and increased splenic cellularity following infection with clone 13 (150). These data demonstrate that the RLR and nucleic acid-sensing TLR pathways play distinct roles in the regulation of virus-specific $CD8^+$ T cell responses. Whereas RLR signaling is required early following either acute or chronic LCMV infection, nucleic acid-sensing TLRs are required only later during chronic infection to prevent persistence of virus and $CD8^+$ T cell exhaustion.

MAVS deficiency leads to impaired type I IFN production early during infection

Because type I IFNs are known to play important roles in virus clearance and $CD8^+$ T cell responses to LCMV, we sought to analyze the contribution of RLRs and nucleic acid-sensing TLRs to type I IFN production during acute and chronic LCMV infections.

In order to determine the peak of type I IFN production, we performed a kinetic analysis by quantitative RT-PCR of type I IFN in spleens following infection with either LCMV Arm or clone 13 (Fig. 4A). Type I IFN production was transient, detectable as early as 8 h p.i., reaching peak levels at 16-24 h, and decreasing to near baseline levels by 72 h p.i. for both Arm and clone 13, consistent with previous reports (189). To identify type I IFN-producing cells, we used Mob (IFN-β) reporter mice, in which an internal ribosome entry site (IRES)-YFP cassette is inserted into the endogenous *Ifnb1* locus downstream of the stop codon, preserving regulatory elements in the 3'-untranslated region, thus

marking IFN-β-producing cells (185). When Mob mice were analyzed on day 1 p.i., at the peak of the type I IFN response, the majority of spleen cells (~90%) expressing YFP had surface markers consistent with plasmacytoid DCs (pDCs) (Fig. 4B). Because production of type I IFN by pDCs in response to virus has been shown to be dependent on expression of nucleic acid-sensing TLRs, (190), we generated Mob mice carrying the *Unc93b1*$^{3d/3d}$ mutation (Mob-3d) and analyzed the ability of pDCs to produce IFN-β at day 1 following infection with LCMV Arm or clone 13 (Fig. 4C). In response to either strain, approximately 45% of pDCs in Mob mice expressed YFP at day 1 p.i., and this was dependent on nucleic acid-sensing TLRs, consistent with results seen using MyD88-deficient *Ifna6* reporter mice (177). Furthermore, the transient expression of IFN-β by pDCs was equivalent in mice infected with either Arm or clone 13 at multiple times examined (Fig. 4D). Dependency of pDC IFN-α/β expression on nucleic acid-sensing TLRs in response to LCMV infection was verified by quantitative RT-PCR in sorted pDCs from LCMV-infected 3d mice, and levels were equivalent to those in *Myd88*$^{-/-}$ pDCs (Fig. 4E). Unexpectedly, when levels of IFN-β and IFN-α were determined in whole spleens of mice infected with LCMV Arm or clone 13 at day 1 p.i. by quantitative RT-PCR, there were no differences in levels of type I IFN transcripts in 3d mice compared to WT (Fig. 4F). These results are corroborated by recent reports showing that depletion of pDCs and loss of nucleic acid-sensing TLR signaling have no effects on levels of type I IFN in acute LCMV infection (180, 191). In contrast, there were large decreases in both IFN-α and IFN-β in *Mavs*$^{-/-}$ mice (Fig. 4F). For both acute and chronic infections, IFN-α expression was affected to a greater degree than IFN-β, and type I IFN expression in chronic infection was affected to a larger extent by MAVS-deficiency than

in acute infection. Therefore, although nucleic acid-sensing TLRs, in pDCs, and the RLR pathway are both activated in response to infection, the RLR pathway is the primary innate pathway contributing to early type I IFN production in both acute and chronic LCMV infection.

The RLR pathway is important for both early virus control and $CD8^+$ T cell activation

Because both RLR and nucleic acid-sensing TLR-dependent pathways are activated early in response to LCMV infection, we assessed the contribution of these pathways to early virus control and $CD8^+$ T cell activation, which may affect the long-term ability of mice deficient in either of these pathways to limit chronic infection. To determine the necessity of RLR and nucleic acid-sensing TLR pathways for early restriction of virus replication, we analyzed the level of virus in sera of mice infected with LCMV Arm or clone 13 at days 1, 3, and 5 p.i. Deficiency in MAVS resulted in significantly increased viral loads at almost all time points examined for both acute and chronic infection (Fig. 5A). In contrast, loss of nucleic acid-sensing TLRs had no appreciable effect on levels of virus for either acute or chronic infection.

In order to determine the effect of these pathways on early activation of virus-specific $CD8^+$ T cells, we adoptively transferred wild-type CFSE-labeled $CD45.1^+$ TCR Tg P14 cells, which recognize the LCMV GP33 epitope in the context of $H\text{-}2D^b$, into $CD45.2^+$ WT, *Mavs*$^{-/-}$, or 3d recipient mice. Adoptively transferred mice were then infected with LCMV Arm or clone 13 and analyzed for proliferation and expression of various activation markers at 48 h p.i. (Fig. 5B). CD69 is induced in T cells upon

stimulation with either antigen or IFN-α/β (192). However, we did not observe significant differences in CD69 expression on P14 cells transferred into *Mavs*$^{-/-}$ mice compared to those transferred into WT or 3d recipients (Fig. 5B). This may be due to the fact that direct effects of TCR stimulation on CD69 expression may compensate for the relatively lower IFN levels. Shortly following activation, antigen-specific CD8^{+} T cells also express CD25, the high-affinity IL-2 receptor α chain (193, 194). While in response to acute or chronic LCMV infection P14 cells transferred into WT and 3d mice expressed high levels of CD25, P14 cells transferred into *Mavs*$^{-/-}$ recipients showed a broader range of CD25 expression with both high and low-expressing cells (Fig. 5B). Granzyme B expression was also lower in *Mavs*$^{-/-}$ recipients, consistent with the observed phenotype in virus-specific CD8^{+} T cells at day 8 p.i. with LCMV Arm (Fig. 5B and Fig. 2A). In contrast to the observed defects in the activation of P14 cells transferred into *Mavs*$^{-/-}$ mice, these cells had no defect in proliferation, as measured by CFSE dilution, when compared to WT recipients (Fig. 5B). Conversely, P14 cells that were transferred into 3d mice exhibited no aberrant activation compared to WT recipients, but demonstrated slightly decreased proliferation with respect to WT and *Mavs*$^{-/-}$ recipients (Fig. 5B). Additionally, ~10% of donor P14 cells in 3d mice had not proliferated, compared to ~1-2% in WT and *Mavs*$^{-/-}$ mice, suggesting there may be a slight defect in recruitment of antigen-specific T cells in the absence of nucleic acid-sensing TLRs.

To determine how differences in early activation and viral load affected later CD8^{+} T cell responses, we assessed the numbers and function of P14 cells at day 5 and 8 p.i. with LCMV Arm (Fig. 5C) or clone 13 (Fig. 5D) following transfer into WT, *Mavs*$^{-/-}$, or 3d mice. Similarly to results seen with endogenous LCMV-specific CD8^{+} T cells at

day 8 p.i. (Fig. 2A and E), P14 cells in *Mavs*$^{-/-}$ recipients demonstrated decreased functionality at day 5 (Arm) and day 8 (Arm and clone 13) of LCMV infection by intracellular staining for both granzyme B and the effector cytokines IFN-γ and TNF-α (Fig. 5C and D). Although numbers of P14 cells in *Mavs*$^{-/-}$ mice were roughly equivalent to those in WT recipients at day 5 after acute infection, by day 8 they exhibited a roughly ten-fold decrease in relative numbers (Fig. 5C). P14 cells in *Mavs*$^{-/-}$ recipients exhibited a similar, though less dramatic, defect in chronically infected mice (Fig. 5D). In contrast, P14 cells transferred into 3d mice had levels of granzyme B, IFN-γ, and TNF-α expression similar to WT in both acute and chronic LCMV infections (Fig. 5C and D). Total numbers of P14 cells in 3d mice were slightly decreased compared to WT mice at day 5 and 8 p.i. with Arm, but showed a more pronounced, approximately three-fold decrease during clone 13 infection at day 8 (Fig. 5C and 4D) similar to results seen with endogenous D^bGP33-specific cells (Fig. 2B and 2F). These results demonstrate that MAVS deficiency impacts both early virus control and $CD8^+$ T cell activation. In contrast, nucleic acid-sensing TLRs do not contribute to early virus control but have a discernible impact on early recruitment and proliferation of antigen-specific $CD8^+$ T cells.

Nucleic acid-sensing TLRs are required for optimal antibody responses during chronic infection

Although both *Mavs*$^{-/-}$ and 3d mice failed to control low dose clone 13 infection with the same kinetics as WT mice (Fig. 1B and C), the differences in the kinetics of viral titers and LCMV-specific $CD8^+$ T cell dysfunction suggested a different mechanism by which

nucleic acid-sensing TLRs contributed to control of a persistent infection. One possibility is that viral recognition by nucleic acid-sensing TLRs may be responsible for low-level type I IFN production at later time points that contributes to virus control. However, we were unable to detect significant levels of type I IFN at various time points later during chronic infection by quantitative RT-PCR (data not shown), consistent with previous reports (189, 195). In addition to T cell-mediated immunity, antibodies have also been shown to be necessary for resolution of chronic LCMV infection (159). Therefore, we investigated the contributions of the RLR and nucleic acid-sensing TLR pathways to LCMV-specific antibody responses. Following acute infection with LCMV Arm, levels of LCMV-specific IgG were not significantly different at day 15 p.i. in either *Mavs*$^{-/-}$ or 3d mice, but at days 30 and 45 p.i., LCMV-specific IgG titers were approximately three- and two-fold decreased in 3d mice, respectively, compared to WT mice with no differences seen for *Mavs*$^{-/-}$ mice (Fig. 6A). In contrast, during chronic infection with LCMV, 3d but not *Mavs*$^{-/-}$ mice exhibited defective LCMV-specific IgG production throughout the course of infection that was more severe than during acute infection (Fig. 6B). At day 15 p.i., LCMV-specific IgG titers in clone 13 infected 3d mice were approximately ten-fold lower than those in WT mice, and at days 30 and 44 p.i., 3d mice had approximately twenty-fold less LCMV-specific IgG. In mice infected with 2×10^6 PFU clone 13, LCMV-specific antibody titers were similarly affected, with no differences between WT and *Mavs*$^{-/-}$ mice, but approximately fifteen- to one-hundred-fold lower titers of anti-LCMV IgG at days 30, 45 and 60 p.i. in 3d mice compared to WT (Fig. 6C). These results demonstrate that impaired antibody production likely contributes to persistence of virus in mice deficient in signaling through nucleic acid-

sensing TLRs. Furthermore, larger differences in antibody levels between WT and 3d mice in chronic when compared to acute infection suggests that continued stimulation through nucleic acid-sensing TLRs may play a role in maintaining antibody responses during chronic viral infection.

Discussion

Our results demonstrate clearly that both nucleic acid recognition pathways are involved in the recognition of LCMV and contribute to control of a chronic viral infection, but through distinct mechanisms. Recognition of viruses via innate pathways may exert their influence on chronic infection in a variety of ways: prevention of early virus replication and spread; initiation of adaptive immune responses; and maintenance of adaptive immune responses and virus control later in infection. Although the first two are not unique to chronic viral infections, they are nonetheless important for establishment of infection. It was apparent from our studies that although both the nucleic acid-sensing TLR pathway and the MAVS-dependent RLR pathway are engaged early during LCMV infection, the RLR pathway was of primary importance for the induction of type I IFNs, limiting early virus replication, and producing effective $CD8^+$ T cell responses during both acute and chronic LCMV infections. This is in contrast to a report demonstrating compensation by RLR and TLR pathways for type I IFN and $CD8^+$ T cell responses to influenza (196). The effect of the RLR pathway on control of LCMV infection is consistent with decreased levels of type I IFN, although effects from differences in other innate mediators cannot be excluded. *Mavs*$^{-/-}$ mice exhibited increased viral titers early during the course of infection consistent with studies in type I IFN receptor-deficient mice (154, 155). Importantly, viral clearance in *Mavs*$^{-/-}$ mice was not impaired as greatly as that in type I IFN receptor-deficient mice, consistent with our results showing other sources of type I IFN.

IFN-α/β has been shown to act as a 'signal 3' for $CD8^+$ T cells and can induce the expression of granzyme B and CD25, among other genes (197, 198). Type I IFN

receptor-deficient $CD8^+$ T cells also express lower levels of granzyme B in response to LCMV infection, demonstrating a T cell-intrinsic requirement for type I IFN signaling (115). Interestingly, differences in CD25 expression can also affect levels of granzyme B, so virus-specific $CD8^+$ T cells in *Mavs*$^{-/-}$ mice may have a compounded defect through both decreased IFN-α/β and IL-2 signaling (193, 194). It has also been shown that exposure to type I IFN or IL-2 affects cell death or proliferation, respectively, in LCMV-specific $CD8^+$ T cells (115, 193). It will be interesting to see whether this contributes to decreased numbers of antigen-specific $CD8^+$ T cells in *Mavs*$^{-/-}$ mice. Whether virus-specific $CD8^+$ T cell dysfunction in *Mavs*$^{-/-}$ mice results more from defects in priming or is caused by elevated levels of antigen remains unclear, though both likely contribute. However, given the well-known role for $CD8^+$ T cells in controlling chronic viral infections (199), and the normal antibody responses in *Mavs*$^{-/-}$ mice, it is likely that defective $CD8^+$ T cell responses are a major cause of ineffective control of both acute and low dose chronic LCMV infections in *Mavs*$^{-/-}$ mice. Interestingly, despite early severe LCMV-specific $CD8^+$ T cell defects, *Mavs*$^{-/-}$ mice control a high dose of clone 13 with kinetics similar to WT mice. The differences seen when using the lower and higher doses of clone 13 may result from the kinetics of $CD8^+$ T cell exhaustion, which becomes faster in WT mice with high dose inoculation.

In contrast to the multitude of early virus control and $CD8^+$ T cell deficits in *Mavs*$^{-/-}$ mice, 3d mice demonstrated normal control of early virus replication for both acute and chronic infections and had relatively normal early $CD8^+$ T cell responses. This is in contrast to *Myd88*$^{-/-}$ mice which fail to control acute LCMV infection and have a $CD8^+$ T cell-intrinsic expansion defect (179), demonstrating that this effect is

independent of nucleic acid-sensing TLRs. There was, however, a slight recruitment and proliferation defect early following infection when P14 cells were transferred into 3d mice. Because antigen-presenting cells, such as various subsets of conventional DCs, express a wide array of TLRs, this may indicate a role for nucleic acid-sensing TLRs in optimal activation and homing of antigen-presenting cells (2). However, the extent that this affected $CD8^+$ T cell numbers and function is unclear. It has been shown that when different antigen doses were given, this affected recruitment of antigen-specific $CD8^+$ T cells, but all recruited cells divided efficiently and demonstrated similar effector functions (200). Additionally, assuming ~10% take of transferred P14 cells, the precursor frequency of D^bGP33-specific P14 cells in our transfer experiment was approximately a thousand-times greater than numbers of endogenous cells, thereby greatly increasing competition for antigen (201). Therefore, recruitment of endogenous $CD8^+$ T cells in 3d mice may not be similarly affected, though this may account for slightly reduced numbers of D^bGP276-specific T cells, a subdominant epitope for which competition may be more stringent (163, 202).

Although production of type I IFN by pDCs was dependent on nucleic acid-sensing TLRs, type I IFN has not been detected at later time points of chronic infection following transient production early in the response (189, 195). We were also not able to detect IFN-producing cells in Mob reporter mice at day 8 or 15 p.i. with clone 13 (data not shown), indicating an unlikely role for type I IFN in the maintenance of immune responses in 3d mice. In contrast, our data indicate a significant role for nucleic acid-sensing TLRs in controlling chronic infection through anti-viral antibody production. This is particularly interesting given a recent study showing that virus-specific $CD4^+$ T

cells gradually differentiate into T follicular helper (Tfh) cells during chronic LCMV infection, driving germinal center (GC) B cell development and antibody responses that assist in controlling persistent infection (175). Similar to our results in 3d mice, mice that lack CXCR5, which is necessary for Tfh cell interaction with GC B cells, had severe defects in antibody production that were more pronounced in chronic compared to acute LCMV infection (175). One possibility is that chronic stimulation of nucleic acid-sensing TLRs on DCs may drive Tfh cell differentiation which in turn drives antibody responses, and through the production of IL-21 can also function to maintain $CD8^+$ T cell responses (171-173). Additionally, DCs in 3d mice may have disruptive presentation of exogenous antigens, which could impair the development of Tfh cells (20). Another, non-exclusive, possibility is that nucleic acid-sensing TLRs are required intrinsically for anti-viral B cell function. This could either be as an adjuvant effect from directly engaging TLR7 on virus-specific B cells (132), or through the maintenance of B cell function, as 'exhausted' B cells have recently been described during chronic infection of individuals with HIV (203). Due to the expression of TLR3 in DC subsets and TLR7 and 9 in both DCs and B cells (184), it will be important to determine which nucleic acid-sensing TLRs, in which cell types are necessary. Given the intertwined nature of DCs, Tfh cells, and B cells, it is likely that recognition of virus through these TLRs exerts its effect on antibody responses in multiple ways. Although our results imply different requirements for nucleic acid-sensing TLRs in the antibody response during acute and chronic viral infection, further studies are needed to determine if continuous TLR stimulation is necessary to maintain antibody responses. Since it is clear that different innate viral recognition pathways differentially impact adaptive immune responses, further understanding of the roles these

pathways play in other chronic viral infections such as HIV, HCV, and HBV may lead to more targeted immune interventions.

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Footnotes

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Figure 1

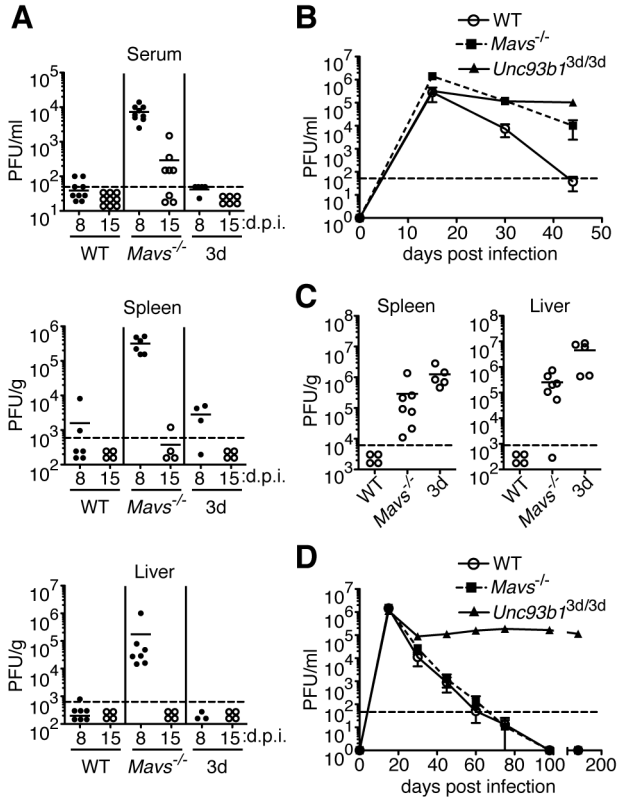

Figure 1. Both nucleic acid-sensing TLRs and the RLR pathway are necessary for control of a chronic viral infection, but only the RLR pathway is required for control of an acute infection. WT, *Mavs*$^{-/-}$ and *Unc93b1*$^{3d/3d}$ (3d) mice were infected with LCMV Arm (A) or clone 13 (B and C) and viral titers were assessed by plaque assay. (A) Viral loads in serum spleen and liver on 8 and 15 days post infection (d.p.i.) with Arm are shown (n=3-9). (B) Longitudinal analysis of viral titers in sera of mice infected with 2×10^5 PFU clone 13. Data shown are mean ± SEM. (C) Viral titers in spleen and liver at day 44 following infection with clone 13. (D) Longitudinal analysis of viral titers in sera of mice infected with 2×10^6 PFU clone 13. Graphs show mean ± SEM (n=4-5 mice per group). Data in A are pooled from three or more independent experiments. Data shown in B and C are representative of three independent experiments

(n=4-8 mice per group). Data in D are representative of two independent experiments. For A and C, the mean is shown as a solid line with data points representing individual mice. Dotted lines indicate the limits of detection. PFU, plaque-forming units.

Figure 2

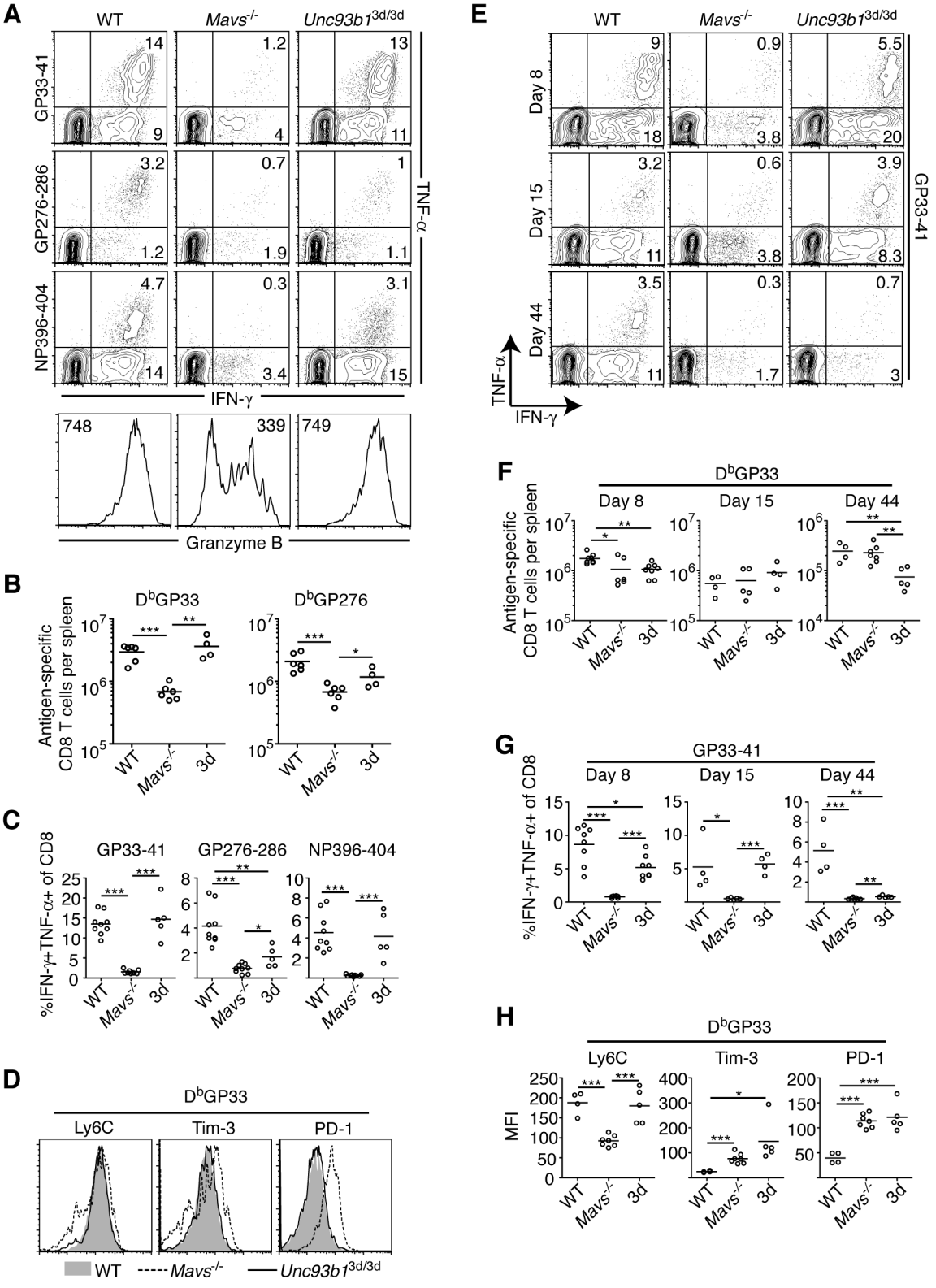
A
WT
Mavs-/-
Unc93b13d/3d
GP33-41
GP276-286
NP396-404
TNF-α
IFN-γ
Granzyme B
B
DbGP33
DbGP276
Antigen-specific CD8 T cells per spleen
C
GP33-41
GP276-286
NP396-404
%IFN-γ+TNF-α+ of CD8
D
DbGP33
Ly6C
Tim-3
PD-1
E
Day 8
Day 15
Day 44
F
G
H
MFI

Figure 2. Differential effects of the loss of innate virus-sensing pathways on virus-specific CD8$^+$ T cell function in acute and chronic viral infection. (A-D) Analysis of virus-specific CD8$^+$ T cell responses in WT, *Mavs*$^{-/-}$ and *Unc93b1*$^{3d/3d}$ (3d) mice infected with LCMV Arm at day 8 p.i. (A) (Top) Splenocytes were incubated with the indicated peptides for 5 hr and the frequency of responding CD8$^+$ T cells was determined by intracellular staining for IFN-γ and TNF-α. Numbers within plots indicate frequency of cytokine-producing cells within that quadrant. (Bottom) Expression of granzyme B by D^{b}GP33-tetramer$^+$ CD8$^+$ T cells was analyzed by intracellular staining. Numbers within histograms indicate the mean fluorescence intensity (MFI). (B) Total D^{b}GP33- and D^{b}GP276-specific CD8$^+$ T cells were quantified in spleens of infected mice. (C) The frequency of CD8$^+$ T cells that are IFN-γ$^+$TNF-α$^+$ in response to peptide stimulation as described in (A) was determined. (D) Expression of Ly6C, Tim-3, and PD-1 on D^{b}GP33-specific CD8$^+$ T cells was analyzed by flow cytometry in WT (shaded), *Mavs*$^{-/-}$ (dashed line) and 3d (solid line) mice. Data in A-D represent three or more independent experiments (n=4-9 mice per group). (E-H) Analysis of virus-specific CD8$^+$ T cell responses in mice infected with 2×10^5 PFU LCMV clone 13 at days 8, 15, and 44 following infection. (E) Expression of IFN-γ and TNF-α by CD8$^+$ T cells at the indicated times post-infection was determined as described in (A) for GP33-41 peptide. (F) Total numbers of D^{b}GP33-specific CD8$^+$ T cells were determined in spleens of infected mice by tetramer staining. (G) The frequency of CD8$^+$ T cells producing both IFN-γ and TNF-α in response to GP33-41 peptide at different times post-infection as shown in (E) was analyzed. (H) The MFI of Ly6C, Tim-3, and PD-1 is shown for D^{b}GP33-specific CD8$^+$ T cells at day 44 post-infection. For E through H, data are from two to four independent

experiments: day 8 (n=6-8); day 15 (n=4-5), and day 44 (n=4-8) for each group. Graphs show the mean as a line and each data point represents an individual mouse. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (two-tailed unpaired Student's *t*-test).

Figure 3

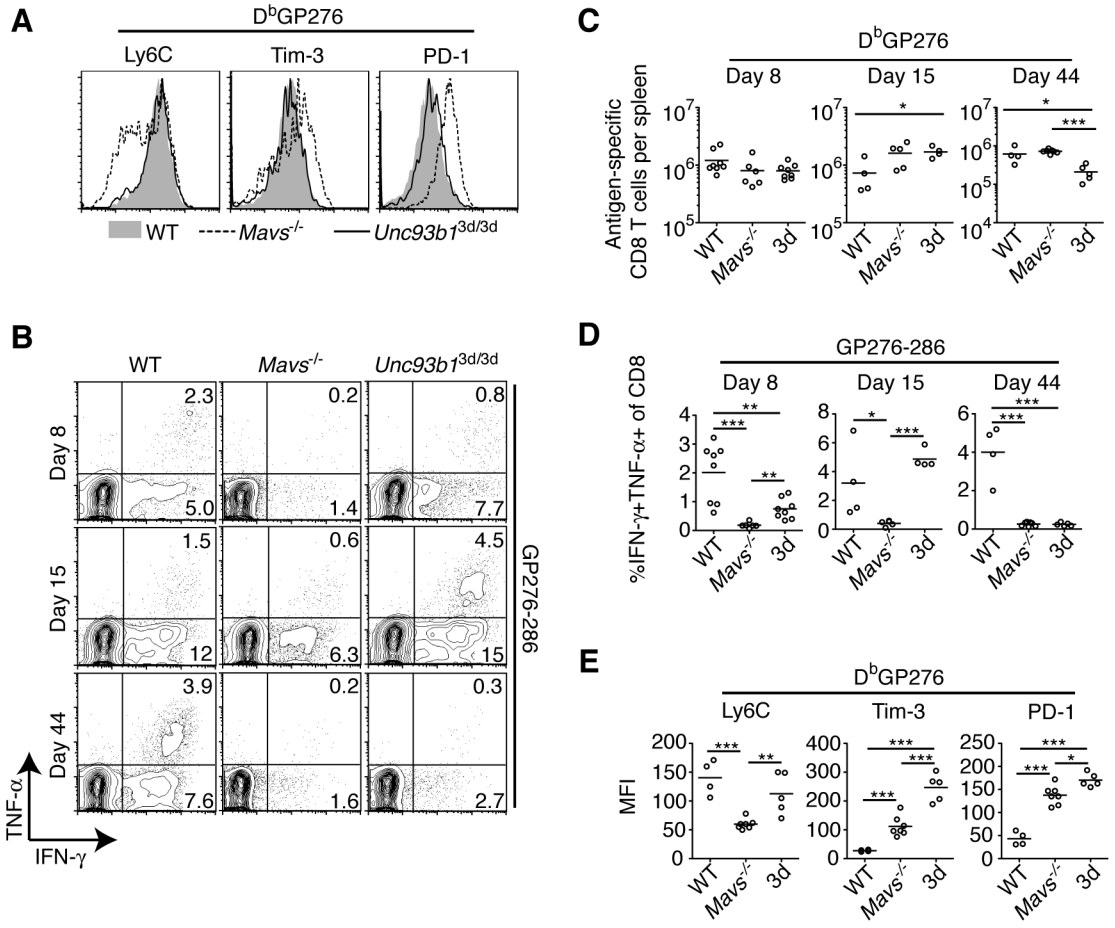

Figure 3. Similar CD8$^+$ T cell dysfunction is seen for other epitopes in acute infection of *Mavs*$^{-/-}$ and chronic infection of *Mavs*$^{-/-}$ and 3d mice. (A) Surface staining was done to analyze expression of Ly6C, Tim-3 and PD-1 on D^{b}GP276-tetramer$^+$ splenocytes on day 8 following infection with LCMV Arm. Histograms show levels of surface expression for WT (shaded), *Mavs*$^{-/-}$ (dashed line), and *Unc93b1*$^{3d/3d}$ (3d) (solid line) mice. (B) Expression of IFN-γ and TNF-α by CD8$^+$ T cells in response to GP276-286 peptide was analyzed by intracellular staining at the indicated times following infection with LCMV 2 × 10^5 PFU clone 13. Numbers in plots indicate the frequency of cytokine-producing CD8$^+$ T cells within that quadrant. (C) The numbers of D^{b}GP276-

specific $CD8^+$ T cells in the spleen were quantified by tetramer staining at the indicated times after clone 13 infection. (D) The frequency of $CD8^+$ T cells making both IFN-γ and TNF-α was analyzed by intracellular staining at the indicated times following infection with clone 13. (E) The MFI of Ly6C, Tim-3 and PD-1 was analyzed at days 8, 15, and 44 following infection with clone 13 by flow cytometry. Data in A represent three independent experiments (n=4-9). Data in B-E represent two to four independent experiments: day 8 (n=6-8); day 15 (n=4-5), and day 44 (n=4-8) for each group. For all graphs, horizontal bars indicate the mean and data points represent individual mice. *$P <$ 0.05, **$P < 0.01$ and ***$P < 0.001$ (two-tailed unpaired Student's *t*-test).

Figure 4

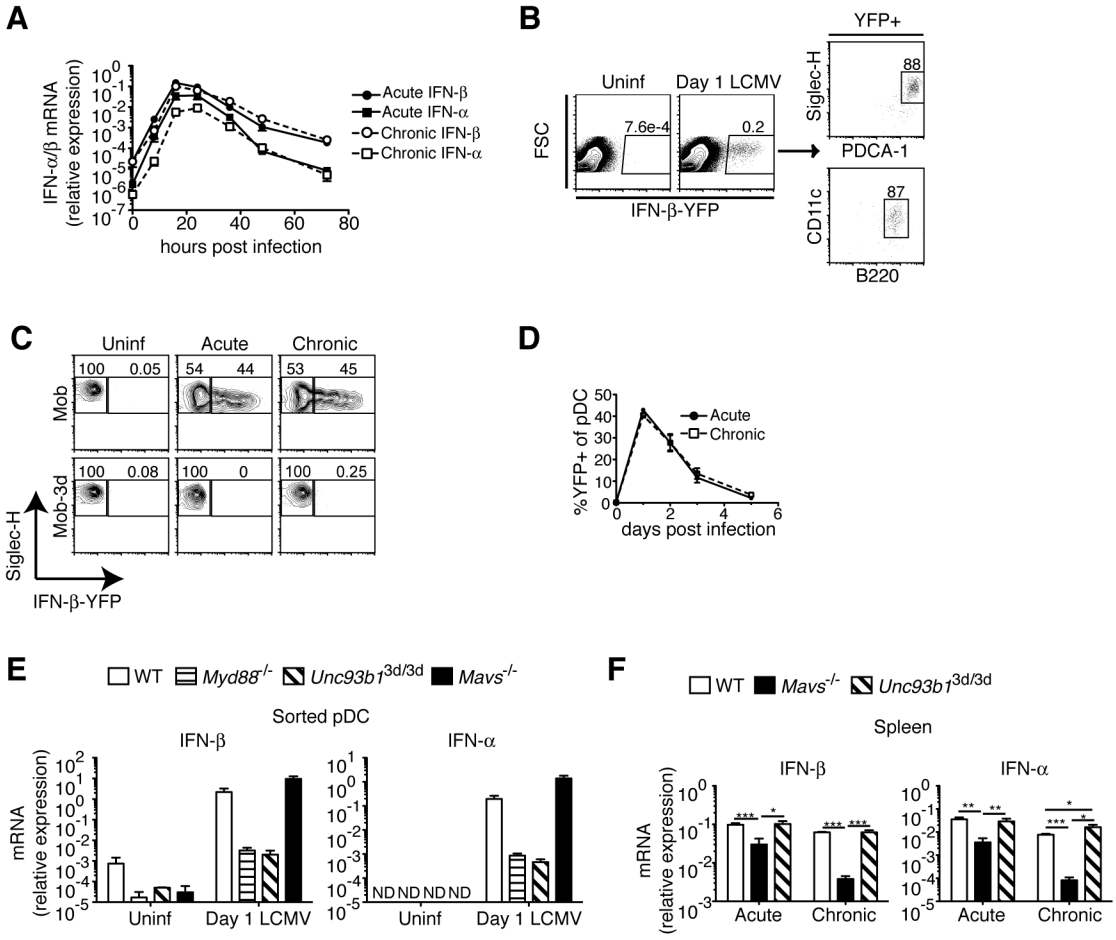

Figure 4. Both the nucleic acid-sensing TLR and RLR pathways are activated early following infection with LCMV. Mice were infected with either LCMV Arm (acute) or 2×10^6 PFU clone 13 (chronic) and production of type I IFN was analyzed. (A) Expression of mRNA for IFN-β and IFN-α was analyzed in spleens of infected mice at the indicated time points by quantitative RT-PCR. Data are representative of two independent experiments and show mean ± range (n=2 for each time point). (B) Mob (IFN-β) reporter mice were analyzed for reporter expression by flow cytometry. Phenotyping of YFP^+ cells in spleen at day 1 p.i. is shown for a representative experiment. (C) Reporter expression in pDCs from uninfected or day 1 Arm (acute) or

clone 13 (chronic) infected Mob and Mob-3d mice were analyzed by flow cytometry. Density plots are gated on PDCA-1^{hi}, Siglec-H^{+} splenocytes. (D) The frequency of YFP^{+} pDCs in spleens at the indicated time points following infection in Mob mice are shown (mean ± SEM). Data in C and D represent two independent experiments (n=2-5 mice per group). (E) IFN-β and IFN-α mRNA expression was analyzed by quantitative RT-PCR in sorted pDCs from uninfected or day 1 LCMV Arm-infected mice of the indicated genotypes. Data for each group are averages of 2-5 independently sorted samples (n=2-4 mice per sample). (F) IFN-β and IFN-α mRNA expression was analyzed by quantitative RT-PCR in spleens of the indicated mice at day 1 p.i with either Arm (acute) or clone 13 (chronic). Data for each group are averages of individual mice pooled from eight different experiments. For RT-PCR, mRNA expression was normalized to GAPDH. All data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed unpaired Student's *t*-test). ND, not detected.

Figure 5

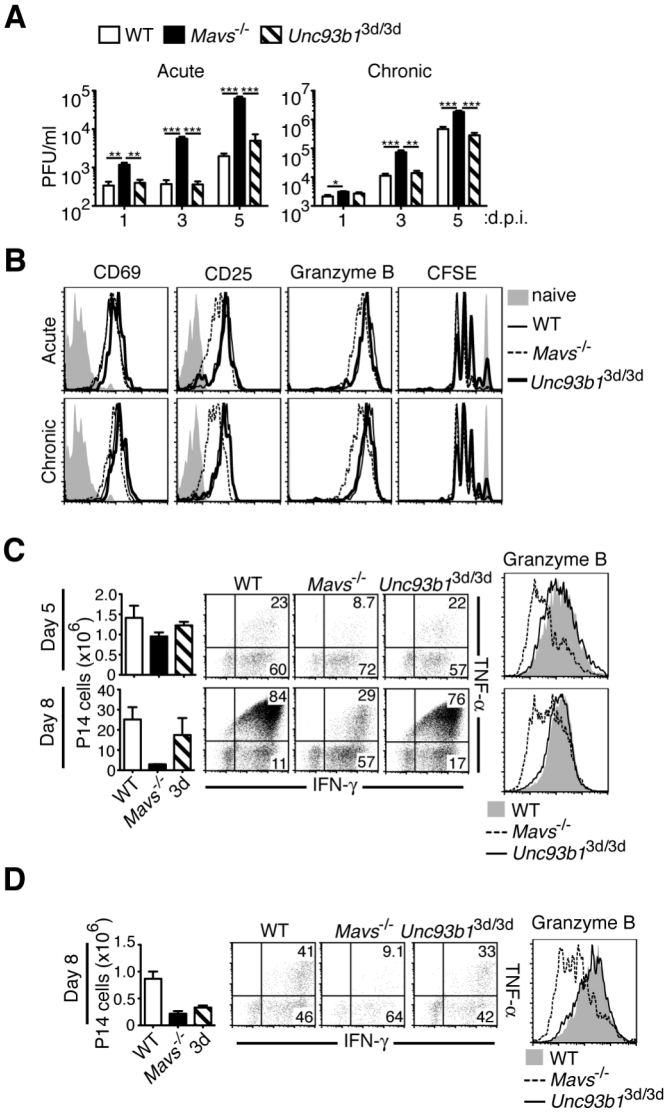

Figure 5. The RLR pathway is important for early control of viral replication and optimal CD8$^+$ T cell activation. (A) WT, *Mavs*$^{-/-}$, and *Unc93b1*$^{3d/3d}$ (3d) mice were infected with LCMV Arm (acute) or 2 × 10^6 PFU clone 13 (chronic) and viral titers in serum analyzed at days 1, 3, and 5 following infection by plaque assay (n=4-5). (B) CFSE-labeled CD45.1$^+$ P14 cells transferred into CD45.2$^+$ WT (solid thin line), *Mavs*$^{-/-}$ (dashed line), or 3d (thick solid line) recipient mice and infected one day later with LCMV Arm (acute), 2 × 10^6 PFU clone 13 (chronic), or left uninfected (shaded

histograms). 48 h p.i. cell division by CFSE dilution, and the expression of CD69, CD25, and granzyme B on transferred cells were determined by flow cytometry. Histograms are gated on CD45.1^{+} P14 cells. Expression levels and CFSE dilution in uninfected mice are shown in shaded histograms. (C and D) CD45.1^{+} P14 cells were adoptively transferred into CD45.2^{+} WT, *Mavs*$^{-/-}$, or 3d recipient mice and infected the next day with LCMV Arm (C) or 2 × 10^{5} PFU clone 13 (D). Graphs on the left show the number of P14 cells in spleens in each recipient group at the indicated times following infection. Expression of IFN-γ and TNF-α (density plots) and granzyme B (histograms) were determined by intracellular staining at days 5 (Arm) and 8 (Arm and clone 13) following infection. Plots and histograms are gated on transferred P14 cells. Data in A-D are representative of two or more independent experiments. For A, graphs show mean ± SEM. For C and D, graphs show mean ± range for a representative experiment (n=2). $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-tailed unpaired Student's *t*-test).

Figure 6

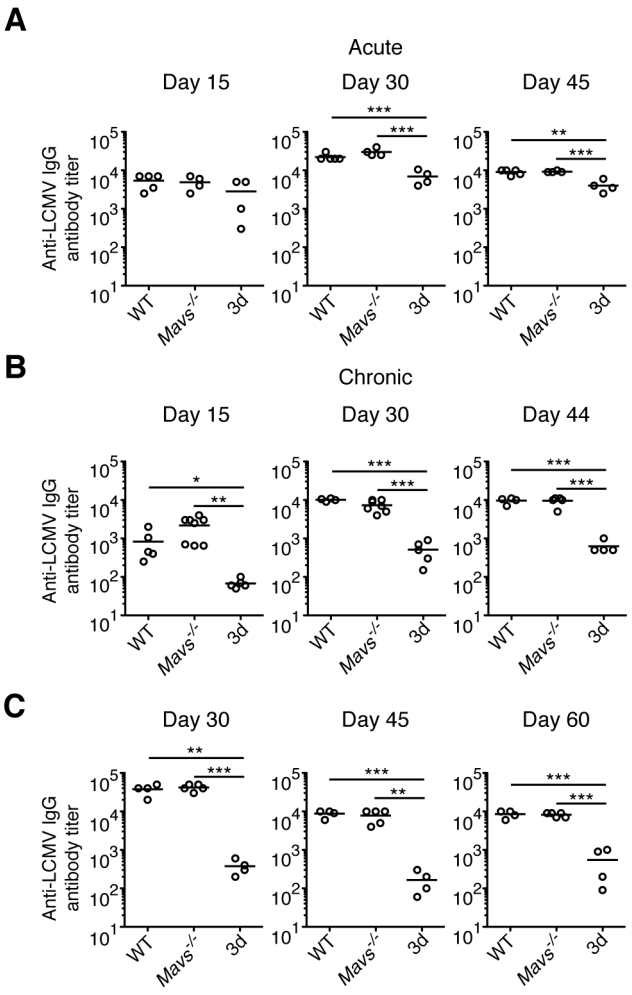

Figure 6. Nucleic acid-sensing TLRs are necessary for optimal virus-specific antibody responses in a chronic viral infection. WT, *Mavs*$^{-/-}$ and *Unc93b1*$^{3d/3d}$ (3d) mice were infected with LCMV Arm (acute) or clone 13 (chronic) and LCMV-specific IgG antibody titers in sera were measured by ELISA. (A) Antibody titers were analyzed in Arm-infected mice at day 15, 30 and 45 following infection (n=4-5). (B) Antibody titers at days 15, 30 and 44 following infection with 2×10^5 PFU clone 13 were determined in the indicated mice (n=4-8). (C) Antibody titers at days 30, 45 and 60 p.i. in indicated mice infected with 2×10^6 PFU clone 13 (n=4-5). Data are representative of

two or more independent experiments. Graphs show the mean as a line and each data point represents an individual mouse. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ (two-tailed unpaired Student's *t*-test).

Chapter 3

B cell-intrinsic TLR7 signaling is required for optimal B cell responses during chronic viral infection

Abstract

The importance for activation of innate immunity by pattern recognition receptors in forming an effective adaptive immune response is well known. Toll-like receptors (TLRs) have been demonstrated to be critical for antibody responses to a variety of immunizations. In particular, recent evidence suggests that B cell-intrinsic TLR signaling is required for optimal responses to virus-like antigens, but mechanisms by which TLR signaling impacts antibody responses during infection *in vivo* is unclear. In the present study, we demonstrate that deficiency of TLR7 in B cells alone is sufficient to significantly impact antibody responses during chronic viral infection. This effect was independent of T follicular helper cells, and resulted in a loss of plasma cells generated later, but not early, in the response. The defect in plasma cell formation appeared to be secondary to a qualitative effect of TLR signaling on the germinal center (GC) B cell response. GC B cells in TLR7-deficient mice proliferated to a lesser extent and had increased frequencies of light zone, relative to dark zone, GC B cells. These results suggest that B cell-intrinsic TLR signaling *in vivo* likely affects plasma cell output by altered selection of antigen-specific B cells in the germinal center.

Introduction

In recent years, there has been much interest in harnessing the activation of innate immunity for prevention and treatment of both acute and chronic infections of major concern to global public health (204). However, much remains to be understood about how engagement of different innate immune receptors contributes to protective immune responses. How these varying recognition pathways contribute to protective adaptive immune responses is further complicated by their broad expression among many immune cell types, and further, by non-hematopoetic cells (184). This is of particular interest as vaccines, such as live-attenuated viruses, can activate different classes of innate immune pattern recognition receptors including both Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs) (205). Therefore, an understanding of the role innate immune receptors play in the induction of protective immune responses in the context of a live infection can lend crucial insight to both basic biology and vaccinology.

Infection of mice with lymphocytic choriomengitis virus (LCMV) has served as a useful model to interrogate immune responses during the course of both acute and chronic viral infections. Whereas infection with the Armstrong (Arm) strain of LCMV results in acute infection that is eliminated approximately 8-10 days postinfection (p.i.), infection with the related variant clone 13 leads to persistent infection which lasts for two or more months (141). Additional manipulation of the immune response during chronic LCMV infection through either genetic means or $CD4^+$ T cell depletion leads to sustained high levels of viremia throughout the course of the life of the animal and has demonstrated requirements for both $CD4^+$ T cells and B cells, in addition to $CD8^+$ T cells for long-term virus control (156, 157, 159, 160, 170, 176). Although B cells may

contribute to clearance in non-antibody dependent ways (160, 176), antibody-dependent requirements have also been demonstrated (159). Furthermore, chronic LCMV infection drives differentiation of $CD4^+$ T cells into T follicular helper (Tfh) cells, and Tfh cell expression of the chemokine receptor CXCR5 was necessary for optimal antibody responses and viral clearance (175).

Recently, in an analysis of the role innate pattern recognition receptors (PRRs) play in the clearance of acute and chronic LCMV infections, we and another group identified defective antibody responses during chronic LCMV infection when virus recognition by nucleic acid-sensing TLRs, and in particular TLR7, was deficient (206, 207). Furthermore, TLR7 was shown to be required for efficient germinal center (GC) B cell formation, and *Tlr7*$^{-/-}$ B cells were less capable of forming antibody-producing cells (207), although whether B cell-intrinsic loss of TLR7 was sufficient for decreased antibody responses in *Tlr7*$^{-/-}$ mice, as a whole, was not addressed. These results are consistent with other recent studies describing a requirement for B cell-intrinsic TLR signaling for GC B cell formation in response to virus-like particles (VLP) and retroviral infection (132, 208). However, the general mechanism by which TLR signaling contributes to B cell responses *in vivo*, in particular during the course of a natural virus infection, remain largely ill defined.

In order to more precisely address the mechanism by which TLR7, and potentially TLR3, may impact the outcome of chronic infection with LCMV and the formation of B cell responses in general, we have undertaken more detailed studies to examine at what point, and for which cell types, TLR signaling is critical. Herein, we show that B cell-intrinsic TLR7 is necessary for control of chronic LCMV infection and optimal antibody

responses. On closer examination, chimera experiments revealed a GC B cell defect, but also a subsequent, secondary, defect in plasma cell formation. In the absence of competition, lack of TLR7 led to a delayed, but significant, decrease in plasma cell formation. Although we observed little quantitative impact of TLR7-deficiency on total GC B cell numbers, *Tlr7*$^{-/-}$ GC B cells proliferated to a lesser extent and were skewed in their distribution between light zone (LZ) and dark zone (DZ) cells. These results demonstrate that qualitative differences in the germinal center response in the absence of TLR signaling lead to defective plasma cell and antibody formation.

Materials and Methods

Mice

C57BL/6 (CD45.2$^+$) and B6.BoyJ mice (CD45.1$^+$) were purchased from the Jackson Laboratory or the National Cancer Institute. *Unc93b1* "3d" mice on a C57BL/6 background were purchased from the Mutant Mouse Regional Resource Center (University of California, Davis, CA) (20). *Tlr3*$^{-/-}$ and *Tlr7*$^{-/-}$ mice on the C57BL/6 background were purchased from the Jackson Laboratory (13, 17). Dr. M Wabl (University of California, San Francisco, CA) generously provided $J_H^{-/-}$ mice (209). All experiments were done in accordance with University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

Virus infection and titration

Mice were infected intravenously (i.v.) with 2×10^6 PFU LCMV clone 13, or LCMV Armstrong where stated. Virus was propagated on BHK cells and titration by plaque assay was carried out on Vero cells as previously described (141).

Bone marrow chimeras

Bone marrow chimeras were generated by lethally irradiating 6-8 week old B6.BoyJ (CD45.1$^+$) mice with γ-radiation from a Cesium source. B6.BoyJ mice were reconstituted with either a 50:50 mixture of WT (CD45.1$^+$) and *Tlr7*$^{-/-}$ (CD45.2$^+$) bone marrow, or a mixture of 85% $J_H^{-/-}$ and 15% WT, *Unc93b1*$^{3d/3d}$, or *Tlr7*$^{-/-}$ bone marrow. Mice were maintained on antibiotics for 6 wk during reconstitution then bled to determine the relative frequencies of B220$^+$ cells from each donor population, at which time they were removed from antibiotics and maintained in the absence of antibiotics until experimentation.

Flow cytometry, antibodies, and intracellular staining

All antibodies were purchased from BioLegend except for anti-mouse CD16+CD32 (2.4G2 mAb) and straptavidin-QDot605 (University of California, San Francisco Antibody Core Facility); anti-mouse GL-7 and B220 (eBioscience), anti-CXCR4 and anti-mouse IgD (BD Biosciences) and FITC-conjugated goat anti-mouse IgG (Caltag). Control hCLIP-tetramer and MHC class II tetramer (NIH tetramer core facility, Emory University, Atlanta, GA) staining for LCMV GP_{66-77}-specific $CD4^+$ T cells was performed as previously described (123). Intracellular staining was performed as previously described for cytokine staining, with the following modifications: cells were blocked with anti-mouse CD16+CD32 prior to staining and FITC-conjugated anti-mouse IgG was used in place of anti-cytokine antibodies (163). Flow cytometry was performed on LSRFortessa or FACSCalibur (BD Biosciences) cytometers and analyzed with FlowJo 9.0.2 software (TreeStar).

BrdU labeling and caspase activation

5-bromodeoxyuridine (BrdU) labeling and caspase activation in cells was performed as previously described (210). For BrdU assays, mice were given a single intraperitoneal dose of 3 mg BrdU (Sigma), in sterile phosphate-buffered saline, 2 h before being euthanized. A FITC BrdU Flow Kit (BD Pharmingen) was used to detect BrdU according to the manufacturer's instructions. To detect caspase activation, cells were incubated 30 min to 1 h at 37 °C and 5% CO_2 with FITC-VAD-FMK followed by washing and surface staining with antibodies for phenotypic analysis according to manufacturer's instructions (CaspGLOW; BioVision).

ELISA

Serum titers of LCMV-specific IgG were determined by ELISA as previously described (206).

Determination of antibody-secreting cells

LCMV-specific antibody-secreting cells (ASC) were quantified as previously described with slight modifications (211), using HA-mixed cellulose ester membrane 96-well plates (Millipore). Briefly, LCMV-infected BHK cell lysate was diluted and added to wells and then incubated overnight (O/N) at 4 °C. The next day, plates were washed once with PBS containing 0.1% Tween 20 (Bio-Rad), then blocked with 5% FBS in PBS for 30+ min. Following red blood cell lysis, three-fold dilutions of spleen and bone marrow cell preparations were added and incubated for 4 h at 37 °C and 5% CO_2. Antigen-bound IgG was detected first with biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch) followed by subsequent incubation with horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch), both O/N at 4 °C. Antibody-secreting cells were visualized by using freshly prepared chromogen substrate in which 0.5 ml of 3-amino-9-ethylcarbazole (Sigma) dissolved in dimethylformamide (8 mg/ml) was added to 9.5 ml 50 mM acetate buffer (pH 5.0), passed through a 0.45 μm filter, and 5 μl of 30% H_2O_2 added immediately before use. The reaction was terminated using tap water, and spots were analyzed with an EliSpot Reader System and EliSpot 3.5 software (AID).

Statistical analysis

All statistical analysis was performed by using a two-tailed unpaired Student's t-test, or one-way ANOVA where noted, with Prism software (GraphPad Software, Inc.).

Results

TLR7 but not TLR3 is important for clearance and antibody responses during chronic LCMV infection

We previously observed that mice containing a missense mutation in *Unc93b1* (3d), which affects signaling through TLR3, 7, and 9, when chronically infected with LCMV had relatively normal initial $CD8^+$ T cell function, but developed a poor LCMV-specific IgG response, which likely contributed to a failure to clear virus and subsequent $CD8^+$ T cell exhaustion (206). In contrast, although there was a slight defect in LCMV-specific IgG responses during acute infection in 3d mice, antibody responses were dependent on TLRs to a much lesser extent. As another report identified TLR7 as required for antibody responses in chronic infection (207), we sought to ascertain the role of TLR7 in antibody responses during acute infection (Fig. 1A). Consistent with our previous report, when we assessed titers of LCMV-specific IgG by ELISA at day 60 following acute infection, 3d and *Tlr7*$^{-/-}$ mice had slightly lower levels of IgG when compared to wild-type (WT) mice, though in the case of TLR7, this was not statistically significant.

It has been previously shown that both 3d and *Tlr7*$^{-/-}$ mice have greatly reduced levels of IgG in sera when compared to WT mice during chronic infection, and this correlates with inability to control virus replication long-term (206, 207). Although TLR3, and other nucleic acid-sensing TLRs are not required for control of acute LCMV infection (206, 212), the contribution of TLR3 to control of chronic LCMV infection has yet to be elucidated. In order to determine the necessity for TLR3 signaling for LCMV-specific antibody responses and clearance of chronic infection, we infected either WT mice, or mice deficient in various nucleic acid-sensing TLRs and assessed LCMV-

specific IgG and viral loads in sera of infected mice (Fig. 1B and C). We found that at day 67 p.i. with LCMV clone 13, antibody titers were reduced ten-fold or more in 3d, *Tlr7*$^{-/-}$, and *Tlr3*$^{-/-}$*Tlr7*$^{-/-}$, but not *Tlr3*$^{-/-}$ mice (Fig. 1B). In contrast, *Tlr3*$^{-/-}$ mice had levels of LCMV-specific IgG that, though differing from those in WT mice in a statistically significant manner, this difference was generally less than two-fold in magnitude. When we assessed virus clearance at approximately two months p.i., whereas WT mice cleared virus by this time point, 3d mice and those deficient in TLR7 maintained high viral loads (Fig. 1C). In addition, mice doubly deficient in TLR3 and 7 had levels of virus similar to that in both 3d and *Tlr7*$^{-/-}$ mice. In contrast, *Tlr3*$^{-/-}$ mice had normal kinetics of virus clearance. This result, along with the similar virus titers between 3d, *Tlr7*$^{-/-}$, and *Tlr3*$^{-/-}$ *Tlr7*$^{-/-}$ mice indicate that TLR3 is not necessary, and appears to play no additive or synergistic role in clearance of chronic LCMV infection. Additionally, TLR-dependent clearance of chronic LCMV infection seems to be directly proportional to the ability of mice to mount normal antibody responses, suggesting a critical role for this TLR-mediated function.

TLR7 is required in B cells for optimal LCMV-specific IgG responses

We previously hypothesized that nucleic acid-sensing TLRs may be required in a B cell-intrinsic manner to generate optimal antibody responses (206). We therefore sought to determine to what extent B cell-intrinsic TLR7 deficiency contributes to overall levels of LCMV-specific IgG, as other cell types, which may be involved in antibody responses, can also express TLR7 (184). To address the contribution of nucleic acid-sensing TLRs within B cells for LCMV-specific antibody responses, we generated bone marrow

chimeras consisting of 85% $J_H^{-/-}$ bone marrow, which cannot form B cells, and 15% WT, 3d, or *Tlr7*$^{-/-}$ bone marrow. Thus, B cells in these chimeric mice were derived entirely from the non-$J_H^{-/-}$ bone marrow, with all other cell subsets being WT. Assessment of B cell reconstitution in these mice prior to experimentation exhibited similar proportions of $CD19^+B220^+$ cells, indicating that the B cell compartment was restored in all chimeric mice (Fig. 1D). When we assessed titers of LCMV-specific IgG at day 30 p.i. in chronically infected chimeric mice, we found that antibody levels in *Tlr7*$^{-/-}$ and 3d chimeric mice were both reduced approximately ten-fold compared to WT, although this was not statistically significant, likely due to large variances in antibody responses in the chimeras. Therefore, TLR7 deficiency solely within B cells is sufficient to reduce LCMV-specific IgG levels to a large extent.

T follicular helper cell differentiation is not impaired in the absence of TLR7

In addition to B cell-intrinsic defects in *Tlr7*$^{-/-}$ mice, the formation of Tfh cells, which help drive GC responses and the production of antibody, may also be defective. This is of particular interest in LCMV infection since chronic infection has been shown to drive the differentiation of $CD4^+$ T cells into Tfh cells (175), and IL-6-dependent control of chronic LCMV infection was shown to be due to effects on Tfh cell differentiation (195). Furthermore, the expression of *Bcl6* and differentiation of Tfh cells is driven early by dendritic cells and later by B cells, both of which may be impacted in the absence of TLR7 signaling (126-130). We therefore assessed the formation of Tfh cells in *Tlr7*$^{-/-}$ and WT mice during acute and chronic LCMV infection. We analyzed Tfh cell responses by assessing the formation of Tfh cells among the endogenous $CD4^+CD44^{hi}$GP66-77-

tetramer^{+} population, using non-antigen specific hCLIP-tetramer as a negative control for our tetramer gating strategy (Fig. 2A). Tfh cells were identified as SLAM^{lo}CXCR5^{hi}PD-1^{hi} as previously described (127). We found that Tfh cell differentiation at days 8 and 15 during chronic infection, and day 15 during acute infection were not significantly impacted (Fig. 2B and C). The total numbers of endogenous Tfh cells per spleen were approximately equal in WT and *Tlr7*$^{-/-}$ mice regardless of infection or time point (Fig. 2C). Although the frequency of SLAM^{lo}CXCR5^{hi} Tfh cells varied somewhat between WT and *Tlr7*$^{-/-}$ mice, it is unlikely that such small differences in frequency represent a major impediment to Tfh cell differentiation (Fig. 2B and C). Based on these results, it is unlikely that decreased antibody responses in *Tlr7*$^{-/-}$ mice are a result of insufficient Tfh cells in these mice.

B cell-intrinsic TLR7 signaling is required for efficient germinal center B cell and plasma cell formation

Because loss of TLR7 signaling within B cells alone was sufficient to substantially affect LCMV-specific IgG responses (Fig. 1E), we sought to address at what stage during the B cell response TLR7 was required. Briefly, following stimulation by T cells, B cells are recruited into the GC reaction during which GC B cells undergo iterative rounds of selection leading to the production of long-lived plasma cells (213). In order to determine at what stage *Tlr7*$^{-/-}$ B cells are defective, and to isolate this defect to B cells, we generated 1:1 bone marrow chimeras in which WT mice were irradiated and reconstituted with 50% WT and 50% *Tlr7*$^{-/-}$ bone marrow marked by different CD45 congenic markers. As a control, 1:1 WT chimeras were also generated using WT bone marrow

containing either CD45 congenic marker. Prior to immunization, the proportions of $B220^+$ cells in blood derived from each donor population were determined. We first sought to assess the impact of TLR7 deficiency on the formation of GC B cells. At days 8 and 14/15 p.i. with LCMV Arm (acute) and clone 13 (chronic), we determined the frequencies of GC B cells derived from either WT or *Tlr7*$^{-/-}$ populations. We then calculated the ratios of WT (control) to WT (test) or *Tlr7*$^{-/-}$ (test) GC B cells and normalized it to the ratio within $B220^+$ cells of the same non-immunized chimeric mice, such that the data are expressed as the fold change following immunization in the relative representation by cells from either congenic donor (Fig. 3A and B). In this case, no preference for WT (control) or *Tlr7*$^{-/-}$ would be represented by a value of 1. If WT cells constitute a larger proportion of the GC B cell population than *Tlr7*$^{-/-}$ cells, this would result in a value greater than 1. We found that at either day 8 or 14/15 p.i., there was a large preference in GC B cells derived from WT, as opposed to *Tlr7*$^{-/-}$ B cells in WT/*Tlr7*$^{-/-}$ chimeric mice (Fig. 3B). Additionally, the preference for WT-derived GC B cells increased over time in both acute and chronic infections, but to a much lesser extent during acute infection. Interestingly, the requirement for TLR7 was much greater in chronic compared to acute infection. For example at day 14 or 15 p.i., the proportion of WT cells increased three- to four-fold over that of uninfected mice following acute infection, but was increased fifteen-fold at this time point during chronic infection. By comparison, a relative increase in WT ($CD45.1^+$) GC B cells was not seen in control WT/WT chimeras, indicating that this preference was not due to the difference in congenic markers used.

We then examined the formation of plasmablasts and plasma cells in the same chimeras (Fig. 3C and D). We determined the fold change in WT (control) relative to WT (test) and *Tlr7*$^{-/-}$ populations in the same way as we did for GC B cells. Similarly to results seen for GC B cells, we found that the preference for WT over *Tlr7*$^{-/-}$ was again increased over time and to a higher degree in chronic compared to acute infection (Fig. 3D). We further noticed that the magnitude of the difference in requirement for TLR7 appeared to be greater for plasmablasts and plasma cells than GC B cells, particularly for the formation of long-lived plasma cells in the bone marrow during chronic infection. In order to address whether this was the case, we compared the frequencies of WT or *Tlr7*$^{-/-}$ plasmablasts and plasma cells to those of GC B cells in the same mouse. We hypothesized that if there were no defect in the ability of *Tlr7*$^{-/-}$ GC B cells to form plasma cells, then the WT/*Tlr7*$^{-/-}$ frequency distribution of plasmablasts or plasma cells and GC B cells in the same chimeric mice should be equivalent. Therefore, if no additional effects on plasma cell differentiation were observed, this would mean that the ratio of plasma cells to GC B cells would be the same and equal 1. However, if there were an additional defect, then the frequency of plasma cells for the same population should be lower than that of GC B cells, and therefore this ratio would be less than 1. Using this method of analysis, we determined that, as expected, there were no differences in WT/WT chimeras in the plasma cell/GC B cell ratio (Fig. 3D). However, in WT/ *Tlr7*$^{-/-}$ chimeras, *Tlr7*$^{-/-}$ GC B cells appeared to be less capable of forming plasma cells, with the exception of day 8 during chronic infection. These data indicate that TLR7 signaling is required intrinsically within B cells for the optimal formation of GC B cells and further for effective differentiation into long-lived plasma cells.

TLR7 signaling is crucial for the formation of long-lived plasma cells, but not GC B cells in non-chimeric mice

Because our experiments with chimeras indicated a significant intrinsic requirement for TLR7 in B cells for the efficient formation of GC B cells and plasma cells, we perfomed a more detailed analysis of the antibody response in *Tlr7*$^{-/-}$ mice in order to identify the nature of the defect. At days 8, 15 and 30 during acute or chronic LCMV infection, we analyzed the frequencies of GC B cells and plasma cells. Surprisingly, despite the severe defects in *Tlr7*$^{-/-}$ GC B cells in chimeric mice, we found no significant differences in the frequency of GC B cells in spleens of WT or *Tlr7*$^{-/-}$ mice at any of the time points examined during either acute or chronic infection, although the percentage of GC B cells among IgD^{-} cells was slightly lower at day 8 in chronic infection (Fig. 4A). In contrast, when we compared the plasmablast and plasma cell responses between WT and *Tlr7*$^{-/-}$ mice during acute and chronic infection, we found near WT levels of plasma cells at day 8 p.i. in *Tlr7*$^{-/-}$ mice, but by day 15 this decreased to about two-thirds of WT during acute infection, and about one-fourth of WT during chronic infection, and these differences were sustained at day 30 (Fig. 4B). Furthermore, the magnitude of the plasmablast and plasma cell response was larger during chronic infection, and was sustained to a greater extent than that during acute infection.

We compared the total numbers of GC B cells in spleens of WT and *Tlr7*$^{-/-}$ mice during acute and chronic infection (Fig. 4C). For the most part, total numbers of GC B cells were similar between the two groups at all time points during acute and chronic infection, consistent with similar frequencies of GC B cells (Fig. 4A). Although total

numbers of GC B cells at day 15 were slightly elevated in *Tlr7*$^{-/-}$ mice during chronic infection, this was not statistically significant. However, consistent with a slight reduction in frequency during chronic infection, numbers of GC B cells at day 8 p.i. in both groups of mice were reduced in chronic infection, compared to those in acute (Fig. 4C). These results indicate that although *Tlr7*$^{-/-}$ B cells are restricted in their ability to form GC B cells while in competition with WT cells, an inability to form GC B cells in *Tlr7*$^{-/-}$ mice is an unlikely explanation for the severe defects in antibody production seen during chronic infection, although a slight lag in the formation of GC B cells, or the quality thereof, may contribute.

We assessed the numbers of plasma cells in WT and *Tlr7*$^{-/-}$ mice during acute and chronic infection. Consistent with the differences in frequencies, WT and *Tlr7*$^{-/-}$ mice had similar numbers of plasma cells at day 8 during the immune response, although there was small reduction in *Tlr7*$^{-/-}$ mice (Fig. 4D). However, by day 15, responses in *Tlr7*$^{-/-}$ mice were reduced approximately two-fold during acute and three-fold during chronic infection. Unlike acute infection, in which numbers were relatively similar at day 30, the reduction in splenic plasma cells in *Tlr7*$^{-/-}$ mice was also present at day 30 p.i. We also assessed numbers of LCMV-specific IgG-producing plasma cells in WT and *Tlr7*$^{-/-}$ mice by ELISpot assay (Fig. 4E). At day 8 p.i., the total numbers of IgG-producing plasma cells in spleen were relatively equivalent between WT and *Tlr7*$^{-/-}$ mice during chronic infection. However, by day 15 p.i., the presence of IgG-producing plasma cells in spleens of *Tlr7*$^{-/-}$ mice were reduced approximately two-fold, and this deficit continued to day 30 p.i. In contrast, and consistent with results from flow cytometric analysis (Fig. 4D), although numbers of LCMV-specific IgG-secreting cells in spleens were reduced about

two-fold during acute infection at day 15 in *Tlr7*$^{-/-}$ mice, numbers were similar to those in WT mice by day 30 (Fig. 4E). In addition to defective plasma cell formation in the spleen at days 15 and 30 p.i., we also found lower numbers of LCMV-specific ASCs in bone marrow at these time points in *Tlr7*$^{-/-}$ mice, with the exception of day 15 during chronic infection, when little difference was observed. Therefore, although the loss of TLR7 significantly impacts the formation of plasma cells during both acute and chronic LCMV infections, this effect seems to be somewhat magnified during chronic infection in both the magnitude of the decline in numbers compared to WT mice, but also the duration for which it persists.

TLR signaling is important for GC B cell proliferation and plasma cell survival

Although total numbers of spleen GC B cells were not affected in *Tlr7*$^{-/-}$, relative to WT mice, the large reduction in plasma cell formation indicated that these cell populations might be qualitatively different with respect to survival or proliferation. We first analyzed cell proliferation in GC B cells by incorporation of BrdU following a 2 h pulse (210). While we found a small, approximately 5%, difference in BrdU incorporation between WT and *Tlr7*$^{-/-}$ GC B cells at day 8 p.i. during chronic infection, by day 15, approximately half the frequency of GC B cells incorporated BrdU in *Tlr7*$^{-/-}$, relative to WT mice (Fig. 5A). In contrast, at day 15 during acute infection, fewer GC B cells were labeled with BrdU, and there was a smaller difference between WT and *Tlr7*$^{-/-}$ GC B cells, indicating that chronic infection may drive differences in GC B cell proliferation between WT and *Tlr7*$^{-/-}$ GC B cells.

We assessed cell death by using a fluorescent pan-caspase inhibitor that binds to activated caspases (210). When we analyzed the frequency of GC B cells that were labeled with this inhibitor, we observed a slight increase in *Tlr7*$^{-/-}$ GC B cells at day 8, but a small decrease at day 15 during chronic infection, and no effect during acute infection (Fig. 5B). Thus, as there seemed to be no consistent differences between WT and *Tlr7*$^{-/-}$ GC B cells in the frequency of cells with activated caspases, an increase in cell death by *Tlr7*$^{-/-}$ GC B cells is unlikely to lead to defective plasma cell formation. We then analyzed the frequency of CD138^{+}IgD^{-} plasmablasts and plasma cells that had caspase activation (Fig. 5C). Wheras only 1-2% of plasma cells at days 8 and 15 during chronic infection in WT mice were labeled positive for caspase activation, the frequency increased to approximately 4% in *Tlr7*$^{-/-}$ CD138^{+}IgD^{-} cells at day 8 and 5% at day 15. Although there was also a difference at day 15 during acute infection, this did not appear to be as large in magnitude. Therefore, the decrease in GC B cell proliferation in *Tlr7*$^{-/-}$ mice was associated temporally with defective plasma cell formation, with a larger decrease in GC B cell proliferation correlating with higher frequencies of plasmablasts and plasma cells with activated caspases.

TLR7 signaling affects the distribution of light zone and dark zone GC B cells

During antigen-driven selection in the GC, GC B cells are divided between a light zone (LZ), in which selection of high affinity B cells occurs, and the dark zone (DZ), during which positively selected GC B cells proliferate (213). Expression of the chemokine receptor CXCR4 is critical for localization of GC B cells to the DZ, and the expression of this receptor is associated with cell division (214, 215). Although the demarcation

between LZ and DZ cells is difficult to do based solely on the expression of CXCR4 alone, additional use of the makers CD86 and CD83 has provided a useful tool for differentiating between these populations with LZ cells being $CXCR4^{lo}CD86^{hi}CD83^{hi}$ and DZ cells being $CXCR4^{hi}CD86^{lo}CD83^{lo}$ (216). Therefore, given the differences in proliferation between WT and *Tlr7*$^{-/-}$ GC B cells, we assessed whether the relative frequencies of DZ and LZ GC B cells were different. At day 15 p.i. during acute LCMV infection, WT and *Tlr7*$^{-/-}$ GC B cells were similar in their frequencies of LZ and DZ GC B cells (Fig. 6A and B). In contrast, during chronic LCMV infection, the frequency distribution of LZ and DZ GC B cells in *Tlr7*$^{-/-}$ GC B cells relative to that in WT was skewed towards increased proportions of LZ GC B cells (Fig. 6A and B). As expected from these results, the ratio of DZ to LZ GC B cells was equivalent in WT and *Tlr7*$^{-/-}$ mice at day 15 following acute infection, but was lower in *Tlr7*$^{-/-}$ GC B cells at this time during chronic infection (Fig. 6C). Consistent with the DZ being where GC B cell proliferation occurs, we found that $BrdU^{+}$ GC B cells in both WT and *Tlr7*$^{-/-}$ mice expressed slightly higher levels of CXCR4 (data not shown). Therefore, consistent with our results that *Tlr7*$^{-/-}$ GC B cells proliferate to a lesser extent during the course of chronic LCMV infection (Fig.5A), TLR7 signaling is important for driving proliferation of GC B cells in the DZ.

Discussion

In the present study, we demonstrate that B cell-intrinsic TLR7 signaling is required for optimal antibody responses during the course of both acute and chronic LCMV infection, but to a greater extent in chronic infection. In the absence of TLR7, mice failed to form an effective long-lived plasma cell response, though early plasmablast and plasma cell responses were relatively unimpaired. This defect was linked to an altered GC response in which *Tlr7*$^{-/-}$ GC B cells proliferated less and had an abnormal LZ versus DZ distribution, with a slight increase in plasmablast and plasma cell caspase activation. These findings provide evidence that in the context of virus infection, TLR signaling in B cells augments long-lived plasma cell formation by alteration of the GC reaction.

Recently, both we and another group identified a necessity for nucleic acid-sensing TLRs in the control of infection with the chronic strain of LCMV, likely due to defective antibody responses in the absence of TLR signaling (206, 207), although we observed that this affect was not as significant during acute infection (206). Experiments using bone marrow chimeric mice revealed defective GC B cell formation during chronic infection in the absence of TLR7 signaling when in competition with WT cells (207). Examination of B cells from these chimeras also demonstrated that *Tlr7*$^{-/-}$ B cells were less capable of forming antibody-secreting cells. However, the role of TLR7 in antibody responses during acute infection, whether or not TLR7 signaling in B cells alone was sufficient for antibody responses, and whether other TLRs, such as TLR3, further contributed during chronic infection were not investigated. In this study, we demonstrate that TLR7, and not TLR3 is required for LCMV-specific antibody responses and clearance of chronic LCMV infection, and is required to a lesser extent for antibody

responses during acute infection. Despite LCMV forming dsRNA replication intermediates, it appears that signaling via TLR3 does not cooperate with TLR7, as *Tlr3*$^{-/-}$ *Tlr7*$^{-/-}$ and 3d mice were comparable to *Tlr7*$^{-/-}$ in virus load and antibody titer. Furthermore, when B cells alone were deficient in their ability to respond to TLR7 ligands, antibody responses were greatly decreased. These results were somewhat surprising given a recent study showing that immunization with multiple TLR ligands was synergistic in driving antibody responses when both were engaged on the same B cell (217), but may reflect the restriction of TLR3 expression among B cells to those of the marginal zone, and a limited role for TLR3 expression in cDCs for driving antibody responses (218).

Consistent with an earlier report, we found that in competition, *Tlr7*$^{-/-}$ cells were severely impaired in their ability to form GC B cells in competition with WT cells (207). Furthermore, our studies revealed that in the same chimeric mice, TLR7-deficient plasma cells were underrepresented relative to their proportion among GC B cells. Interestingly, the increase in preference for WT, over *Tlr7*$^{-/-}$ GC B cells and plasma cells increased over time, and was greater in chronically infected mice. Therefore, it seems as though chronic infection in particular exacerbated the need for TLR7 signaling in GC B cell and plasma cell development. Consistent with a subsequent defect in plasma cells in *Tlr7*$^{-/-}$ mice, we observed a slight increase in the frequency of plasma cells marked by a reagent that binds to activated caspases. However, as this was only 4-5% of all splenic plasma cells, it is unclear to what extent cell death alone is responsible for decreased plasma cell numbers in *Tlr7*$^{-/-}$ mice.

Although it was previously shown that *Tlr7*$^{-/-}$ mice had decreased frequencies of GC B cells relative to WT mice (207), we did not find significant differences in total numbers or GC B cells in spleens of *Tlr7*$^{-/-}$ versus WT mice, and minimal, if any, differences in frequencies. In contrast, despite robust formation of early plasmablasts and plasma cells at day 8 p.i., *Tlr7*$^{-/-}$ mice showed impaired long-lived plasma cell formation in both spleen and bone marrow, which was greater in both magnitude and duration in chronic, compared to acute, infection. This is similar to results seen in MyD88-deficient mice following infection with mouse polyoma virus (219). The effect of TLR7 on antibody responses and plasma cell formation was independent of an effect on endogenous Tfh cell numbers, despite recent studies demonstrating the importance of Tfh cells and IL-21, a cytokine produced by Tfh cells, for control of chronic LCMV infection (171-173, 175, 195). In light of a study demonstrating that administration of virus-like particles (VLPs) containing RNA was able to rescue antibody production by *Il21r*$^{-/-}$ B cells (137), through direct action on B cells in a MyD88-dependent manner (132), this may indicate that Tfh cells are necessary, but not sufficient, for optimal antibody responses during chronic LCMV infection when B cells lack TLR7. Alternatively, although Tfh cell numbers were unaffected, Tfh cells may be functionally impaired when B cells lack TLR7.

Upon investigation of the GC B cell response, TLR7 was shown to affect the proliferation, but not survival of GC B cells. This was due to a relative decrease in *Tlr7*$^{-/-}$ GC B cells in the DZ relative to the LZ. It has been previously reported that lipopolysaccharide (LPS) treatment of B cells increased proliferative responses of these cells when transferred *in vivo*, and that these cells preferentially localized to the DZ of

GCs, compared to non-LPS-treated cells (220). However, these cells were treated *in vitro* prior to transfer, and LPS treatment was shown to up-regulate expression of homing receptors such as CCR7, L-selectin, CXCR4, and CXCR5, as well as expression of the GC B cell marker GL-7. Therefore, it was unclear whether or not TLR signaling is generally required for DZ localization, or if these results reflected an altered homing and activation status of these cells with respect to non-treated cells prior to transfer. Here, we demonstrate during the course of a chronic viral infection, a requirement for TLR7 for optimal GC B cell proliferation and normal LZ versus DZ distribution.

How exactly this requirement for TLR7 signaling affects LZ versus DZ GC B cell formation is unclear, but could possibly be the result of changes in levels of genes differentially expressed between these subsets, such as those required for cell cycle entry (216). Furthermore, how this translates to the formation of long-lived plasma cells is unclear, but as proliferation and somatic hypermutation occur in the DZ (213), and long-lived plasma cells are preferentially selected from high-affinity GC B cells (221-223), decreased proliferation in *Tlr7*$^{-/-}$ GC B cells may result in a smaller pool of somatically hypermutated B cells that are less efficient than TLR7-sufficient cells at forming long-lived plasma cells. Although this may partly explain the exacerbated defect in chimeric mice, this likely does not account for defective responses in the absence of competition. Considering that *Tlr7*$^{-/-}$ plasma cell formation in chimeras was more affected than GC B cell formation, and the plasma cell and antibody defects in *Tlr7*$^{-/-}$ mice were more exacerbated than the defect in GC B cell proliferation, these results suggest that TLR7 signaling may additionally affect plasma cell output, possibly through survival or programming. Mechanistically, this may be through a qualitative difference in the ability

of *Tlr7*$^{-/-}$ GC B cells to interact with and be positively selected by Tfh cells, which helps drive clonal expansion (216). Together, these results indicate that manipulation of TLR signaling in B cells may provide therapeutic benefit in generating antibody responses during immunization or as treatment for chronic viral infections.

Acknowledgments

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Footnotes

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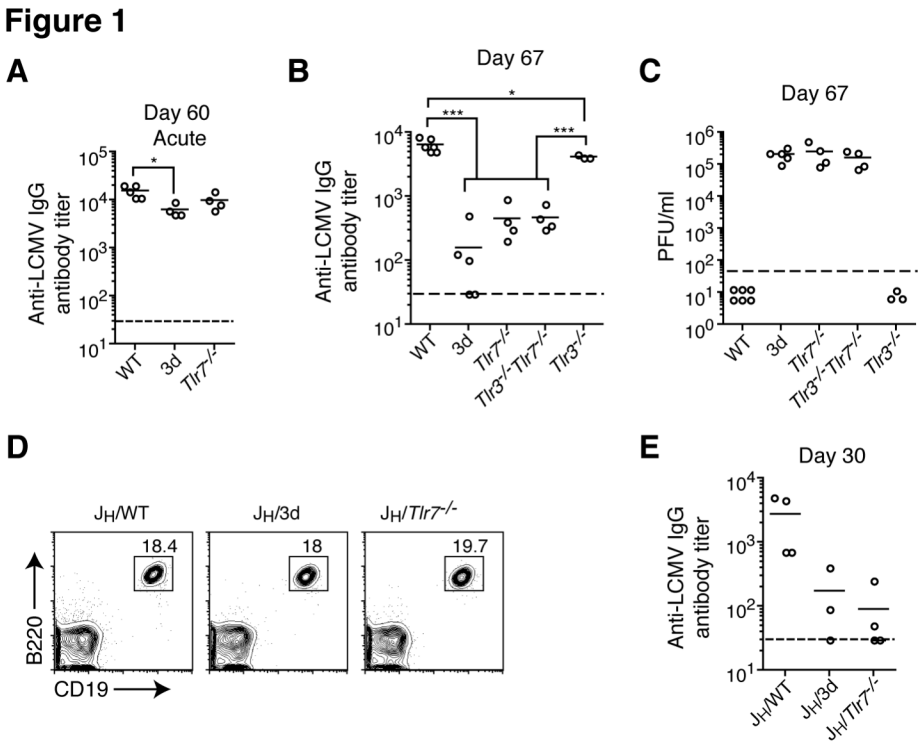

Figure 1. LCMV-specific IgG responses in chronically infected mice are dependent on B cell-intrinsic TLR7 signaling. (A) WT, 3d, or *Tlr7*$^{-/-}$ mice were infected with LCMV Arm and anti-LCMV IgG responses were measured by ELISA on day 60 p.i. (B) The indicated mice were infected with LCMV clone 13 and LCMV-specific IgG levels were determined by ELISA at day 67 p.i. (C) Titers of LCMV in sera at day 67 of mice infected in (B) were measured by plaque assay. (D) Bone marrow chimeras reconstituted with 85% $J_H^{-/-}$ (B cell-deficient) bone marrow and 15% WT, 3d, or *Tlr7*$^{-/-}$ bone marrow were bled at approximately six weeks and reconstitution of the B cell compartment was confirmed by flow cytometry. (E) Levels of LCMV-specific IgG in sera of chimeric mice were determined at day 30 p.i. with LCMV clone 13 by ELISA. Data in A-C are representative of two or more independent experiments. Data in D-E are representative of two independent sets of chimeric mice using either 3d or *Tlr7*$^{-/-}$ bone marrow. Dotted

lines indicate levels of detection. For ELISA, if samples fell below the limit of detection they were assigned a value of 30, which was the level of least dilution. Each data point represents an individual mouse with a bar indicating the mean. Statistics were calculated using one-way ANOVA. $*P < 0.05$ and $***P < 0.001$.

Figure 2

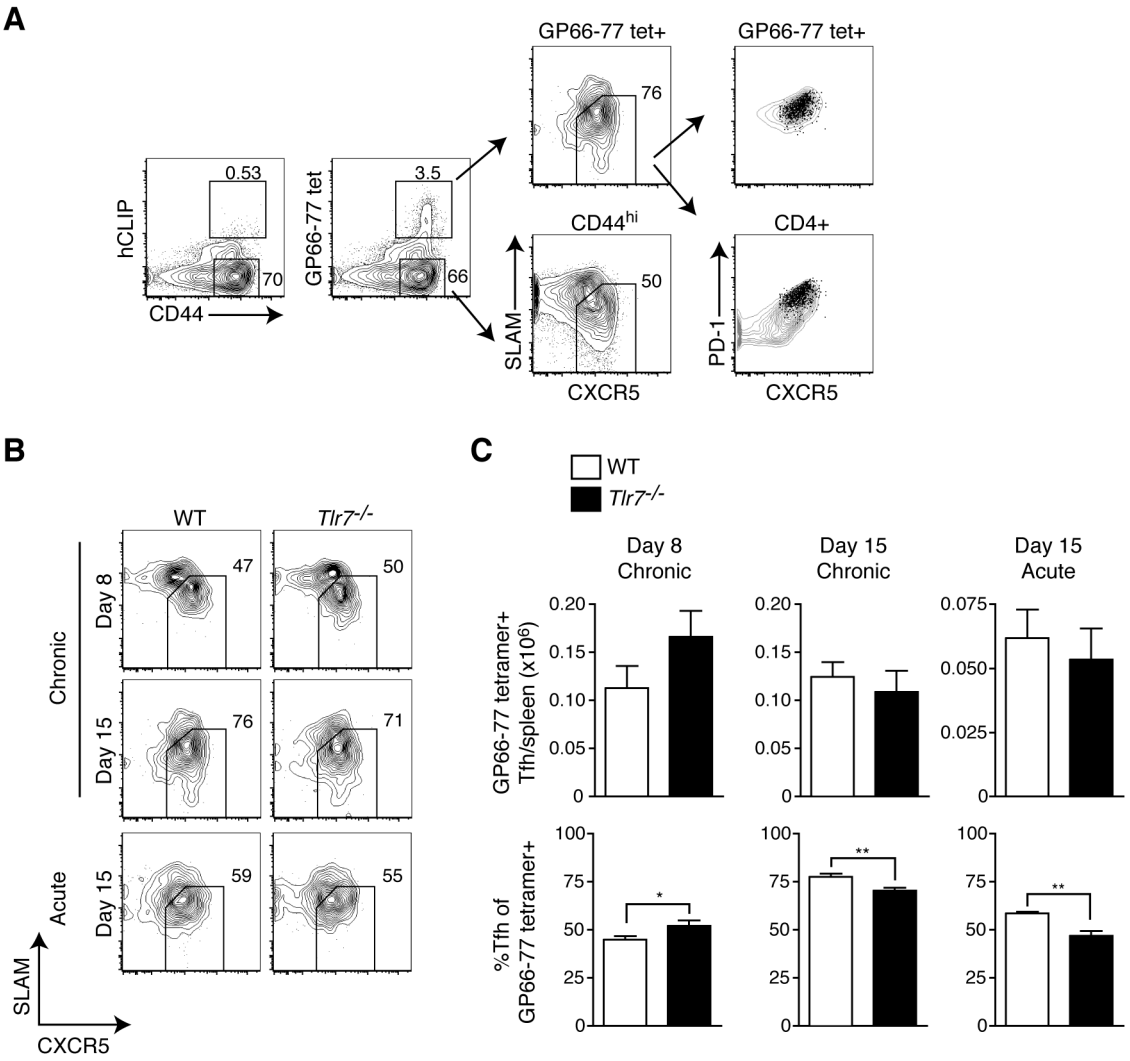

Figure 2. TLR7-dependent antibody responses are not a result of Tfh cell deficiency. WT or *Tlr7*$^{-/-}$ mice were infected with LCMV Arm (acute) or clone 13 (chronic) and the numbers and frequency of endogenous GP66-77 tetramer^{+} SLAM^{lo}CXCR5^{+} Tfh cells were determined in spleens of infected mice at the indicated time points. (A) A sample gating strategy for identifying endogenous Tfh cells by flow cytometry is shown. Staining is shown for tetramer control (left); SLAM^{lo}CXCR5^{+} CD4^{+} T cells in GP66-77 tetramer^{+} versus CD44^{hi}, non-tetramer^{+} populations; and the expression of PD-1 and

CXCR5 in GP66-77 tetramer^{+}SLAM^{lo}CXCR5^{+} (dot plots) versus all GP66-77 tetramer^{+} CD4+ T cells (right, top) or all CD4^{+} T cells (right, bottom) shown as contour plots. (B) Representative contour plots for Tfh cells are shown in WT and *Tlr7*$^{-/-}$ mice for the indicated time points during acute or chronic LCMV infection. Cells showing in plots were gated on CD4^{+}CD44^{hi}GP66-77 tetramer^{+} cells. (C) Numbers of CD44^{hi}GP66-77 tetramer^{+} Tfh cells in spleens of WT and *Tlr7*$^{-/-}$ mice are shown (top). The frequencies of SLAM^{lo}CXCR5^{+} cells among the GP66-77 tetramer^{+} population are depicted (bottom). Data in B-C for LCMV Arm are from two experiments (n=4-6 mice per group). Data for LCMV clone 13 day 8 are from two experiments (n=5-6 mice per group) and for day 15 are from five experiments (n=11 mice per group). Statistics were calculated using two-tailed unpaired Student's *t*-test. Graphs show mean ± SEM. **P* < 0.05 and ***P* < 0.01.

Figure 3

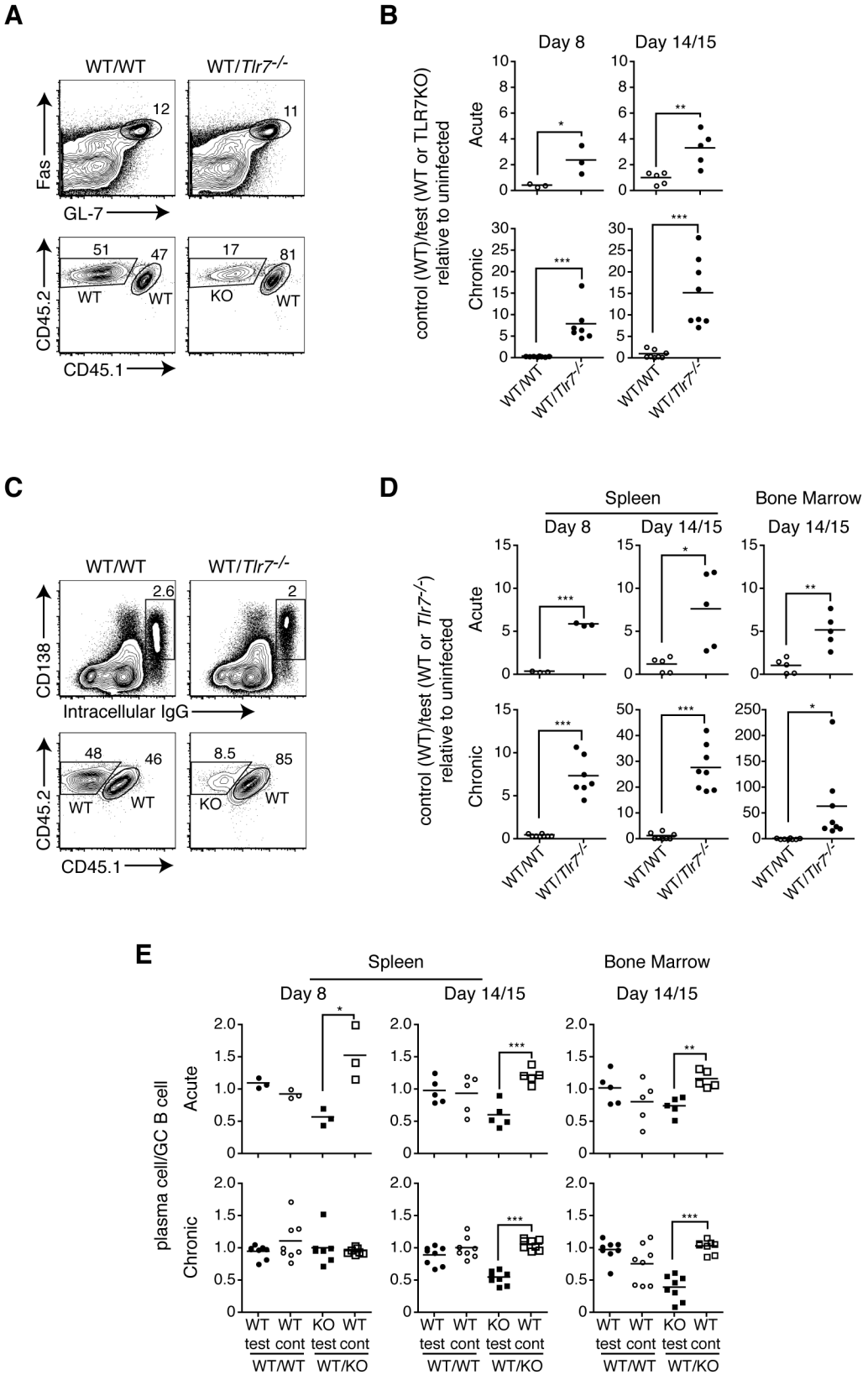
A
WT/WT
WT/Tlr7-/-
Fas
GL-7
CD45.2
CD45.1
B
Day 8
Day 14/15
Acute
Chronic
control (WT)/test (WT or TLR7KO) relative to uninfected
C
CD138
Intracellular IgG
D
Spleen
Bone Marrow
E
plasma cell/GC B cell
test cont
WT/WT
WT/KO

Figure 3. TLR7 signaling is required for effective GC B cell and plasma cell formation in mixed bone marrow chimeras. WT/WT or WT/*Tlr7*$^{-/-}$ 1:1 bone marrow chimeras were infected with LCMV Arm (acute) or clone 13 (chronic) and the frequency of GC B cells and plasma cells at days 8 and 15 p.i. from either donor was determined. (A) A representative example is shown for flow cytometric analysis of WT or *Tlr7*$^{-/-}$ donor-derived GC B cell populations. Cells depicted in contour plots showing GC B cells (top) were gated on CD4^{-}B220^{+} cells. Later experiments gated on CD4^{-}B220^{+}IgD^{-} cells, with similar results. Cells depicted in plots showing CD45.1 versus CD45.2 expression were gated on GC B cells (bottom). (B) The ratio of GC B cells derived from WT control to that derived from WT or *Tlr7*$^{-/-}$ test populations was determined and normalized to the B cell ratio in the same uninfected chimeric mouse to calculate the fold change relative to the WT control population. Results are shown for LCMV Arm (top) and clone 13 (bottom) for the indicated time points. (C) Sample contour plots to identify IgG-producing plasma cells in spleen are shown (top). Cells shown in plots were gated on IgD^{-} B cells. CD45.1 versus CD45.2 expression identifying WT or *Tlr7*$^{-/-}$ donor-derived plasma cells is shown (bottom). (D) The fold change in WT control-derived IgG-producing plasma cells was calculated as described in (B) and determined at the indicated time points in spleen and bone marrow for Arm (top) or clone 13 (bottom) LCMV-infected chimeric mice. (E) In the same experiments as A-D, the proportions of WT or *Tlr7*$^{-/-}$ donor-derived plasma cells was normalized to the proportion of corresponding WT or *Tlr7*$^{-/-}$ donor-derived GC B cells in the same chimeric mouse for LCMV Arm (top) and clone 13 (bottom) infection. Data in A-E represent collective data from three sets of independently derived bone marrow chimeras (n=3 or more mice per group). Each data

point represents an individual mouse with a bar denoting the mean. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Figure 4

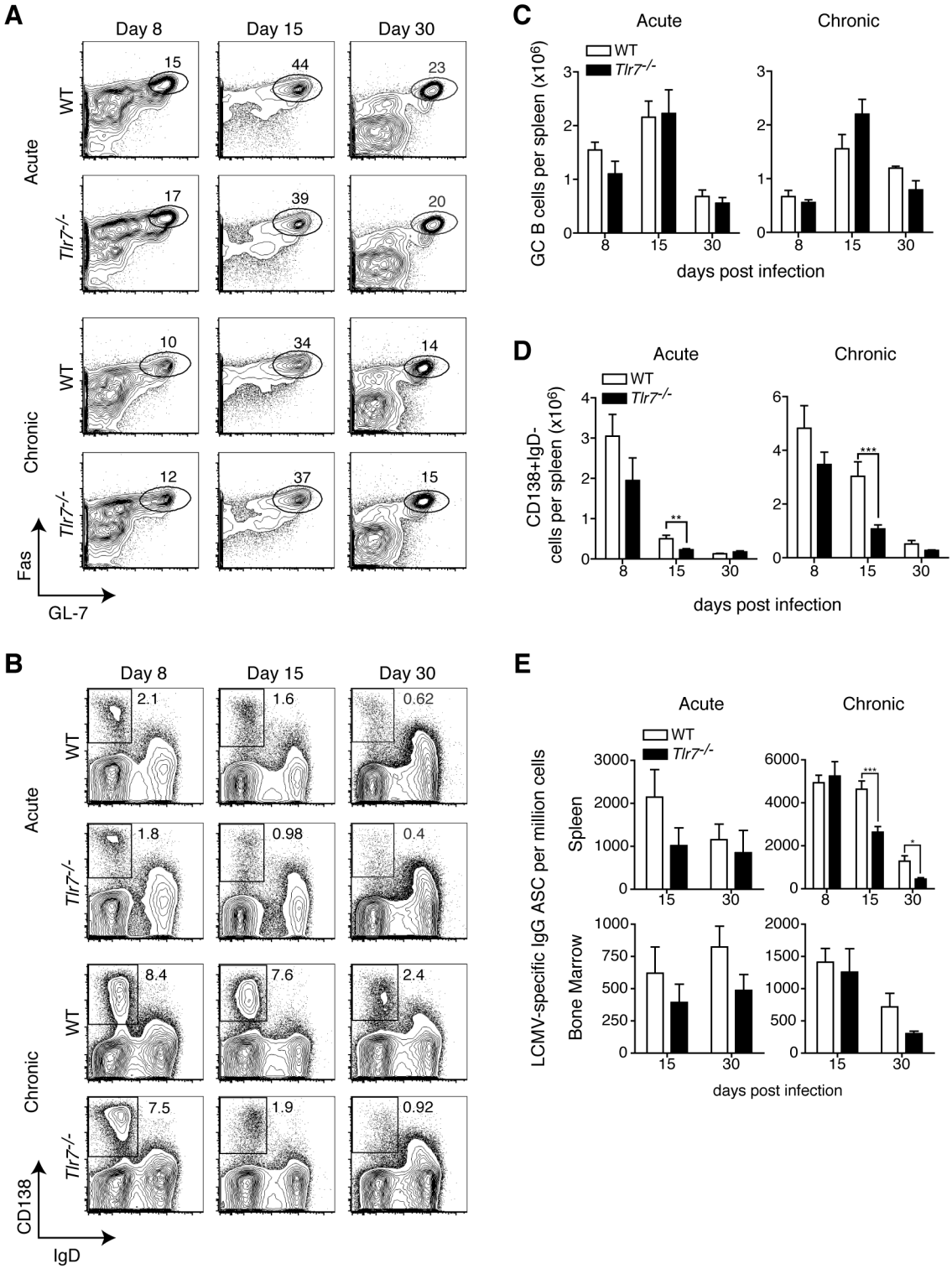
A
Day 8
Day 15
Day 30
Acute
Chronic
WT
Tlr7-/-
15
44
23
17
39
20
10
34
14
12
37
15
Fas
GL-7
B
2.1
1.6
0.62
1.8
0.98
0.4
8.4
7.6
2.4
7.5
1.9
0.92
CD138
IgD
C
GC B cells per spleen (x10^6)
days post infection
D
CD138+IgD- cells per spleen (x10^6)
E
LCMV-specific IgG ASC per million cells
Spleen
Bone Marrow

Figure 4. TLR7 signaling is required for formation of plasma cells, but not GC B cells, in non-chimeric mice. WT or *Tlr7*$^{-/-}$ mice were infected with LCMV Arm (acute) or clone 13 (chronic) and the frequencies and numbers of GC B cells and plasmablasts and plasma cells in spleens were determined. (A) Representative flow cytometry analysis of spleen GC B cells for acute or chronically infected WT and *Tlr7*$^{-/-}$ mice is shown for days 8, 15 and 30 p.i. Cells shown in plots were gated on CD4^{-}B220^{+}IgD^{-} cells. (B) Spleen plasma cell gating for the same mice as in (A) is shown. (C) Numbers of GC B cells in spleens of WT and *Tlr7*$^{-/-}$ mice were calculated at the indicated time points following infection with LCMV Arm or clone 13. (D) For the same mice as in (C), total numbers of plasmablasts and plasma cells in spleen were calculated. (E) Numbers of LCMV-specific IgG secreting cells were enumerated in WT or *Tlr7*$^{-/-}$ mice during acute or chronic infection at the indicated time points. Data in A-E are compiled from two or more independent experiments (n=3-20 mice per group). Statistics were calculated using two-tailed unpaired Student's *t*-test. Graphs show mean ± SEM. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Figure 5

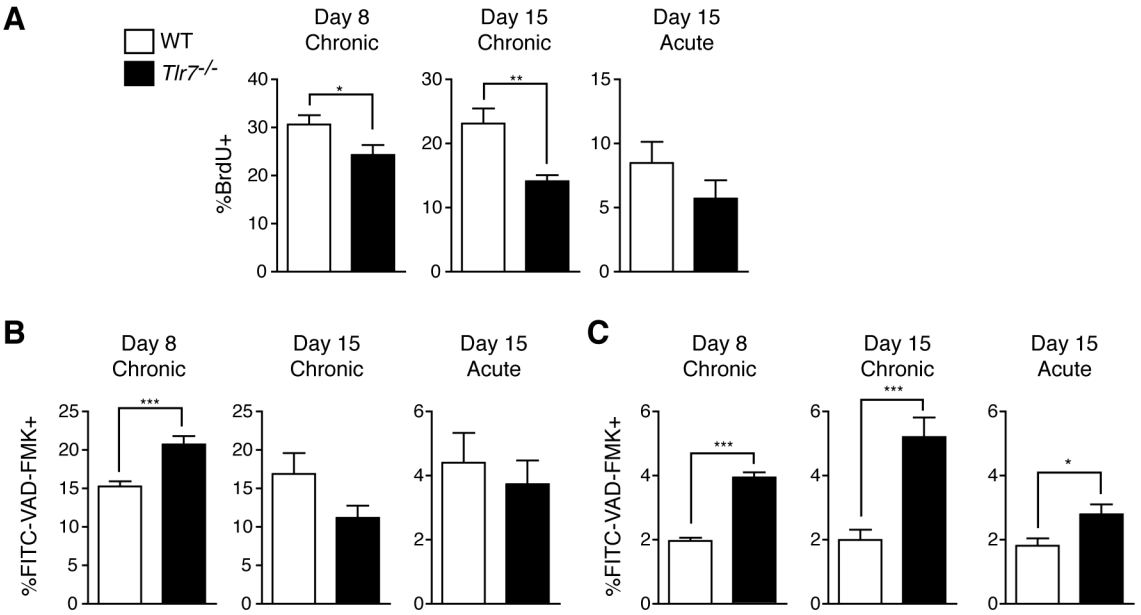

Figure 5. Decreased GC B cell proliferation and increased plasma cell death during chronic LCMV infection. (A) Proliferation of GC B cells in spleens was measured by BrdU incorportation in WT or *Tlr7*$^{-/-}$ mice following infection with LCMV Arm (acute) or clone 13 (chronic). The data is expressed as the frequency of GC B cells that incorporate BrdU during a 2 h pulse. (B) and (C) Caspase activation in GC B cells (B) and spleen plasmablasts and plasma cells (C) was measured by FITC-VAD-FMK, which binds to active caspases. The frequencies of GC B cells or CD138^{+}IgD^{-} plasmablasts and plasma cells that were marked by FITC-VAD-FMK are shown. Data in A-C are from three or more independent experiments (n=5-16 mice per group). Statistics were calculated using two-tailed unpaired Student's *t*-test. Graphs show mean ± SEM. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Figure 6

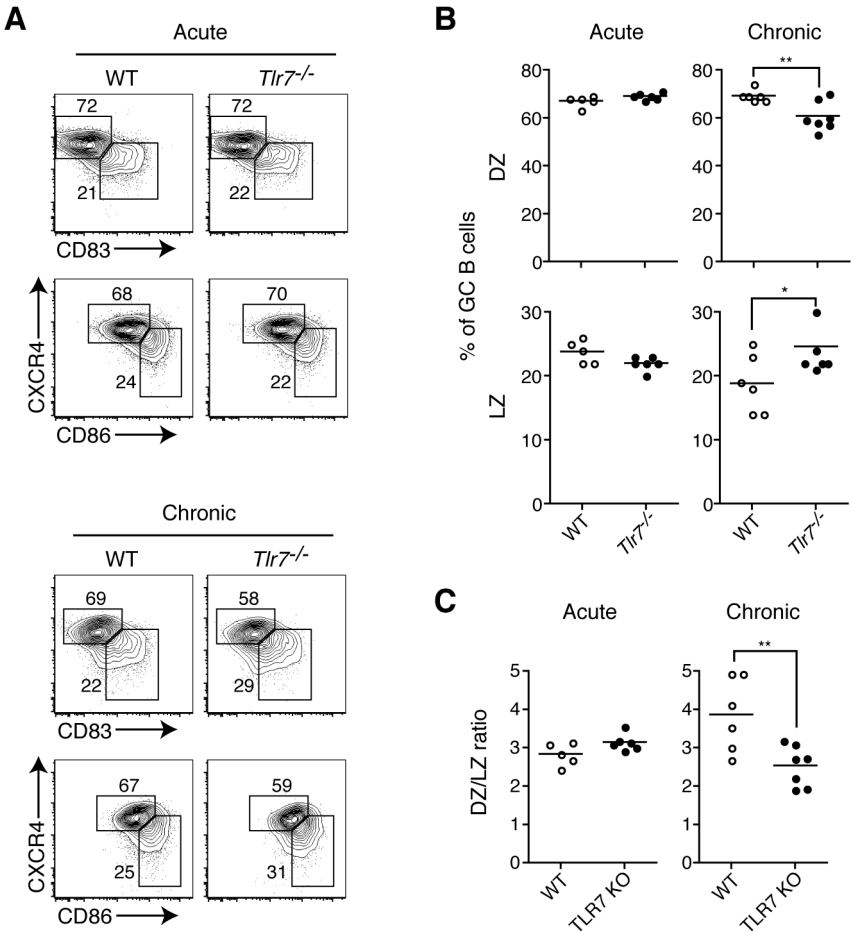

Figure 6. TLR7 deficiency alters GC B cell light zone/dark zone distribution in chronic, but not acute infection. WT and *Tlr7*$^{-/-}$ mice were infected with LCMV Arm (acute) or clone 13 (chronic) and the distribution GC B cells among light zone (LZ) and dark zone (DZ) populations at day 15 p.i. were assessed by flow cytometry. (A) Representative contour plots are shown for GC B cell LZ and DZ populations at day 15 following acute (top) or chronic (bottom) LCMV infection, using two different strategies. Cells shown in contour plots were gated on live (DAPI^{-}), CD4^{-}B220^{+}IgD^{-}Fas^{+}GL-7^{+} cells. (B) Frequencies of LZ and DZ GC B cells. (C) Ratios of DZ to LZ GC B cells from mice in (A) were calculated. Data in A-C are from two to three independent experiments

(n=5-7 mice per group). Statistics were calculated by using a two-tailed unpaired Student's *t*-test. Data points on graphs represent individual mice with the means indicated by bars. $*P < 0.05$, $**P < 0.01$.

Chapter 4

Regulation of plasmacytoid dendritic cell responses following initial activation during virus infection

Abstract

Plasmacytoid dendritic cells (pDCs) have been well studied as they are capable of secreting large amounts of type I interferon (IFN), and have been implicated both in protection from infection, but also autoimmune diseases characterized by inappropriate production of type I IFNs. However, the fate of pDCs following initial activation, and their contribution to secondary or heterologous immune responses has not been well characterized. In this study, we demonstrate that pDCs are rapidly lost following virus infection in a manner consistent with intrinsic activation through Toll-like receptors (TLRs). In addition, pDCs that were present at later times of infection were impaired in their ability to make type I IFNs regardless of the nature of virus infection. This unresponsive state was independent of previous cell-intrinsic TLR-dependent activation, and was not rescued by genetic deletion of adaptors known to transduce signals from pDC inhibitory receptors. These results suggest that pDC responses following activation are tightly regulated through both activation-dependent and -independent mechanisms.

Introduction

Plasmacytoid dendritic cells (pDCs) have long been recognized as specialized type I interferon (IFN)-producing cells, which are capable of secreting large amounts of type I IFN in response to virus infection (87, 191). Normally, in the context of virus infection, type I IFN is protective, as evidenced by increased pathogenesis in mice lacking the type I IFN receptor (IFNAR), or in humans with deficient type I IFN responses (101, 102, 155, 224). However, in the context of autoimmunity, pDCs can be activated by self-nucleic acids and aberrant pDC activation and type I IFN can contribute to the pathogenesis of autoimmune diseases such as systemic lupus erythematosus and psoriasis (87, 191, 225-227). Therefore, an understanding of pDC activation and the mechanisms by which aberrant pDC activation is controlled have garnered great attention.

Plasmacytoid dendritic cells are activated following engagement by pattern recognition receptors (PRRs) that recognize nucleic acids. In particular, pDCs utilize Toll-like receptor (TLR)-7 and TLR9, which recognize ssRNA and DNA, respectively (5, 14-18, 87, 191). Induction of type I IFNs downstream of TLR7 and 9 is dependent on the adaptor MyD88, and phosphorylation of the transcription factor interferon regulatory factor 7, which induces transcription of type I IFNs (76, 85). Additionally, these nucleic acid-sensing TLRs normally localize to endosomes, and the proper subcellular localization of these TLRs, which is dependent on the ER resident protein UNC93B, is necessary for their function (20, 21). Mice containing a missense mutation in the gene encoding UNC93B, *Unc93b1*, are incapable of signaling through TLR7, TLR9, or TLR3, which is also localized to the endosome, and are termed ‘3d’ due to a failure to respond to ligands to these three TLRs. Furthermore, pDC activation is modulated by various

activating and inhibitory receptors, most of which use the adaptors DAP12 and FcRγ (87, 191).

Following activation, the fate of pDCs is unclear. It has been demonstrated that pDC numbers are reduced following infections with various viruses or administration of TLR ligands (189, 228-230). Type I IFN led to loss of pDCs, and cell death was rescued to an extent in IFNAR-deficient mice. However, although this was described as separate from cell-intrinsic activation, a similar reduction was observed in *Myd88*$^{-/-}$ mice, and it therefore remains unclear to what extent cell-intrinsic activation may play a role in early loss of pDCs following infection. Additionally, pDCs do remain following infection, but appear to be unresponsive to either high levels of viremia (in the case of chronic infection) or administration of the TLR9 ligand, CpG (189). However, the precise mechanism by which pDC function is regulated at these later times following infection remains unknown.

In order to more precisely address these questions regarding pDC function *in vivo*, we have employed the use of an IFN-β reporter mouse (Mob) in which an internal ribosome entry site (IRES)-YFP cassette is inserted downstream of the endogenous *Ifnb1* locus in a manner that preserves the *Ifnb1* message and does not alter IFN-β production (185), and which we have shown is effective at marking type I IFN-producing pDCs in response to lymphocytic choriomeningitis virus (LCMV) infection (206). We demonstrate that pDCs were activated in a TLR7-dependent manner, and that decreased pDC numbers following infection, and increased caspase activation in pDCs was dependent on TLR7 signaling. Consistent with increased cell death in activated pDCs, IFN-β-YFP expression was associated with increased caspase activation in pDCs. Upon

examination of the pDC activation status following LCMV infection, we found that pDCs were not activated during chronic infection and were reduced in responsiveness to CpG as long as 30 days following acute LCMV infection. Unresponsiveness to CpG was common across other virus infections as well, and surprisingly, in the case of LCMV, was not dependent on previous TLR7-mediated activation, presence of pDCs during the initial phase of the immune response, or the adaptors DAP12 and FcRγ. These results demonstrate clearly that both cell-intrinsic and cell-extrinsic factors combine to prevent prolonged pDC activation and may have important implications regarding the role of pDCs during conditions of chronic inflammation or as participants in the immune response to secondary infections.

Materials and Methods

Mice

C57BL/6 were purchased from the Jackson Laboratory and maintained in our mouse colony. *Unc93b1* "3d" mice on a C57BL/6 background were purchased from the Mutant Mouse Regional Resource Center (University of California, Davis, CA) (20). *Tlr7*$^{-/-}$ mice on the C57BL/6 background were purchased from the Jackson Laboratory (17). *Myd88*$^{-/-}$ mice on a C57BL/6 background were obtained from Dr. A. DeFranco (University of California, San Francisco, CA (UCSF)) and maintained in our mouse colony (187). Dr. R. Locksley provided Mob (IFN-β) reporter mice, which were maintained in our mouse colony (UCSF) (185). Blood dendritic cell antigen 2 (BDCA2)-diphtheria toxin receptor (DTR) transgenic (Tg) mice were purchased from the Jackson Laboratory (88). Mob-*Tlr7*$^{-/-}$ and Mob-BDCA2-DTR Tg mice were generated by crossing Mob with *Tlr7*$^{-/-}$ or BDCA2-DTR Tg mice. *Tyrobp*$^{-/-}$ *Fcer1g*$^{-/-}$ (DAP12+FcRγ double knockout (DKO)) mice were obtained from Dr. M. Nakamura and Dr. C. Lowell (UCSF) (231). All experiments were done in compliance with University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

Viral infections

LCMV Armstrong and clone 13 stocks were propagated in BHK cells and virus titration carried out on Vero cells as previously described (141). For LCMV infections, mice were infected with 2×10^6 PFU LCMV Armstrong (Arm) or clone 13 intravenously (i.v.) where indicated. Dr. Raul Andino (UCSF) provided vesicular stomatitis virus (VSV) (Indiana strain), and Dr. Lewis Lanier (UCSF) provided mouse cytomegalovirus

(MCMV) (Smith strain). For VSV infections, mice were infected with 2×10^5 PFU i.v. For MCMV infections, a dose of 5×10^4 PFU i.v. was used.

CpG and diphtheria toxin (DT) treatments

CpG oligodeoxynucleotide (ODN) 1018 (5′ -TGACTGTGAACGTTCGAGATGA-3′), a type B CpG containing a phosphorothioate backbone (Dynavax, Inc.), was generously provided by B. Hou and A. DeFranco (UCSF) (132). For CpG treatment, mice were injected with 10 μg CpG complexed with DOTAP (Roche) by an i.v. route. Per mouse, CpG-DOTAP was made by adding 10 μg CpG to 100 μl PBS and 20 μl DOTAP to 80 μl PBS separately before diluted CpG was added to the DOTAP in a dropwise manner. CpG-DOTAP complexes were allowed to form for 10-15 min prior to injection. Mice were analyzed 6 h following treatment with CpG. For experiments with BDCA2-DTR Tg mice, DT (Sigma) was administered intraperitoneally (i.p.), 150 ng/mouse in PBS, on days -1, 1, 3, and 5 p.i..

Flow cytometry, antibodies and chemokine receptor staining

All antibodies were obtained from BioLegend except for anti-mouse CD16+CD32 (2.4G2 mAb), straptavidin-QDot605, anti-CD19 and anti-CD4 (University of California, San Francisco Antibody Core Facility), anti-mouse PDCA-1, CD80, CD11b and B220 (eBioscience), and anti-mouse TCRβ, CD11b and CD3ε (BD Biosciences), biotin-conjugated goat anti-human IgG (Jackson ImmunoResearch). Dr. J. Cyster provided mouse CCL19-human IgG1 Fc fusion protein (CCL19-Fc). For CCR7 staining, cells were first re-sensitized by incubating at 37 °C for 30 min. Cells were then stained with CCL19-Fc, followed by biotin-conjugated goat anti-human IgG (in staining buffer with 2% normal mouse and normal rat sera to neutralize antibodies cross-reacting with mouse

or rat Ig). Antibodies for surface staining were then added along with fluorophore-conjugated streptavidin. Flow cytometry was performed on LSRFortessa or FACSCalibur (BD Biosciences) cytometers and analyzed with FlowJo 9.0.2 software (TreeStar).

Cell Sorting

Plasmacytoid DCs were sorted from the spleens of uninfected, day 1, or day 8 LCMV clone 13-infected mice. Enrichment of pDCs and the sorting strategy have been previously described (206).

Caspase activation

To detect caspase activation, cells were incubated for 30 min to 1 h at 37 °C and 5% CO_2 with Red-VAD-FMK followed by washing and surface staining with antibodies for phenotypic analysis according to the manufacturer's instructions (CaspGLOW; BioVision).

Quantitative RT-PCR

Isolation of RNA from tissues and cDNA synthesis was performed as previously described (206). Gene expression was determined by quantitative PCR using FastStart Universal SYBR Green Master (ROX; Roche) on ABI 7300 or QuantStudio 12K Flex Real-Time PCR Systems (Applied Biosystems). Expression of mRNA is shown relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sets for IFN-β, IFN-α and GAPDH (206), LCMV S Segment (232), and IL-6, IL-12p40 and TNF-α (131) have been previously described.

Statistical analysis

All statistical analyses were performed using either a two-tailed unpaired Student's t-test or two-way ANOVA, as indicated, with Prism software (GraphPad Software, Inc.).

Results

Plasmacytoid DCs are activated and undergo cell death in a TLR7-dependent manner

In order to better understand the pDC response early following virus infection, we used IFN-β reporter mice (Mob), which we have previously shown to become highly expressed in pDCs following infection with LCMV in a manner dependent on nucleic acid-sensing TLRs (206). Reporter expression dependence on nucleic acid-sensing TLRs was consistent with results using sorted pDCs, indicating that Mob mice can be useful to assay pDC function *in vivo*. As pDCs do not express TLR3 (5), and LCMV does not contain any known DNA replication intermediates that may stimulate TLR9, we anticipated that pDC activation in response to LCMV infection was dependent on TLR7. To confirm that pDC activation in response to LCMV infection *in vivo* was dependent on TLR7, we generated Mob-*Tlr7*$^{-/-}$ mice and assayed IFN-β reporter expression by flow cytometry at 1 day postinfection (p.i.), which corresponds roughly to the peak of both the systemic type I IFN and pDC responses (206). Consistent with our previous results using Mob-*Unc93b1*$^{3d/3d}$ (3d) mice, and a previous report showing TLR7-dependent production of type I IFN by pDCs in LCMV-infected mice when cultured *ex vivo* (233), we found that whereas approximately 40% of pDCs expressed the IFN-β reporter in Mob mice, Mob-*Tlr7*$^{-/-}$ pDCs failed to turn on reporter expression (Fig. 1A). We further assessed pDC activation in Mob or *Tlr7*$^{-/-}$ mice by determining the expression of the co-stimulatory molecules CD80 and CD86 and the chemokine receptor CCR7, which are induced upon activation in pDCs (Fig. 1B). We found that CD86, CD80 and CCR7 were all induced after activation of pDCs on day 1 p.i. in Mob mice. YFP^{+} pDC most highly

expressed CD80 and CCR7, whereas expression of CD86 was only marginally increased, if at all, above that of YFP^- pDCs. In contrast, expression of CD80 and CCR7 were not increased following infection in *Tlr7*$^{-/-}$ pDCs. Interestingly, CD86 expression on pDCs was less sensitive to TLR signaling, which likely explains why there was no obvious difference in CD86 expression in YFP^+ versus YFP^- pDCs, consistent with a previous report (233). In regards to CD80 and CCR7, we observed substantial increases in expression on YFP^- pDCs, which was not present on *Tlr7*$^{-/-}$ pDCs, despite the absence of detectable reporter expression. This indicates that these pDCs may represent a population that received suboptimal stimulation and are only partially activated in a TLR7 dependent manner.

It has been previously reported that pDC numbers decrease following viral infection (189, 229, 230). We therefore sought to determine the role that activation through TLRs plays in determining pDC numbers and survival following infection with LCMV. We first determined the absolute number of pDCs at 20 and 45 h following infection with LCMV, and normalized those to the numbers of pDCs in littermate uninfected mice, such that we could get an accurate reflection of the change in numbers irrespective of other factors such as mouse age, sex or size (Fig. 1C). We found that the number of pDCs in WT mice decreased to almost half that in uninfected mice by 20 h p.i., and that by 45 h, were only about a quarter of the original number. In contrast, 3d mice, and mice deficient in MyD88 or TLR7 all maintained levels of pDCs on par with those in uninfected mice. Consistent with increased cell death in WT, but not TLR7 signaling-impaired mice, we found a significant increase in caspase activation at 20 h in WT, but not other mice (Fig. 1D). This led us to speculate whether pDC activation was a

direct correlate of caspase activation. In order to determine this, we analyzed caspase activation in YFP^+ and YFP^- pDCs in Mob mice at 20 and 45 h following infection (Fig. 1E). At both time points, we observed significant increases in caspase activation in YFP^+, relative to YFP^-, cells. This was particularly striking at 45 h; however, the relatively low proportion of YFP^+ cells, in which caspase activation is more greatly increased, at this time point is a likely explanation for why there is no substantial increase in caspase activation in this population, as a whole, at 45 h (Fig. 1D). Taken together, these data demonstrate that in response to infection with LCMV, pDCs become activated in a TLR7-dependent manner, and those pDCs that become activated are more likely to activate caspase-mediated cell death.

Plasmacytoid DCs are refractory to further TLR stimulation during chronic and acute LCMV infections

We have previously shown that type I IFN production by pDCs in Mob reporter mice during both acute and chronic LCMV infections is transient, with near basal levels of IFN-β reporter expression by day 5 p.i. (206). We therefore sought to determine whether pDCs might potentially be activated at later time points during infection in a situation where viral ligand is persistent. We infected Mob mice with LCMV clone 13, which causes a persistent infection in WT mice that lasts for approximately two months (141), and determined whether pDCs were activated in spleens at day 8 following infection (Fig. 2A). We found that pDCs were not activated at day 8, despite the presence of large amounts of viral ligand at this time. Consistent with an inactivated state, pDCs at this time expressed low levels of CD80, although they did express CD86, which is not as

dependent on TLR signaling (Fig. 1B). We observed similar results at day 15 of infection, and in lung or liver (data not shown). As we previously noted that YFP$^-$ pDCs might also be activated to a certain extent (Fig. 1B), we wanted to determine whether these pDCs were making type I IFNs or other cytokines. Therefore, we sorted pDCs from uninfected, day 1 or day 8 LCMV clone 13-infected mice and determined the expression of various cytokines by quantitative RT-PCR (Fig. 2B). Whereas various cytokines, including type I IFNs, IL-6, IL-12 and TNF-α, were all easily detectable at high levels in pDCs sorted from day 1 infected mice, levels of these cytokines were comparable to that in uninfected mice in pDCs sorted from mice at day 8 p.i.

As a chronic infection may be complicated due to ongoing infection and sustained inflammation, we sought to further characterize pDC responsiveness during acute infection with LCMV. It was previously reported, that pDCs in LCMV Arm-infected mice do not respond to CpG stimulus when cultured *ex vivo* until day 30 (189). As the type I IFN response to CpG *in vivo* is primarily dependent on pDCs (93, 98), we asked whether pDCs were likewise refractory to *in vivo* administration of CpG. We determined this by analyzing YFP expression in pDCs from Mob mice following treatment of mice with CpG in uninfected, day 8, 15, or 30 LCMV Arm-infected mice (Fig. 2C). We used a B-type CpG, which when in complex with DOTAP, is capable of inducing type I IFN responses in pDCs, but not conventional DCs (77). Similar to the previous report (189), we found that pDCs did not respond to CpG *in vivo* until day 30 p.i. with LCMV Arm (Fig. 2C). However, we did find reduced responses in day 30-infected mice compared to uninfected controls, although these responses were in the low range of what we typically observe with *in vivo* CpG administration.

Plasmacytoid DC refractoriness is a common trait across viral infections

It was previously reported that pDCs in LCMV Arm-infected mice at day 5 p.i. are refractory to stimulus with CpG (189). Furthermore, when mice were infected with LCMV clone 13, levels of serum type I IFNs were reduced in response to VSV and MCMV infection. When mice were instead infected with MCMV, reduced levels of serum type I IFNs were also seen following subsequent infection with LCMV clone 13. However, as type I IFN responses to LCMV (mediated by MDA5 and MAVS), VSV (mediated by STING and RIG-I), and MCMV (mediated by TLR3) can be affected by other PRRs and cell types other than TLR7 and TLR9 and pDCs (22, 54, 190, 206, 234), we sought to ascertain whether pDCs, specifically, were refractory to secondary stimulus across these various infections. Therefore, we infected Mob mice with LCMV Arm, MCMV, or VSV and treated the infected mice with CpG on day 5 (Fig. 3A). We found that in all cases, responses of pDCs to CpG were abrogated, relative to the response in uninfected mice, regardless of the virus used for the primary infection. Although CpG induced slightly more expression of YFP in pDCs from LCMV-infected mice, compared to MCMV or VSV, this may be a reflection of higher levels of YFP expression at day 5 in non-CpG-treated, LCMV-infected mice.

To determine whether the innate response at the tissue level was similarly impaired, we performed quantitative RT-PCR on samples isolated from spleens of mice treated with CpG to measure expression levels of IFN-β, IFN-α and TNF-α mRNA (Fig. 3B). Whereas CpG strongly induced the expression of all three of these cytokines in uninfected mice, levels in infected mice were reduced to levels near that in non-treated mice. A marginal response to CpG was seen in mice infected with VSV, but this was still

well below the response observed in uninfected mice. Therefore, suppression of type I IFN, as well as other inflammatory responses such as TNF-α, in pDCs is likely a common feature of virus infections.

Cell-intrinsic activation is not a requirement to induce pDC refractoriness

It is currently unknown what factors confer unresponsiveness of pDCs to further stimulus following virus infection. We hypothesized that TLR recognition of viruses by pDCs would induce a refractory state in a cell-intrinsic manner. Thus, if pDCs could no longer respond to the initial viral infection, they may still be able to be activated in response to a secondary TLR stimulus. To test this hypothesis, we used the knowledge that recognition of LCMV by pDCs is dependent on TLR7, and pDCs in Mob-*Tlr7*$^{-/-}$ mice do not become activated to make type I IFN (Fig. 1A). However, Mob-*Tlr7*$^{-/-}$ mice are still able to respond to CpG (which is recognized by TLR9) (Fig. 4A). Therefore, we infected Mob and Mob-*Tlr7*$^{-/-}$ mice with LCMV Arm and assayed YFP expression in pDCs at day 5 p.i. following treatment with CpG. To our surprise, pDCs in Mob-*Tlr7*$^{-/-}$ mice were still abrogated in their response to CpG when previously infected with LCMV (Fig. 4A). This defective IFN response was further confirmed by quantitative RT-PCR for IFN-β, IFN-α, and LCMV S segment in spleens of these mice (Fig. 5B). Interestingly, the inability of pDCs to make type I IFN responses at day 5 of LCMV infection is not due to availability of ligand, as these mice contain high levels of LCMV genomic RNA, consistent with rapid inhibition of pDC responses following initial activation.

We next hypothesized that pDCs may become exposed to some factor during the early innate inflammatory response that causes them to be refractory. This may explain

why after day 1 following infection, the frequency of responding pDCs in Mob mice gradually declines to approximately 1-2% by day 5 (206). In order to test this, we used BDCA2-DTR Tg mice, in which pDCs can be depleted by administration of DT (88). It was previously reported that DT treatment resulted in pDC depletion that lasted for 2-3 days (88). However, this was shown in the steady-state, so recruitment of 'new' pDCs may be different under inflammatory conditions. Therefore, we first verified the kinetics of pDC renewal after DT induced depletion in LCMV-infected mice. Consistent with the earlier report, pDCs increased in frequency 2 days following DT administration, reaching levels found in non-depleted mice by 3 days after DT treatment (data not shown). In order to determine whether or not newly recruited pDCs could now respond to CpG, we first crossed BDCA2-DTR Tg to Mob mice in order to assay IFN-β production *in vivo*. We then depleted pDCs with DT at days -1, 1, 3, and 5 following LCMV Arm infection, such that by day 8, when the pDC compartment is restored, most of these pDCs will not have been present during the initial 5 days of the immune response. We first confirmed pDC depletion at day 6, following the final administration of DT, and determined that the frequency of pDCs was reduced approximately 75% (Fig. 4C). Then, at day 8, a time point at which pDCs do not normally respond to CpG (Fig. 2C), we assessed pDC activation by IFN-β-YFP reporter expression (Fig. 4D). Although pDCs in uninfected reporter mice were able to express YFP following CpG treatment, pDCs in both depleted and non-depleted mice were incapable of responding to CpG. As CD86 seemed to be expressed on pDCs in a manner that was non-TLR, but infection-dependent (Fig. 1A), and may be a marker of pDCs receiving exogenous stimuli, we assessed CD86 expression on pDCs in day 8 untreated and CpG-treated mice (Fig. 4E). Despite pDCs in these mice

expressing no IFN-β, CD86 expression was increased at day 8 relative to uninfected mice, and was further increased by CpG treatment, indicating that these pDCs are responding to some exogenous stimulus.

Loss of pDC DAP12 and FcRγ adaptors has no effect on refractoriness to CpG stimulation

Currently, most known receptors involved in modulating type I IFN secretion by pDCs in mice and humans lack their own intracellular signaling domains and use one of two immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor molecules: DAP12 or FcRγ (87, 191). In mice these include the activating receptor PDC-TREM (235), and the inhibitory receptor Siglec-H (236), both of which utilize DAP12. Therefore, in order to address whether any of these receptors, which associate with DAP12 or FcRγ, provide signaling that leads to an unresponsive state, we infected DAP12+FcRγ DKO mice with LCMV Arm and assessed CpG responsiveness at day 5 by quantitative RT-PCR for type I IFN in spleens of compared to uninfected mice (Fig. 5). Although slight decreases were seen in IFN induction in uninfected mice, we observed no difference in type I IFN message levels in day 5 LCMV Arm-infected mice regardless of CpG treatment. These results indicate that inhibition of pDC responsiveness may be independent of most known activating or inhibitory receptors on pDCs.

Discussion

The results described herein demonstrate multiple mechanisms by which pDC functions are regulated throughout the course of virus infection. Early following infection, pDC numbers rapidly decrease, likely through a manner dependent on caspases, the activation of which correlates with the level of pDC activation. In addition, pDCs, both fail to make cytokines and type I IFN in the presence of continued virus ligand (as during chronic infection with LCMV), but also long after infectious virus is undetectable (as in acute infection with LCMV), and are unable to respond to subsequent stimulus with CpG. This refractory state was induced in response to infection with other viruses as well, and surprisingly, appeared to be independent of previous pDC activation, presence during the innate inflammatory response, or the ability to signal via adaptors required for transducing signals from a number of receptors known to both positively and negatively regulate pDC type I IFN responses.

It has previously been reported that pDCs undergo cell death during herpes simplex virus (HSV)-1 infection in a type I IFN-dependent manner (230). As recombinant type I IFN alone was enough to lead to reductions in pDC numbers and elevation of activated caspases, it is clear that type I IFN signaling through the IFNAR is sufficient to induce cell death. However, we and others have previously reported that type I IFN levels in 3d mice, in which pDCs are not activated, were relatively similar to those in WT (180, 206), although other groups have observed slight reductions in type I IFN in *Tlr7*$^{-/-}$ mice in response to LCMV (207, 233, 234). Even so, reported levels of IFN-α in *Tlr7*$^{-/-}$ mice at the peak of the response to LCMV of 15-20 ng/ml in serum (207), are 3- to 4-fold higher than that in HSV-1 infection (4-5 ng/ml) (230). Therefore, despite

substantial levels of type I IFN in these mice, pDC numbers were relatively well-maintained following LCMV infection in *Myd88*$^{-/-}$, 3d, and *Tlr7*$^{-/-}$ mice (Fig. 1C), with little increase in caspase activation (Fig. 1D). We further noted that caspase activation was higher in pDCs that expressed the IFN-β-YFP reporter, indicating that caspase activity was correlated with cell-intrinsic activation status of pDCs (Fig. 1E). This discrepancy may be accounted for by noting that pDC activation and type I IFN production is increased by IFNAR signaling (228). As *Myd88*$^{-/-}$ and IFNAR-deficient mice in the previous study both showed similarly impaired type I IFN production and decreased caspase activation in pDCs (230), it is difficult to distinguish between the two effects and IFNAR signaling may, in fact, increase cell death in a manner additive or synergistic with that induced by TLR-dependent activation.

Understanding the function of pDCs under situations of chronic stimulus is of great interest, as pDCs can become activated during autoimmune diseases such as psoriasis and systemic lupus erythematosus, and pDC responses may be either protective or detrimental during chronic infections such as HIV (191, 226, 227, 237, 238). Here, we show *in vivo* that pDC cytokine-secreting functions are not constantly activated in the course of a chronic viral infection, and responsiveness is suppressed long after even acute infection, consistent with a previous report (189). Furthermore, this property was applicable to other RNA and DNA viruses, known to activate pDCs. Therefore, it is unclear how pDCs may be activated to make type I IFN in other circumstances. It is possible that, at least in the case of autoimmunity, that the interaction of nucleic acids with other proteins, such as LL37, may override such a refractory state (226, 227). It may also be that these conditions do not produce a factor necessary to induce

unresponsiveness. Presumably, this is an extrinsic factor, since pDCs that could not respond to LCMV, due to TLR7-deficiency, were also refractory. Though this could be part of the inflammatory milieu, it was previously shown that LCMV-infected mice lacking the ability to make or respond to type I IFN, IL-10, TNF-α, IFN-γ, or lacking T cells failed to make type I IFN in response to CpG when stimulated *ex vivo*(189). It remains a possibility that a cadre of inflammatory cytokines could induce such a state, and they are redundant to such an extent that loss of one alone is insufficient to restore responsiveness to CpG. As cleavage of TLR7 and TLR9 is necessary for their function (23-25), it is also a possibility that extrinsic signals alter the processing of these TLRs, such that they can no longer be activated.

With the idea that pDCs may be exposed to something during the initial inflammatory phase of the immune response that renders them refractory, we took the approach of depleting pDCs through the first 5 days of the response. This may also serve to mimic an initial loss, followed by restoration of pDC numbers. Importantly, pDC depletion has no effect on the kinetics of clearance of acute LCMV infection (234) As LCMV Arm is typically cleared by approximately 8 days, and it takes 3 days for pDCs to be fully restored, most pDCs at this time should have been minimally exposed to high levels of inflammatory stimuli. However, to our surprise, pDCs restored late in the immune response were still incapable of type I IFN production in response to CpG. One explanation may be that even following clearance of infectious virus, LCMV nucleic acid is maintained until day 16 or beyond (234). However, if TLR engagement by nucleic acids alone were sufficient to confer unresponsiveness, then we would expect to see a restoration of responsiveness in *Tlr7*$^{-/-}$ mice, which we do not. Another possibility is that

despite depleting pDCs, signals may act upon pDC precursors rendering them unresponsive. Consistent with this, although cell recovery was suboptimal, we were unable to observe pDC activation when we transferred naïve pDCs into previously infected mice, although if pDCs were transferred prior to infection, activation was observed (data not shown). Therefore, it would be useful to create genetic tools which one could use to identify previously activated, but currently inactivated pDCs, and those pDCs that are newly formed, in order to further dissect the mechanism.

Lastly, we used DAP12+FcRγ DKO mice to explore the possibility that receptors on pDCs that negatively or positively regulate pDC function may be involved in the refractory nature of pDCs to CpG stimulation (87). Support for this hypothesis comes from studies showing that DAP12-deficient macrophages and mice show increased inflammatory cytokine production in response to TLR ligands (239). Furthermore, Siglec-H is a DAP12-associated inhibitory receptor on pDCs and engagement of Siglec-H by a stimulatory antibody reduces pDC secretion of type I IFN (236). Similarly, ablation of Siglec-H genetically increases type I IFN production by pDCs (98). However, we observed no increase in response to CpG in DAP12+FcRγ DKO mice at day 5 following infection with LCMV. Despite this result, it still may be that one of these receptors negatively regulates pDCs such that they cannot respond to secondary stimulus with CpG. For example, although Siglec-H is inhibitory, another DAP12-associated protein, PDC-TREM, is an activating receptor for pDCs (235). Therefore, genetic ablation of adaptors alone may cancel out effects of activating and inhibitory receptors. Ablation of each adaptor alone, or of specific receptors will be needed to further investigate the role of these receptors in modulating the ability of pDCs to make IFN

following initial stimulation. Together, these results imply that modulation of pDC numbers and responsiveness following initial activation limits the ability of pDCs to participate in subsequent immune responses. Therefore, a more thorough understanding of changes in pDCs following activation and the mechanism by which this change in responsiveness occurs may better inform such applications as the timing of vaccines, or the modulation of pDC function in the course of chronic viral diseases or autoimmunity.

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Footnotes

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Figure 1

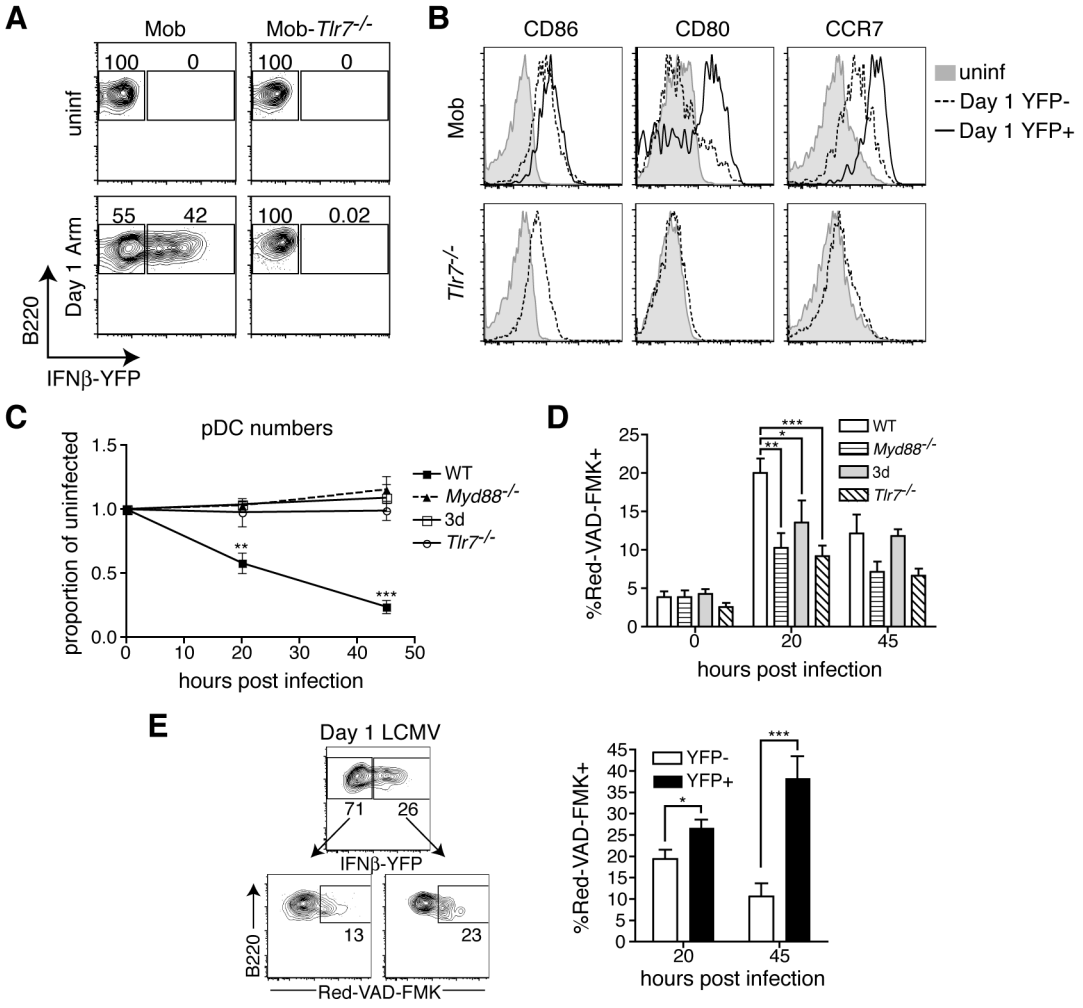

Figure 1. TLR7-dependent pDC activation and cell death. Mice were infected with LCMV Arm and pDC activation and caspase activity were assessed. (A-B) Mob and Mob-*Tlr7*$^{-/-}$ were infected and IFN-β-YFP reporter expression (A), and the expression of CD86, CD80 and CCR7 (B) on pDCs were analyzed at 18-24 h p.i. Cells shown in contour plots in (A) were gated on CD3ε^{-}CD11b^{-}Siglec-H^{+}B220^{+} cells. Cells showing in the histograms in (B) were gated as in (A), but separately for YFP^{+} or YFP^{-} pDCs. (C) Total numbers of PDCA-1^{+}Siglec-H^{+}B220^{+} pDCs were calculated in spleens of the indicated mice at days 0, 1 and 2 following infection. Numbers are normalized to those in

uninfected littermate control mice. (D-E) Caspase activation was determined in Siglec-H^+B220^+ pDCs in the indicated mice at days 0, 1, and 2 p.i. For (E), the left panel shows representative contour plots of VAD-FMK staining gated on YFP^- and YFP^+ pDCs; the right panel shows the proportion of YFP^- and YFP^+ pDCs in Mob mice that are VAD-FMK^+ at the indicated time p.i.. Data in A-B are representative of two or more independent experiments using either Mob-*Tlr7*$^{-/-}$ or Mob-3d mice. Data in C-E are from four experiments (n=3 or greater) for each group and time point. Statistics were calculated by using a two-way ANOVA (C and D) or two-tailed unpaired Student's *t*-test (E). Graphs show mean ± SEM. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Figure 2

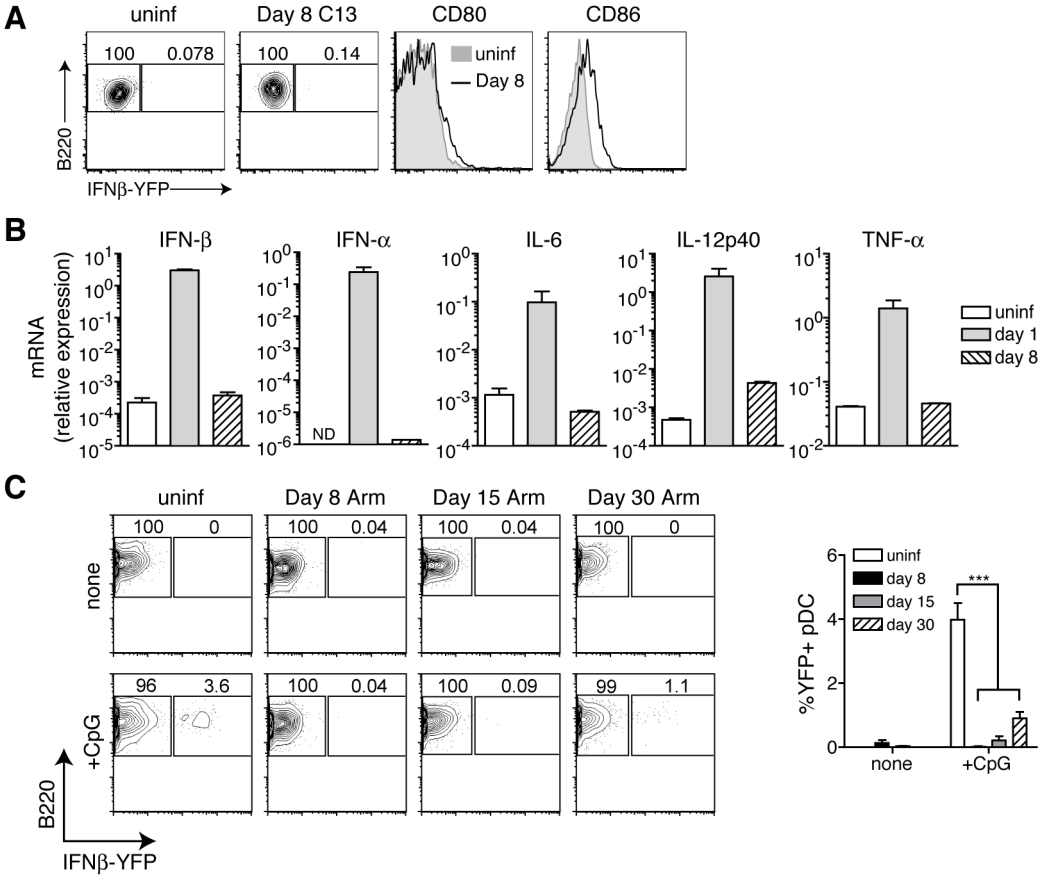

Figure 2. Plasmacytoid DCs are not activated at later time points in chronic infection and are refractory to further stimulus following acute infection. (A) Mob mice were infected with LCMV clone 13 and pDC activation in spleens at day 8 p.i. was determined by IFN-β-YFP, CD80 and CD86 expression. Cells shown in contour plots and histograms were gated on Siglec-H$^+$B220$^+$ cells. (B) Expression of mRNA for various cytokines were analyzed in pDCs sorted from uninfected or infected mice at the indicated time points by quantitative RT-PCR. (C) At days 8, 15 or 30 p.i. with LCMV Arm, mice were treated with CpG and IFN-β-YFP expression in pDCs was assessed 6 h later. Cells depicted in contour plots shown on left were gated on B220$^+$Siglec-H$^+$PDCA-1$^+$ cells and the % of YFP$^+$ pDCs was graphed on right. Data in (A) are representative of

two independent experiments (n=2: uninf; n=4: day 8). For (B), data for each time point are from two different sorted samples, with each sample pooled from 2-3 mice. The data in (C) are combined from three experiments (n=2-6 per group). Statistics were calculated using two-way ANOVA. Graphs show mean ± SEM. ***$P < 0.001$.

Figure 3

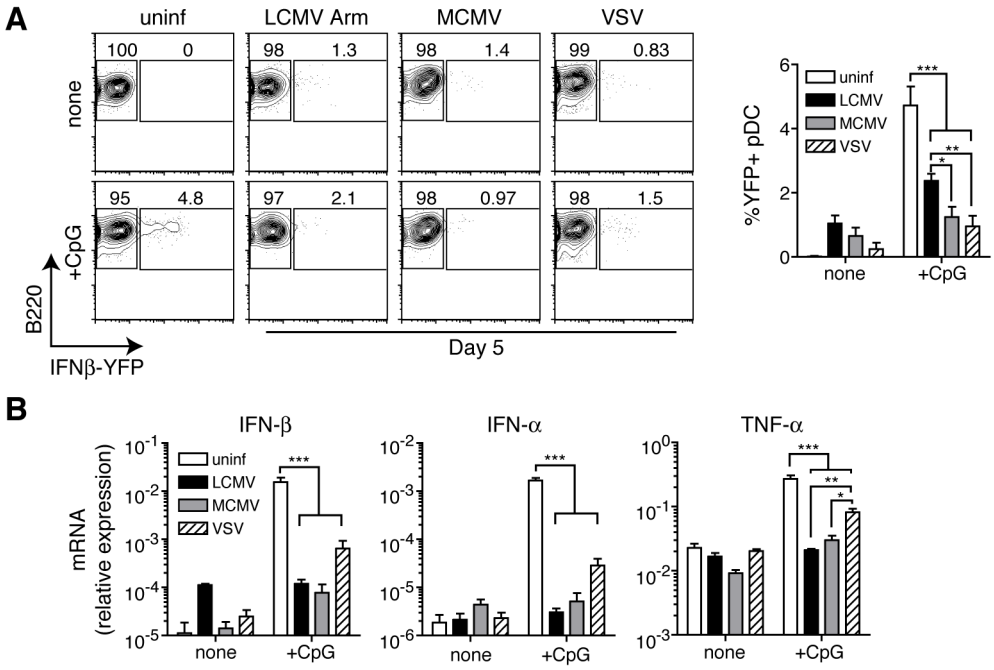

Figure 3. Plasmacytoid DC unresponsiveness is common to infection with multiple viruses. Mice were infected with LCMV Arm, MCMV or VSV and pDC CpG responsiveness was measured on day 5 p.i. 6 h following CpG administration. (A) Representative contour plots of IFN-β-YFP expression in Mob mice are shown in PDCA-1^+Siglec-H^+B220^+ pDCs (left), and the frequency of pDCs that are YFP^+ are shown for mice infected with each virus (right). (B) Expression of IFN-β, IFN-α and TNF-α mRNA was measured by quantitative RT-PCR in spleens of the indicated mice treated with or without CpG. Data in A-B are from two independent experiments (n=4 mice per group). Statistics were calculated by using a two-way ANOVA. Graphs show mean ± SEM. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Figure 4

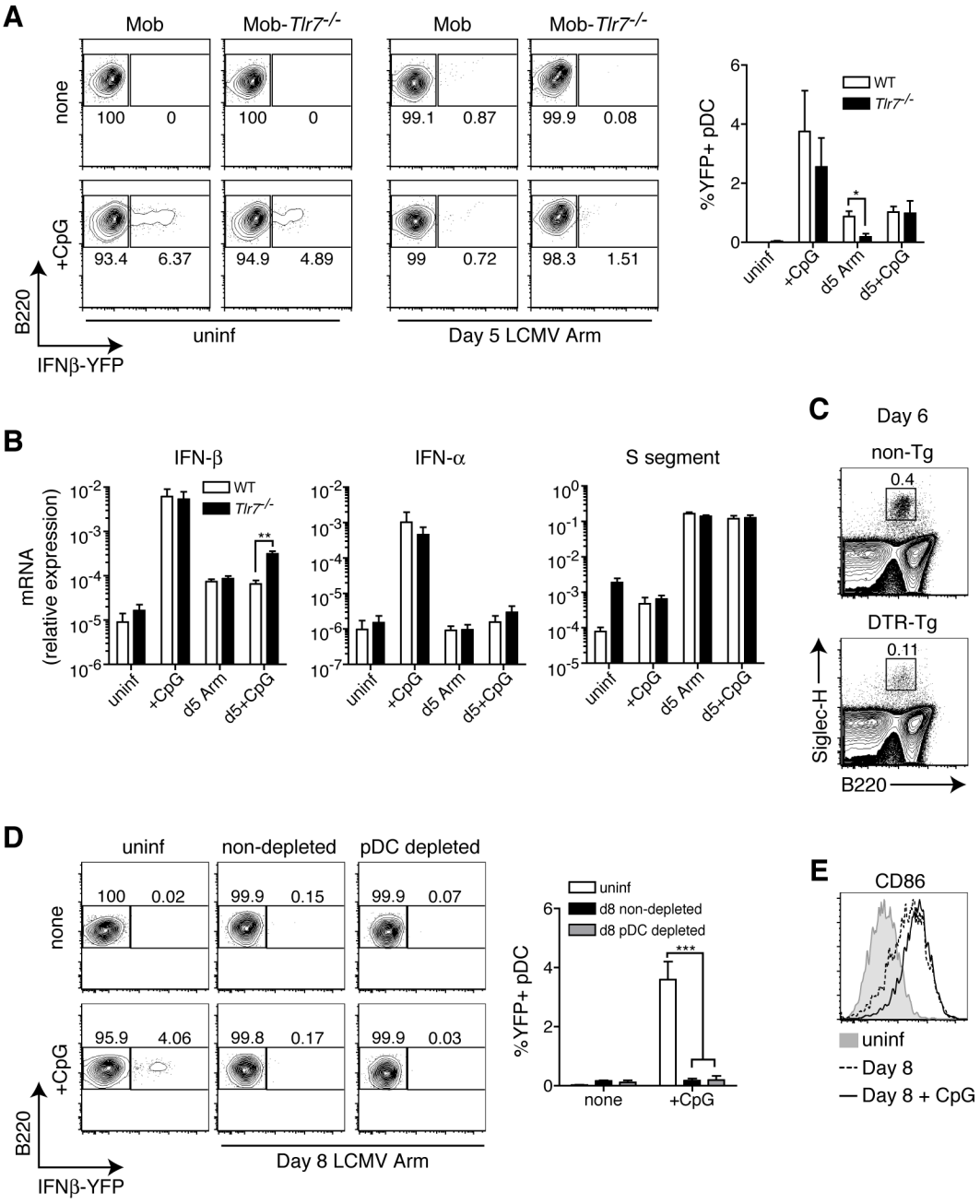

Figure 4. Plasmacytoid DC unresponsiveness is independent of previous TLR-dependent activation or presence during early immune responses. (A-B) Mob or Mob-*Tlr7*$^{-/-}$ mice were infected with LCMV Arm and CpG responsiveness was measured on day 5 p.i. 6 h following CpG administration. (A) Representative contour plots for IFN-

β-YFP expression in PDCA-1^{+}Siglec-H^{+}B220^{+} pDCs are shown on left. The frequency of YFP^{+} in pDCs in each group is shown in graphical form on the right. In one experiment, pDCs are defined as CD11b^{-}B220^{+}Siglec-H^{+}. (B) Expression of IFN-β, IFN-α and LCMV S segment in spleens of the indicated mice with or without CpG treatment was determined by quantitative RT-PCR. (C-E) Activation of pDCs was analyzed in Mob-BDCA2-DTR Tg mice 6 h following CpG treatment on day 8 p.i. Mice were treated with DT on days -1, 1, 3 and 5 p.i. (C) Depletion of pDCs following the last DT treatment was confirmed by flow cytometry at day 6 p.i. Cells shown in contour plots gated on live (DAPI^{-}), CD11b^{-} cells. (D) IFN-β-YFP expression in pDCs on day 8 following CpG treatment was analyzed by flow cytometry. Representative contour plots, gated on live (DAPI^{-}), TCRβ^{-}CD11b^{-}Siglec-H^{+}B220^{+} cells are shown (left). The frequency of YFP^{+} pDCs is shown at right. In one experiment pDCs were gated on live (DAPI^{-}), CD19^{-} PDCA-1^{+}Siglec-H^{+}B220^{+} cells. Both DT-treated, non-Tg and non-DT-treated, Tg mice were used as non-pDC-depleted controls with no differences observed. (E) CD86 expression is shown in uninfected and day 8 LCMV Arm-infected mice with or without CpG treatment. Cells shown in histograms were gated on live (DAPI^{-}), CD11b^{-}CD3ε^{-} Siglec-H^{+}B220^{+} cells. Data in A-B are from two independent experiments (n=3-5 mice per group). Data in C-E are from two independent experiments (n=4 mice per group). Statistics were calculated by using a two-tailed unpaired Student's *t*-test (A and B), or two-way ANOVA (D). Graphs show mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Figure 5

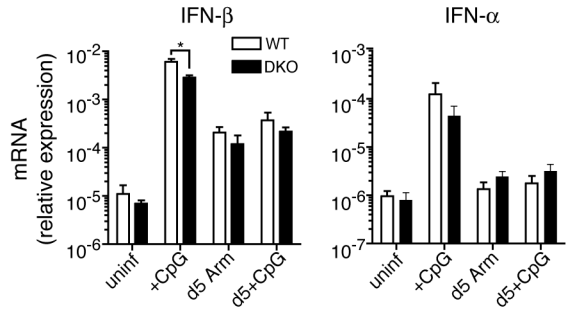

Figure 5. CpG unresponsiveness is maintained in Dap12+FcRγ DKO mice. WT or Dap12+FcRγ DKO (DKO) mice were infected with LCMV Arm and on day 5 treated with CpG. Induction of IFN-β and IFN-α were analyzed in spleens of the indicated mice by quantitative RT-PCR. Data are from two independent experiments (n=3-4 mice per group). Statistics were calculated by using a two-tailed unpaired Student's *t*-test. **P* < 0.05.

Chapter 5

Conclusions and Future Directions

Role of PRRs in the Control of Acute versus Chronic Viral Infection

Summary

In the works presented in Chapters 2 and 3, I described our work investigating the roles various classes of innate immune PRRs play in the clearance of acute or chronic LCMV infection. We discovered that RLRs were primarily required for production of early type I IFN, and for limiting early viral replication during both acute and chronic infection. This was despite the fact that pDCs were activated by nucleic acid-sensing TLRs, the production of which could be visualized using IFN-β reporter mice. In the absence of RLRs, clearance of acute infection with LCMV was delayed approximately a week, whereas nucleic acid recognition by TLRs was inconsequential. Effective $CD8^+$ T cell responses required RLRs in a non-cell-intrinsic manner, presumably through the action of type I IFNs. RLRs also contributed to long-term control of chronic infection with LCMV, but this contribution was obscured when given a high dose inoculum of 2×10^6 PFU, and a high dose of LCMV clone 13 was controlled with kinetics similar to that in WT mice. In contrast, nucleic acid recognition by TLRs was required for eventual clearance of high dose chronic LCMV infection, and this was associated with LCMV-specific antibody responses that were defective to a greater extent during chronic infection.

Upon closer investigation of the role nucleic acid-sensing TLRs played in the anti-LCMV antibody response and clearance of chronic LCMV, we demonstrated that antibody responses and virus control were both dependent on TLR7, but not TLR3. Loss of TLR7 signaling specifically within B cells was sufficient to severely decrease the levels of LCMV-specific IgG. TLR7-deficient B cells were impaired in their ability to form both GC B cells and plasma cells in competition with WT B cells, particularly

during chronic infection. Surprisingly, in the absence of competition, *Tlr7*$^{-/-}$ cells were perfectly capable of forming GC B cells, but despite a normal early plasmablast and plasma cell response, were deficient in the formation of long-lived plasma cells. Though numerically normal in GC B cell numbers, *Tlr7*$^{-/-}$ GC B cells were qualitatively different, as evidenced by a decrease in proliferation of *Tlr7*$^{-/-}$ GC B cells and a skewed distribution of GC B cells between LZ and DZ populations.

The Roles of PRRs in Early Immune Responses and Control of Virus Infections

Virus replication generates multiple PAMPs that can activate multiple PRRs, often with overlapping specificities. Therefore, understanding how different PRRs contribute to immune responses necessary for control of viral infections is of the utmost importance. Much work has been done regarding the roles these various receptors play during virus infection, however, relatively little work has addressed the question of whether PRR engagement is qualitatively different during the context of chronic infection. In this scenario, PRRs could be, theoretically, engaged throughout the course of infection, thereby altering or driving responses required to limit pathology, or conversely, driving immune responses which may increase pathology.

The role of RLRs in protection from viruses has been demonstrated in response to a wide range of infections (119, 186, 240). Likewise, recognition of viruses by nucleic acid-sensing TLRs has been shown to be important for induction of type I IFNs, particularly by pDCs (2, 5). However, a demonstrable requirement for TLRs in eliminating viral pathogens *in vivo* has revealed a more limited requirement. In particular, control of herpes viruses such as HSV-1, HSV-2 and MCMV seem to be dependent on

these TLRs, as well as some other viruses such as influenza, and possibly West Nile virus (20, 22, 196, 241-243). The limited and/or redundant role of TLRs in viral infection is supported by studies in humans in which viral disease in patients deficient in TLR3 and UNC-93B seems to be limited to herpes simplex encephalitis (244). At present, it is not known if RLRs contribute to primary human immunodeficiencies that confer susceptibility to viral infections.

What dictates the proportionate contributions of either RLR or TLR virus-sensing to the control of replication is likely multifactorial, including factors such as virus tropism, dosage, route of infection, viral immune evasion strategies, and the contribution of various PRRs to adaptive immune responses that are required for viral clearance. In regards to LCMV, in addition to our own study, a study published subsequently from the Colonna group demonstrated that the RLR, MDA5, was required for control of acute LCMV infection and type I IFN responses to both acute and chronic LCMV infection (234). These data are in concurrence with another recent study showing that type I IFN responses to acute LCMV infection were dependent on MDA5, but not nucleic acid-sensing TLRs (180). However, in contrast to our study which showed no dependence on TLR signaling for total splenic type I IFN responses or early virus control, the Colonna study and two others showed slight reductions in IFN levels in sera of *Tlr7*$^{-/-}$ mice, presumably due to pDCs (207, 233, 234). However, this effect on type I IFN production appeared transient, and resulted in either a small early increase, or no effect on early virus titers, consistent with a role for RLRs as the primary sensors of LCMV (207, 234).

Despite this evidence that pDCs play little early role in type I IFN production and control of LCMV replication, two studies have argued for a larger pDC role (177, 245).

As mentioned previously, a $CD8^+$ T cell-intrinsic requirement for MyD88 during LCMV infection calls into question results using MyD88-deficient mice (178, 179). The other study relies on conditional deletion of the transcription factor E2-2 in $CD11c^+$ cells (*Itgax*-Cre^+ *Tcf4*$^{flox/-}$) (245). The discrepancy between this and the Colonna study, which used DT-mediated depletion of pDCs in BDCA2-DTR Tg mice, may be due to the strains of chronic LCMV used (LCMV Docile versus LCMV clone 13). Alternatively, conditional E2-2 ablation may adversely affect other cells during the course of the immune response. Although recombination was shown to occur at low levels in B cells, which also express E2-2, this was shown at steady-state, and because activated cells other than DCs can express CD11c, it is unclear whether B cells may be depleted or undergo altered differentiation under inflammatory conditions. Furthermore, although the results in this study were interpreted as caused by a lack of type I IFN, no data were presented demonstrating that type I IFN levels were affected in these mice following LCMV infection. Therefore, at present, it seems likely that pDCs and TLR7 play a limited role early in the immune response to LCMV.

The requirement for RLRs in initiating type I IFN responses to LCMV may be partly explained by virus tropism. Early LCMV replication has been shown, by our group and others, to occur in dendritic cells and macrophages, particularly those of the splenic marginal zone (143, 148, 232). Indeed, depletion of splenic marginal zone macrophages with liposome-clodronate was shown to impact type I IFN responses to LCMV (246). It is well established that, although pDCs do not require signaling through RLRs to produce type I IFN, myeloid cells such as cDCs and macrophages require these PRRs to produce type I IFN (186, 190, 247). Therefore, although pDC produce more IFN on a per cell

basis, the contribution via the RLR pathway from infected cells, which likely greatly outnumber pDCs on the whole, are more important for overall early antiviral responses.

Under what conditions may the pDC contribution to early antiviral responses be greater? One possibility is that pDCs may play a more important role at lower viral doses. In response to MCMV infection, the effect of pDC depletion on virus titers in various tissues was dependent on virus dose, with more effect seen at lower doses (88). Although we still observed a dominant effect of the RLR pathway when using a ten-fold lower dose, it may be that at physiological doses pDC contribute to a greater extent. Another possibility is that the main contribution of pDCs to antiviral defense is very early, as type I IFN responses in either VSV- or MCMV-infected, pDC-depleted mice, were decreased at very early, but not later time points postinfection (88). This may somewhat explain a discrepancy between our study in which we assayed type I IFN production at 24 h, versus the Colonna study which showed a small decrease in *Tlr7*$^{-/-}$ and pDC-depleted mice at 16 h, but not 48 h p.i. Route of infection may be an important additional factor in which cells and pathways are required for type I IFN responses and control of early virus replication. During systemic response to Newcastle disease virus, pDCs were identified as a major producer of type I IFN, but following intranasal administration, alveolar macrophages were largely responsible (247). Lastly, as some viruses are able to employ immune evasion strategies, which inhibit signaling through specific PRRs, other, non-inhibited pathways, may take on greater importance (248).

Interestingly, the early type I IFN response, and control of early virus replication to both acute and chronic LCMV infections were similarly dependent on the RLR pathway. However, moving forward, it will be interesting to separate spatially (in what

cell types) and temporally (at what point in infection) the roles for PRRs in detecting viruses. For example, although recent work using conditional deletion of MyD88 has revealed roles for MyD88 in different cell types during immune responses, conditional RIG-I, MDA5 or MAVS knockouts have yet to be developed to study the roles of these RLRs in different cell types. This may be particularly informative for RIG-I in which one knockout is embryonic lethal (190), and another develops a colitis-like disease characterized by fewer Peyer's patches and aberrant T cell activation (249). Furthermore, most of these studies use mice that lack PRRs from the onset of infection, but it will be interesting to see, particularly in the context of chronic infection, whether inducible conditional ablation of different PRRs may affect disease outcomes when initiated at later stages of infection, implying a role for PRRs not just in the initiation, but the maintenance of immune responses.

Innate Initiation of T Cell Responses

The role of the innate immune system in initiating adaptive immune responses is well known. In particular, a critical role for type I IFN in $CD8^+$ T cell, and to a lesser extent, $CD4^+$ T cell responses has been shown (114, 115). However, the relevant cellular sources for type I IFN, downstream of which PRRs, are less well understood. We showed that in the absence of RLR signaling, priming of WT $CD8^+$ T cells were defective in both acute and chronic LCMV infections, with decreased granzyme B and CD25 expression. These results are consistent with effects of type I IFN on $CD8^+$ T cell priming of effector functions (115, 197, 198). We too observed similar, albeit more profound, defects in granzyme B and CD25 expression when using *Ifnar1*$^{-/-}$ $CD8^+$ T cells transferred into WT

hosts (data not shown). Expansion of transferred *Ifnar1*$^{-/-}$ CD8^{+} T cells was also severely impaired. Taken together, we propose that the absence of RLR signaling in *Mavs*$^{-/-}$ mice acts as a type I IFN hypomorph, in which residual type I IFN produced through the TLR pathway is able to support CD8^{+} T cell expansion to a greater extent than when CD8^{+} T cells are completely 'blind' to the presence of type I IFN. In contrast, when we looked at early CD8^{+} T cell activation when cells were transferred into 3d hosts, we observed no defect in expression of various activation markers, but did observe a minor defect in recruitment, indicating that RLRs and TLRs may have non-overlapping functions in CD8^{+} T cell activation.

Consistent with early defects in CD8^{+} T cell activation, and a lack of restriction of virus replication, we observed impaired CD8^{+} T cell function in *Mavs*$^{-/-}$ mice that was not due to a cell-intrinsic requirement for MAVS signaling. Interestingly, despite early defects, *Mavs*$^{-/-}$ mice cleared virus, and CD8^{+} T cells in these mice formed effective memory cells that were capable of controlling a secondary infection with LCMV clone 13 within 5 days, similar to that in WT mice (data not shown). Consistent with this observation, transferred memory CD8^{+} T cells from *Mda5*$^{-/-}$ mice were similarly as effective as WT memory cells in controlling secondary infection with LCMV Arm at day 2.5 (234). Although we did not explore the mechanism in detail, studies from the Colonna group indicated that residual function in CD8^{+} T cells following acute infection of *Mda5*$^{-/-}$ mice was dependent not on help from another 'signal 3' cytokine, IL-12, but was dependent on CD4^{+} T cell help. Therefore, CD8^{+} T cells seem to integrate a variety of signals for optimal activation.

Presumably, $CD4^+$ T cell activation is adequate in *Mda5*$^{-/-}$ mice; at least insomuch as they are able to provide help to $CD8^+$ T cells. In this regard, it will be interesting to determine whether RLRs are equally as crucial for priming of $CD4^+$ T cells, or if TLRs may be more important. Furthermore, it is unclear whether or not RLR-dependent signaling is able to provide stimulation to $CD8^+$ T cells *in trans*. In other words, if only RLR-deficient cells are able to present antigen to $CD8^+$ T cells, but other RLR-sufficient cells can still provide help via type I IFN or other cytokine *in trans*, is this a qualitatively different form of $CD8^+$ T cell activation? These important questions will need to be addressed in order to better understand the precise ways in which various PRRs contribute to the initiation of T cell responses.

PRR Control of B Cell Responses

The engagement of PRRs in a variety of cell types including DCs and B cells can affect the magnitude and quality of B cell responses and the subsequent production of antibodies. First, PRRs including both RLRs and TLRs can be expressed by cDCs, which are important for initiating T cell responses (2). In particular, the initial expression of Bcl6 in $CD4^+$ T cells, which is necessary for the development of T follicular helper cells, is dependent on the interaction of T cells with DCs bearing their cognate antigen (126-130). Secondly, B cells themselves can also express both TLRs and RLRs, and TLR engagement on B cells can significantly impact the outcome of antibody responses (139). Furthermore, production of type I IFNs or other cytokines downstream of PRR engagement can also influence the antibody response through actions on DCs and on B cell Ig class switching (109, 110).

When we examined the effect of the RLR and TLR pathways on LCMV-specific antibody responses, we found that there was no difference in WT and *Mavs*$^{-/-}$ mice in antibody responses during both acute and chronic LCMV infections. This was despite the RLR pathway being the primary pathway of type I IFN production. These data indicate that, at the very least, total IFN levels in *Mavs*$^{-/-}$ mice during the course of LCMV infection are not limiting for robust production of LCMV-specific IgG. It remains to be determined whether the RLR pathway influences the nature of the antibody response, either through effects on antibody class-switching, or influencing the production of neutralizing antibodies. Although most cells, including B cells, can express RLRs, it is unlikely that RLR engagement within B cells influences antibody production, as B cells are not infected by LCMV, and activation of RLRs generally requires active virus replication.

Although a local role for type I IFN production, mediated by TLR7, cannot be ruled out in impacting the magnitude of the antibody response, a few factors weigh against this possibility. 1) TLR7-mediated type I IFN production is primarily from pDCs. However, pDC type I IFN responses are rapidly lost within days of infection, and are therefore of short enough duration that type I IFN production by pDCs is unlikely to influence the GC response in ways that significantly differ from that produced by the RLR pathway. 2) TLR7 signals were required in a B cell-intrinsic manner. As B cells are generally not regarded as significant producers of type I IFN, and the activation of pDCs and other type I IFN-producing cells are not dependent on B cell activation, this further argues against TLR-mediated IFN production as a mechanism for significantly impacting B cell responses. 3) Work from us and others (189, 195)**,** have shown that type I IFN

production is not detectable at later time points following the initial response to LCMV, during either acute or chronic infection. As type I IFN induction in the absence of TLRs is relatively similar between acute and chronic infection, this alone is unlikely to account for such a divergent requirement for TLRs in the antibody response to chronic versus acute infection.

In contrast, our data argue for a requirement for TLR signaling within B cells for both efficient GC B cell and plasma cell responses. This is consistent with data from other groups describing an intrinsic role for MyD88 or TLRs in the generation of antibody responses to virus-like antigens (132, 207, 208). It was proposed that ligand density on VLPs might influence the TLR-mediated impact on antibody responses, with greater ligand density leading to greater augmentation of antibody responses (132). Although an attractive mechanism, it is unclear how this might impact the difference in TLR dependence for antibody responses in acute versus chronic infection. To my knowledge, it has not been shown that either of the amino acid mutations in clone 13 impacts the density of the virus glycoprotein on the surface of the virion. Therefore, it is likely that the differential magnitude of the effect of TLR7 signaling on B cell function in acute versus chronic infection is likely a function of sustained inflammation or antigen. Consistent with this hypothesis, we found similar effects of TLR7 deficiency on GC B cell and plasma cell formation in both acute and chronic infection, but to a larger extent in the chronic setting. Likewise, sustained antigen may drive differences in proliferation and the relative frequencies of LZ and DZ germinal center B cell populations seen in chronic infection. In fact, both in our observations and in findings reported in a previously published paper (207), at time points following clearance of chronic LCMV

infection in WT mice, *Tlr7*$^{-/-}$ mice, which maintained high viral loads, had significantly higher frequencies of GC B cells, indicating that persistent virus continually drives the GC B cell response. One study in HIV-infected individuals indicated that chronic infection might lead to a form of B cell exhaustion (203). Although little is known about whether B cells can become 'exhausted' and what markers might be associated with such a functional state, it is possible that TLR7 signals may be particularly important in preventing such a state during chronic infection.

One aspect of TLR influence on antibody responses, which has been relatively underappreciated in our studies, is the role of TLR engagement on the expression of MHC and co-stimulatory molecules. As previously mentioned, DCs can influence Tfh cell differentiation, and this may be impacted by TLR signaling. However, we found no major differences in the quantity of endogenous Tfh cells by tetramer staining, during either acute or chronic LCMV infection. It may be that activation of cDCs by RLRs is sufficient to stimulate $CD4^+$ T cell responses in the absence of TLR signaling, either through the robust production of type I IFNs or through the expression of MHC and co-stimulatory molecules, which can also be induced by type I IFN signaling. TLR7-dependent activation of B cells may also influence later Tfh cell responses (126-130). Lack of TLR signaling on either DCs or B cells may therefore influence the quality of Tfh cell help to B cells by undermining the interaction between these cells. Similarly, decreased expression of co-stimulatory molecules or MHC on antigen-activated B cells might influence their ability to be positively selected by follicular dendritic cells or Tfh cells during the GC reaction (213, 216). As interaction with Tfh cells can influence the output of memory B cells and plasma cells from the light zone, this may also account for

a secondary defect in TLR7-deficient plasma cells relative to GC B cells. Therefore, a careful analysis of MHC and co-stimulatory molecule expression on GC B cells and Tfh cells, in particular, will be important in moving forward.

In preliminary experiments, we sorted WT and *Tlr7*$^{-/-}$ LZ and DZ GC B cells at day 15 following infection with LCMV clone 13 in order to determine if TLR7 signaling influences transcription of various factors that have been shown to be differentially expressed by LZ and DZ GC B cells (216, 250). In a verification of our sort methodology, and consistent with previous reports, we found CXCR4 to be expressed more highly in DZ GC B cells and c-Myc to be expressed more highly in LZ GC B cells (data not shown). Although expression of CXCR4 and c-Myc were not different between WT and *Tlr7*$^{-/-}$ LZ or DZ GC B cells, it will be interesting to see if other genes which have been shown to be differentially expressed, such as those encoding cell cycle regulators, may be altered, particularly given differences in BrdU incorporation. Although LCMV currently lacks a good B cell antigen receptor transgenic model to study B cell responses, it will be interesting to see in different systems, how TLR signaling may influence properties of GC B cells in the germinal center, such as their movement throughout, or their ability to undergo cyclic re-entry.

Properties of Plasmacytoid DCs Following Initial Activation

Summary

In the work presented in Chapter 4, we investigated the properties of pDCs upon activation including their initial fate, and their ability to respond to sustained or subsequent stimulus to the TLR9 ligand, CpG. We found that following initial activation,

pDC numbers rapidly declined, and that this decrease was dependent on the ability to signal through TLR7, as *Tlr7*$^{-/-}$, *Myd88*$^{-/-}$, or 3d mice all maintained numbers of pDCs following infection. Activation of pDCs was correlated with increased caspase activation, as pDCs that lacked the ability to respond to virus infection in a cell-intrinsic manner had decreased caspase activation, and caspase activation was higher in pDCs that expressed an IFN-β-YFP reporter and hence, were optimally stimulated.

Upon investigation of pDC activation during chronic LCMV infection, at times that virus was present at high levels, we found that pDCs were not activated to make type I IFNs, or multiple other cytokines. Using acute LCMV infection as a model to study unresponsiveness, we found that the ability of pDCs to respond to CpG *in vivo* was not restored until approximately a month following initial infection. pDCs also did not respond to CpG following infection with either MCMV or VSV. Interestingly, the previous activation state of pDCs seemed to have little impact on the response as *Tlr7*$^{-/-}$ mice, in which pDCs do not make type I IFN in response to LCMV infection, were still incapable of producing type I IFN in response to CpG following LCMV infection. This effect was also unrelieved by either depletion of pDCs throughout the initial 5 days of the immune response, or by the absence of known ITAM-bearing adaptors expressed by pDCs.

Cell Death of pDCs Following Virus Infection

A number of reports have noted that pDC numbers decline following virus infection, including with LCMV (189, 229, 230). A recent study examining the mechanism by which pDCs are lost, points to a pro-apoptotic role for type I IFNs, as IFNAR-deficient

mice have decreased caspase activation and increased numbers of pDCs per spleen following infection with HSV-1 (230). However, in our studies, prevention of pDC death did not necessarily correlate with type I IFN levels, as our results in chapter 2 show that 3d mice have levels of type I IFN similar to that in WT mice. Levels of type I IFN in LCMV-infected mice at day 1 p.i., even in *Tlr7*$^{-/-}$ mice, are also much higher than that in HSV-1-infected mice. Particularly, regarding data in which we used Mob mice to show that caspase activation was higher in YFP^{+} pDCs, even amidst pDCs from the same mouse, which should be exposed to equivalent levels of type I IFN, this more strongly indicates pDC-intrinsic activation as a more important determinant of pDC death, at least in the context of LCMV infection. In fact, in *Myd88*$^{-/-}$ mice, the Colonna group reported similar reductions in pDC numbers as those in IFNAR-deficient mice, though they argued that this effect was intermediate. One point of confusion may be that IFNAR signaling can augment pDC activation (228). Indeed IFNAR-deficient mice infected with HSV-1 failed to up-regulate PDC-TREM, a pDC activating receptor induced upon activation (230, 235). Therefore, more studies will be needed to clarify the role of cell-intrinsic activation through TLRs and activation through IFNAR signaling, though both are likely to contribute. One way in which this might be addressed is to cross Mob mice to *Ifnar1*$^{-/-}$ mice. If these pDCs still turn on YFP expression, but are now reduced in caspase activation, this would argue for an independent role in IFNAR signaling in inducing pDC cell death. It may be that TLR signaling and exogenous type I IFN may synergize to induce pDC cell death, in which case TLR and IFNAR doubly deficient mice would be informative. In both our study, and that by the Colonna group, neither

IFNAR nor TLR deficiency completely restored levels of caspase activation in pDCs following infection to that in uninfected mice. Therefore, multiple signals may contribute.

Upon examination of the mechanism by which cell death occurs in activated pDCs, it was previously shown that this was independent of Fas and FasL or TRAIL-mediated death receptor pathways, and dependent on changes in levels of pro and anti-apoptotic proteins such as increased Bid and Bim and decreased Bcl-xl and Bcl-2, respectively (230). Furthermore, pDC numbers were not reduced as greatly in Bid+Bim DKO mice, although caspase activation was not assessed. In addition to Bid and Bim, it will be important to determine which of these pro and anti-apoptotic proteins is important for mediating cell death of pDCs upon activation, and if this is also rescued to an extent in TLR signaling-deficient pDCs.

Refractory State of pDCs Following Infection

Throughout the course of our studies, two observations led us to investigate whether pDCs could be induced to make type I IFNs or other cytokines following the initial innate type I IFN response. 1) In our studies in chapter 2, we observed rapid down-regulation of pDC IFN responses in Mob reporter mice that were decreased to near baseline levels by day 5 p.i. regardless of infection with acute or chronic strains of LCMV. 2) When we investigated pDC activation in Mob reporter mice during chronic infection, we were unable to find activated pDCs at either day 8 or 15, despite high levels of viremia at this time point. Therefore, along with similar observations previously reported by the Oldstone group (189), this led us to investigate the mechanism by which this might occur.

Considering our results that TLR7-dependent activation signals led to increased caspase activation in pDCs and decreases in pDC number, it seemed likely that activation-dependent signals were also responsible for inducing a state in pDCs such that they are refractory to further stimuliation with other TLR ligands, such as CpG. Our results using Mob and Mob-*Tlr7*$^{-/-}$ reporter mice, indicate that TLR7-dependent activation of pDCs is qualitatively different across the entire pDC population following LCMV infection. Whereas YFP^{+} pDCs expressed high levels of CCR7 and CD80, and this was dependent on TLR7 signaling, YFP^{-} pDCs were intermediate in their expression of these molecules. This could indicate that: 1) YFP^{-} cells are activated sub-optimally and increase expression of some activation markers, but not type I IFN expression; 2) YFP^{-} cells are sub-optimally activated and make type I IFN, but not enough to rise above the level of detection in reporter mice; or, 3) that YFP^{-} pDCs represent a transitory state of pDC activation and are either previously activated pDCs that are no longer making high levels of type I IFN, or are newly activated pDCs that have not yet fully up-regulated type I IFN production. Given these different possibilities, we hypothesized that remaining pDCs in TLR7-sufficient mice may represent either those pDCs that were sub-optimally activated or were pDCs that were previously activated but failed to undergo cell death and were now unresponsive to TLR stimulus. Surprisingly, Mob-*Tlr7*$^{-/-}$ pDCs, following LCMV infection, were unable to make type I IFN in response to CpG administration.

Why is it that previous activation of pDCs in a cell-intrinsic manner is not a determinant of the refractory state? One possible explanation for this is that TLR7-independent modes of pDC activation following LCMV infection also occur. For example, although TLR7 signaling was required to fully induce expression of the co-

stimulatory molecule CD86, pDCs in *Tlr7*$^{-/-}$ mice increased CD86 expression relative to that of uninfected mice, despite being unable to produce type I IFN. This is likely why we and others have seen no differences in CD86 expression among YFP^{-} and YFP^{+} pDCs in Mob mice following LCMV infection (233). As CD86 expression on pDCs is less TLR-dependent, but nonetheless increased by infection, this indicated that pDCs may receive other cell-extrinsic signals that may induce unresponsiveness. To address this question, we used Mob-BDCA2-DTR Tg mice to deplete pDCs throughout the initial 5 days of the immune response with the idea that, since pDC responses waned by day 5 p.i., it was likely a factor produced during the innate phase of the immune response that was responsible. However, pDC depletion during this time was not sufficient to restore responsiveness of pDCs to CpG administration.

In general, pDCs are primarily regulated, in a stimulatory or inhibitory manner, by various receptors that associate with the adaptors DAP12 or FcRγ (87, 191). In mice, Siglec-H, a DAP12-associated, pDC-specific receptor, has been shown to negatively regulate pDC type I IFN secretion (98, 236). However, when we examined pDC response to CpG in mice lacking both DAP12 and FcRγ following infection with LCMV, we again found that these mice were not able to make type I IFN to a similar extent as that in uninfected mice. However, this does not rule out a role for either DAP12 or FcRγ alone, or specific receptors that associate with these adaptors, in regulating the refractory phenotype of pDCs following infection. As these adaptors are involved in signaling downstream of both activating receptors such as PDC-TREM (235) and the inhibitory receptor Siglec-H, it is possible that the effect of loss of the adaptors alone is one that is relatively neutral. It may also be that other non-DAP12 or FcRγ-associated receptors may

compensate when signaling via these receptors is compromised. This remains a possibility as other non-DAP12 or FcRγ-associated receptors that inhibit pDC function have been identified (191).

Therefore, although we have shown that Mob reporter mice can be an invaluable tool for the study of pDC responses *in vivo*, and using this system have contributed importantly to the knowledge of mechanisms that regulate pDC function and responsiveness following infection, much remains to be learned. A molecule or mechanism by which pDC responses to subsequent TLR stimulation are abrogated has yet to be identified. Presumably, as type I IFN production in response to CpG is dependent on TLR9 engagement on pDCs (93, 98), the inability of pDCs to respond to CpG following initial infection is an intrinsic property of pDCs. However, we cannot exclude the possibility that delivery of CpG to pDCs is impaired in LCMV-infected mice. As CpG can be labeled with fluorescent moieties (77), it will be necessary to verify whether or not CpG is properly delivered to pDCs at these later time points. This could partially account for why, although we do not observe pDC activation following initial stimulus, aberrant pDC activation has been associated with various autoinflammatory and autoimmune diseases. For example, nucleic acid complexes with proteins such as HMGB1 and LL37 have been associated with inappropriate pDC activation in various inflammatory settings (226, 227, 251). It may be that these proteins are required to properly deliver stimulatory nucleic acids to pDCs under such conditions and override a refractory state. It may also be that sterile inflammation does not produce a factor that makes pDCs refractory, and is a qualitatively different setting than that of virus infection.

In addition to autoimmunity, chronic stimulation of pDCs has been associated with chronic viral diseases such as during the SIV model of HIV infection (238). As our study and another from the Oldstone group (189) failed to identify activated pDCs during chronic infection with LCMV, and pDCs were decreased in responsiveness following infection with other viruses such as MCMV and VSV, more research is needed to understand the activation status of pDCs *in vivo* during the context of chronic virus infection. In order to address these questions, it may be necessary to develop ways to follow pDC cell fate *in vivo*. For example, such a tool could address questions such as: Are all activated pDCs quickly induced to undergo cell death? What is the rate of pDC renewal following infection? Is there a difference in the previous activation history of pDCs that respond in chronic inflammatory settings? This may be particularly important as adjuvants that activate innate immunity may potentially be used therapeutically to treat chronic infections (252).

Innate Immune Recognition During Chronic Virus Infection

Conclusion

Our studies provide new insight into the roles that innate immune recognition plays in the context of chronic viral infection. Using infection with LCMV as a model, we showed that early innate immune responses and initial recognition of virus infection by various PRRs and innate immune cell types were generally similar during both acute and chronic infections. Activation of $CD8^+$ T cell responses was similarly dependent on the more dominant pathway of innate recognition. As a generalization, it may be that T cell responses are affected to a greater extent by the pathway that more potently induces

immunostimulatory cytokines and antigen presentation. In the case of LCMV, in which type I IFN is the dominant 'signal 3' cytokine, this means that the RLR pathway is more dominant in this regard. The outcome of early priming by innate responses may be particularly important, as latter stimulation of innate immune type I IFN appears, at least in the case of pDCs, is impaired in both chronic and acute settings.

In contrast, our results showed a novel role for TLR recognition by B cells in antibody responses during chronic virus infection. Whereas TLR recognition of virus by B cells augmented GC B cell and plasma cell responses during both acute and chronic infections, it was particularly important for antibody responses during chronic infection. This effect was, at least in part, due to an effect on the proliferation of GC B cells and altered distribution of GC B cells in the GC reaction. In addition to a defect in GC B cell responses in the context of TLR deficiency, we observed a secondary defect in the formation of plasma cells in these mice indicating layers of TLR-dependent regulation of B cell antibody responses. As a whole, these studies can serve as a stepping-stone towards an understanding of how to harness innate immunity during immunization and as treatment during chronic viral infection.

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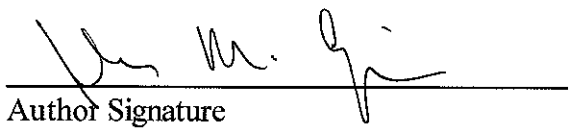

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