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Early Host-Virus Interactions and Late Dendritic Cell Immune Exhaustion in
Persistent Viral Infections

A thesis submitted in partial satisfaction of the requirements

for the degree of Master of Science

in

Biology

by

Aaron Yu-Yuan Chang

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      Professor Elina Zuniga, Chair
      Professor Michael David
      Professor Li-Fan Lu

2013

The thesis of Aaron Yu-Yuan Chang is approved, and it is acceptable in quality

and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013

DEDICATION

I dedicate this thesis to my family—my parents David and Monica and my sisters

Candace and Tiffany, and my girlfriend Diana for all their love and support

outside the lab throughout my studies and pursuit of research.

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ABSTRACT OF THE THESIS

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by

Aaron Yu-Yuan Chang

Master of Science in Biology

University of California, San Diego, 2013

Professor Elina Zuniga, Chair

Dendritic cells play a critical role in orchestrating a robust immune response against viral infections. These specialized cells are responsible for various functions including, the production of type I interferon, (IFN-I) which plays a critical role in both innate and adaptive immune responses against viral infections. Chronic viral infections such as HIV and HCV in humans, and lymphocytic choriomeningitis virus (LCMV) in mice, lead to dendritic cell dysfunctions. Here, we demonstrated that plasmacytoid dendritic cells (pDCs) were a preferentially targeted leukocyte population early after LCMV infection *in-vivo* and that they represented a major leukocyte source of IFN-I. Remarkably, while conventional dendritic cell (cDC) IFN-I production was dependent on intrinsic viral replication, but uninfected pDCs produced IFN-I in a Toll-like receptor (TLR)-7 dependent manner. Moreover, continuous TLR stimulation was sufficient to down-regulate expression of a key pDC transcription factor, E2-2,

and exhaust pDCs for IFN-I production. Treatment with a TLR-7 agonist during development caused pDC developmental and functional defects. Thus, the present study provides new insight into the mechanisms that lead to dendritic cell dysfunction during chronic viral infections and demonstrates that TLR stimulation alone is enough to cause functional defects in pDCs.

INTRODUCTION

Chronic Viral Diseases and Immunosuppression

Chronic viral infections are a major world health issue as infections affect millions of people worldwide each year [1]. Whereas many viruses elicit an acute infection where the virus can be contained and eliminated by the host's immune system, persistent viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) have several immune evasion strategies that promote host immune suppression and exhaustion that lead to viral dissemination and persistence [2]. Often, immune exhaustion and/or depletion associated with persistent viral infection causes immunodeficiency, which leads to enhanced host susceptibility to opportunistic infections by pathogens that are normally non-threatening. For example, HIV-infected patients are more susceptible to pathogens such as cytomegalovirus, mycobacterium, and seasonal and pandemic influenza [2-5]. Moreover, chronic viral infections cause an increased risk for cancers such as the high incidence of hepatocellular carcinoma in HCV patients and increased risk for various cancers including Kaposi sarcoma, non-Hodgkin lymphoma, Hodgkin lymphoma, and lung cancers in HIV patients [6, 7]. The increased susceptibility to pathogens and cancers associated with chronic viral infection can often lead to enhanced morbidity and death [4, 5, 7]. There are key host immune evasion strategies that chronic viruses utilize to establish viral persistence, resulting in host adaptive and innate immune defects. For example, adaptive immune cells such as cytotoxic CD8 T cells and helper CD4 T cells are exhausted and/or depleted through various mechanisms during HIV infection [8].

Furthermore, during HIV and other chronic infections, the co-inhibitory molecule programmed cell death-1 (PD-1) is up-regulated on CD4 and CD8 T cells and is associated with cell anergy [9]. HIV can also deplete CD4 T cells through direct infection and cytolysis, CD8 T cell killing of HIV-infected CD4 T cells, and increased apoptosis due to immune hyperactivation [8, 10]. Because CD4 T cells are important adaptive immune orchestrators which prime B cells for antibody production, activate CD8 T cells for killing, and activate innate leukocytes, the exhaustion and depletion of CD4 T cells leads to heavy abrogation in nearly all arms of the immune system [8, 10]. Lastly, while immune exhaustion in the *adaptive* immune response has been well characterized, less is known about the mechanisms that cause *innate* immune exhaustion.

Dendritic Cells are Important Immune Modulators

To mount an effective immune response against a viral infection, the host must be able to quickly recognize the pathogen and develop robust general and specific defenses against it. Dendritic cells (DCs) are paramount to the recognition of viral pathogens and function to bridge the innate and adaptive immune system [11]. There are different subsets of DCs which have distinct roles in orchestrating a proper defensive response. Conventional dendritic cells (cDCs) specialize in pathogen uptake and antigen presentation to T and B lymphocytes to prime the adaptive immune system for a pathogen-specific response [12]. Due to this strong ability to present antigen, cDCs are known as the major professional antigen presenting cell. There are two distinct subsets of

cDCs in the murine spleen: CD11b$^+$ cDCs and CD8$^+$ cDCs. CD11b$^+$ cDCs specialize in antigen presentation via major histocompatibility complex (MHC) II, primarily to CD4 T cells [13, 14]. CD8$^+$ cDCs function as cross-presenters because they specialize in sampling the extracellular matrix for antigens and present antigen to cytotoxic CD8$^+$ T lymphocytes via MHCI [12, 15].

Lastly, plasmacytoid dendritic cells (pDCs) are a third subset of DCs which comprise approximately 0.1-0.5% of splenic mononuclear cells in mice and peripheral blood mononuclear cells in humans [16, 17]. Although a small portion of DCs, pDCs are highly specialized to produce copious amounts of the potent antiviral mediator type I interferon (IFN-I) upon encounter with antigenic or self nucleic material [18]. Although most heavily regarded for their ability to produce significant levels of IFN-I, they are also capable of producing other cytokines such as the pro-inflammatory cytokines TNF-α, IL-6, and IL-12 [19, 20]. Moreover, pDCs are also capable of antigen presentation to T cells but with less proficiency than the cDCs [20].

DCs can sense infections via toll-like receptors (TLRs), pattern recognition receptors found on mammalian antigen-presenting cells which can bind general motifs common to groups of pathogens [21, 22]. TLRs can be found on the cell surface (TLR1, 2, 4, 5, and 6) and within endosomes (TLR3, 7, 8, 9, and 10) [22, 23]. They recognize molecules such as lipopolysaccharide (LPS) which is found on gram-negative bacteria, dsRNA and ssRNA which is used as the genomic material for large families of viruses, and DNA CpG motifs found in viruses and bacteria [22]. Furthermore, DC subsets express complementary repertoires of

TLRs, suggesting that they may have evolved to recognize different pathogens [21]. For example, cDCs express various TLRs including the endosomal TLR3 and the surface TLR4 to recognize dsRNA and LPS respectively [21]. In contrast, pDCs selectively express high levels of endosomal TLR7, TLR8, and TLR9 which recognize ssRNA (TLR7/8) and DNA CpG motifs (TLR9) that are widely found in viral genomes [21]. There are also non-TLR mechanisms of pathogen sensing such as cytosolic retinoic acid-inducible gene 1 (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) which can sense dsRNA in the cytosol to signal an antiviral response including the production of type I interferon [24].

Type I Interferons are Potent Anti-viral Cytokines

Type I interferons (IFN-I) are a class of potent antiviral cytokines which are important for interfering with viral replication and for triggering a defensive immune response [25, 26]. There are many different subclasses of IFN-I, comprised of several subtypes of IFN-α and IFN-β, all of which have considerable structural homology [25, 27]. IFN-α is primarily produced by leukocytes, especially pDCs, and is comprised of 13 subtypes: IFNA1, IFNA2, IFNA4, IFNA5, IFNA6, IFNA7, IFNA8, IFNA10, IFNA13, IFNA14, IFNA16, IFNA17, and IFNA21 [27]. IFN-β is produced in large quantities by fibroblasts during infection but can also be produced by leukocytes, and is comprised of only 2 subtypes: IFN-β1 and IFN-β3 [25]. All IFN-α and IFN-β subtypes signal through a common receptor: interferon-α/β receptor (IFNAR). The production of IFN-I is a rapidly-induced response, which may be detected within just hours after viral

infection. IFN-I can directly interfere with viral replication by causing reduced protein synthesis, RNA degradation, and increased apoptosis--all of which will inhibit viral replication and reduce the virus' ability to infect new cells [25, 26]. IFN-I mediates much of this antiviral effect through downstream signaling to up-regulate interferon inducible genes. For example, IFN-I up-regulates Mx1 which disrupts cellular functions required for viral replication [28]. IFI16 promotes apoptosis by upregulating pro-apoptotic factors such as BAX and PUMA [29]. IFN-I also functions to activate other cells of the adaptive and innate immune system. For example, IFN-I promotes key innate immune response strategies, such as upregulating MHCI and MHCII on cDCs which leads to enhanced antigen presentation capability and cytotoxic T cell killing [30]. In addition, IFN-I induces NK cell cytotoxicity and enhances NK cell production of the important effector cytokine IFN-γ [31]. Studies have shown that IFN-I also augments macrophage phagocytocis and cytokine production along with promoting DC differentiation from monocytes and enhancing the ability of DCs to induce cytotoxic T lymphocyte expansion [32-34]. In adaptive immunity, IFN-I boosts the primary antibody response through direct effects on DCs, B cells, and T cells [32, 35]. Moreover, IFN-I promotes differentiation of naïve CD4 T cells into IFNγ-producing T cells and maintains survival of activated CD4 T cells [36, 37]. IFN-I is also required for proper CD8 T cell clonal expansion and survival and contributes to CD8 T cell memory formation [38, 39].

There are several pathways used by innate immune cells to mediate IFN-I production in response to virus. pDCs primarily use endosomal TLR7 and TLR9

to recognize ssRNA and CpG DNA motifs respectively (Figure 1A). Recognition of nucleic material by both TLR7 and TLR9 leads to recruitment of the key adaptor protein MyD88 [40]. MyD88 recruitment induces a signaling cascade which leads to phosphorylation and nuclear translocation of the transcription factor IRF7, which promotes IFN-α production [22, 40]. This IFN-α can then act both in an autocrine and paracrine fashion to up-regulate the defensive interferon stimulated genes (ISGs) and create a localized anti-viral defense environment [41, 42]. In contrast, cDCs and macrophages primarily produce IFN-I through TLR3 and RIG-I signaling (Figure 1A). Endosomal TLR3 recognizes dsRNA and recruits the adaptor protein TRIF, inducing a MyD88-independent signaling cascade that leads to phosphorylation and activation of the transcription factor IRF3 to promote IFN-β expression [23]. Similarly, RIG-I recognizes intracellular dsRNA but uses a TRIF-independent pathway to activate IRF3 to promote IFN-β expression [43]. The TLR3 and RIG-I mediated IFN-β can then act in an autocrine fashion to up-regulate intrinsic IRF7 and effectively cause a feedback loop which leads to more copious amounts of IFN-I secretion mediated by IRF7 [42, 44]. Therefore, IRF7 is often thought of as the "master regulator" of IFN-I production [44]. DCs are also capable of production pro-inflammatory cytokines and chemokines in response to pathogen sensing. Utilizing the TLR and MyD88-dependent signaling pathways, these cytokines are induced following activation of nuclear-factor kappaB (NF-kB), a key transcription factor that promotes production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and chemokine responses [46].
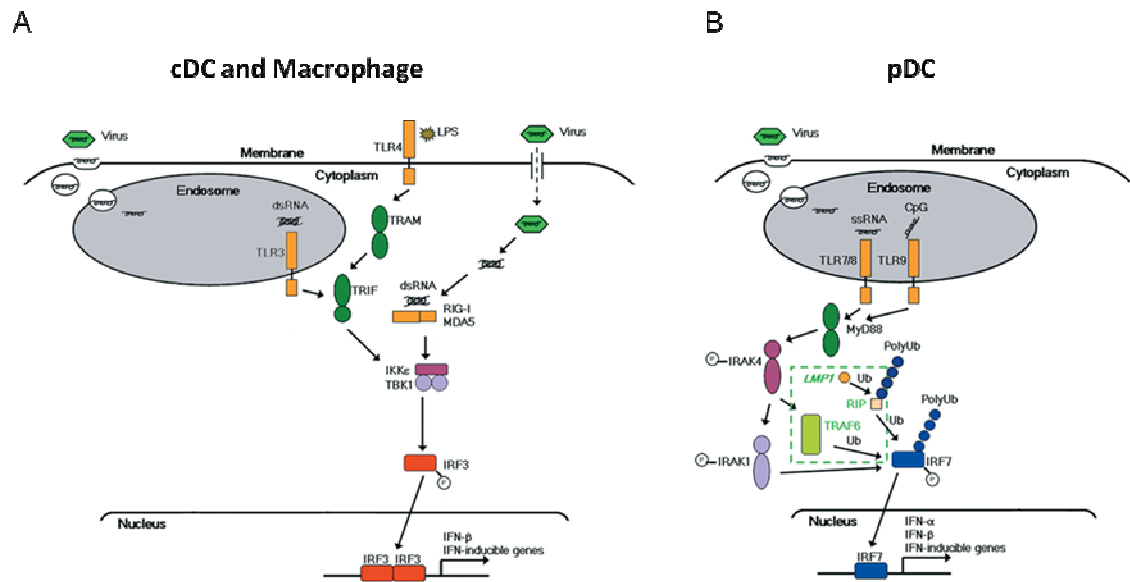
Figure 1. IFN-I Pathway in Innate Leukocytes Cells

IFN-I is produced through distinct signaling pathways [45] (A) cDCs and macrophages use the TLR3 and RIG-I pathways to produce IFN-β while (B) pDCs use the TLR7 and TLR9 pathway to primarily produce IFN-α.

Chronic Viral Infection Leads to Dendritic Cell Dysfunction

Because DCs are a crucial bridge between innate and adaptive immunity, they represent an attractive target for viruses to disrupt host immune response and achieve viral persistence. Numerous studies in HIV and HCV have shown that viral persistence leads to depleted numbers of DCs and DC functional defects [2, 47]. cDCs isolated from HIV-infected individuals show less-efficient stimulation of allogenic T cells when compared to DCs from healthy donors, and HIV-infected DCs fail to become activated when exposed to additional stimuli [48]. pDCs from HIV-infected individuals also have reduced capacity to produce IFN-α [49, 50]. Similarly, during HCV infection, cDCs have reduced capacity to stimulate allogenic T cells and produce less IL-12 in response to stimuli, whereas they produce more of the anti-inflammatory cytokine IL-10 [51, 52]. Limited studies have also shown that cDCs from chronic HCV patients have reduced expression of MHCII and CD86, suggesting decreased capability to present antigen-to or activate other leukocytes [53]. Moreover, pDCs from HCV patients have reduced IFN- α production upon stimulation and reduced ability to activate CD4 T cells [51, 52]. Although HIV and HCV have unique structures and replication mechanisms, they share similarities in characteristic DC depletion and functional defects. This supports the hypothesis that persistent viruses may promote innate immune exhaustion through conserved mechanisms, leading to the unanswered question of what key molecular events lead to DC immune exhaustion and how this can be remedied. Perhaps if proper function of the innate immune system can be restored, we may better treat persistent infections.

Murine LCMV is a Model to Study Chronic Viral Infections

One difficulty of studying the host defense against human persistent viruses is the limited availability of small animal models. Lymphocytic choriomeningitis virus (LCMV) is a powerful tool to study chronic and persistent viral infections within its natural murine host, providing a unique setting to study the interaction between the host immune system and viral infection at the whole organism level. Studies in the LCMV model have identified important factors that translate to human diseases including the discovery of the role of programmed death-I (PD-1) in mediating pathogen-specific CD8 T cell dysfunction in HIV and HCV infection [9, 54]. Studies in LCMV and HIV have identified transforming growth factor-β (TGF-β) a cytokine contributing to CD8 T cell apoptosis [55, 56]. Moreover, during persistent LCMV infection, infected cDCs have reduced MHCII and costimulatory molecules similar to HCV patients [57]. Finally, pDCs isolated from chronic LCMV-infected mice exhibit exhaustion of IFN-I production in response to further stimulation, similar to pDCs from HIV-infected patients [58, 59]. This pDC exhaustion is important because it leads to a defect that impairs the host's ability to respond against secondary infections [58].Thus LCMV can be thought of as the "Rosetta stone" for the investigation of virus-host interactions [60].

LCMV is an enveloped single stranded RNA virus that is regarded as a prototypic arenavirus [61] a family of viruses that includes several highly pathogenic human viruses such as lassa fever virus and junin virus which can

cause deadly hemorrhagic fever [62]. In contrast, LCMV typically results in only mild flu-like symptoms, if any, in otherwise healthy individuals [61]. However, LCMV is a powerful animal model of virus infection, as it is a natural mouse virus that, depending on the lab isolate used, can lead to very different clinical outcomes. For example, LCMV Armstrong 53B (Arm) causes an acute infection resulting from a robust immune response comprised in large part by cytotoxic T lymphocytes and viral clearance within 10 days [63]. LCMV Clone 13 (Cl13) on the other hand, leads to higher viral titer, rapid immune exhaustion, and viral persistence [64], mediated by the induction of a generalized immunosuppression that includes ablation of virus-specific CD8 and CD4 T cells, and reduced antibody responses [64]. It is important to note that although the virus persists during Cl13 infection, it does eventually clear from various compartments such as the serum, for example, 60-80 days post-infection [65]. Cl13 also clears in other organs such as the spleen, but not in some organs such as the kidneys [65]. Interestingly, LCMV Cl13 is an isolate that differs from Arm by two main amino acid substitutions in the viral glycoprotein and polymerase [66]. The mutation in the viral glycoprotein causes Cl13 to have an increased binding affinity to α-dystroglycan (α-DG), its cellular receptor, which among immune cells is expressed preferentially on dendritic cells. The mutation in the polymerase leads to increased replicative capability and thus increased early viremia [67]. Both mutations have been shown to be important for viral persistence and immunosuppression, characteristics which differentiate Cl13 from Arm [67, 68].

Aims and Objectives

While the mechanisms of adaptive immune exhaustion have been well characterized, less is known about the mechanisms of innate immune response and exhaustion in the context of a chronic viral infection. Understanding the early immune response to a chronic viral infection may uncover key mechanisms that may be critical for establishing a persistent infection. In this study, we aimed to define interactions between the host innate immune system and persistent viruses, which lead to innate immune dysfunction and chronic infection. We hypothesize that infection with a chronic virus may inhibit IFN-I production in leukocytes and that continuous antigenic stimulation promotes exhaustion of cytokine secretion. We explored LCMV tropism in leukocytes and examined the effects of direct infection on cellular function. We utilized LCMV *in-vivo* and TLR-7 and TLR-9 agonists (Loxoribine and CpG respectively) *in-vitro* to study mechanisms of IFN-I production and DC exhaustion. The overall objective was two-fold: in chapter 1, we studied the early innate IFN-I response to LCMV, mechanisms of viral tropism in innate leukocyte populations, and how infection affected IFN-I production directly. In chapter 2, we investigated the mechanisms of chronic DC cytokine exhaustion examining continuous stimulation of mature pDCs and stimulation-mediated developmental abnormalities in pDC progenitors as a cause of defect. Together, we hope that this data may add to our understanding of DC and innate immunological defects during persistent viral infections.

Chapter 1: Early Mechanisms of IFN-I Response and LCMV Interactions with DCs

Introduction

The interactions of the host immune system with a virus early during infection are critical and may determine disease outcome. Studies in LCMV suggest that Cl13's enhanced viral replicative capacity compared to Arm is one of the crucial differences that leads to Cl13's persistence [67]. Because IFN-I is important for controlling early viral replication and activating other immune cells, we focused on early defects in IFN-I and IFN-I producing-cells during viral infection [42, 69, 70]. Although the function of IFN-I is well characterized, the virus and host factor interactions that are important to produce a robust IFN-I response or suppress it is less clear. It is known that LCMV Cl13 has preferential binding to cells that express α-DG including DCs [2, 57, 71] and that LCMV infection can directly inhibit IFN-I production by blocking nuclear translocation of IRF3 in DCs [72, 73]. Direct inhibition of IFN-I has also been demonstrated in other viruses. The NS protein of influenza can block IFN-I through blocking ISG15, RIG-I, and IRF3 [74-76]. HCV nonstructural protein 5a (NS5a) can also inhibit the IFN pathway by blocking signal transducers and activators of transcription 1 (STAT1) signaling after ligation of IFN to the IFN-receptor [77], and also through TLR3 and RIG-I pathway inhibition [78-80]. Thus, It is reasonable to believe that direct infection of leukocytes by Cl13 may inhibit IFN-I production *in-vivo.* We are interested in which leukocyte populations are targeted

by Cl13 early after infection, possibly giving the virus a replicative advantage, and how direct infection affects early IFN-I production in DCs.

In the present study, we aimed to first characterize the relative contributions of various IFN-I pathway genes to systemic IFN-I production *in-vivo,* then determined which leukocyte populations were being targeted by Cl13. Lastly, we examined the effects of direct infection on pDC and cDC IFN-I production. We hypothesize that the lack of TLR7, MyD88, and IRF7 would lead to decreased systemic IFN-I during *in-vivo* infection. We also hypothesize that pDCs, which rely exclusively on the TLR7/MyD88/IRF7 pathway for IFN-I production, would be readily infected by Cl13 due to their expression of α-DG and that this enhanced viral targeting would inhibit IFN-I production. We determined that IRF7 is essential for early systemic IFN-I production *in-vivo,* that pDCs are a preferentially targeted leukocyte population early during Cl13 infection, and that pDC IFN-I production is dissociated from intrinsic viral replication. This study provides insight into early host-virus molecular interactions which may be important for determining viral persistence.

Results

Systemic IFN-I Peaks at 24 hrs and is Reduced in IFN-I Pathway Deficient Mice

LCMV infection elicits a robust systemic early IFN-I response in mice which

peaks at 1 day post-infection (p.i.) and is rapidly silenced by 5 days p.i. [58]. To

better understand the early kinetics of the IFN-I response, we performed a more

extensive kinetic within the first 24 hours of Cl13 infection. Using an IFN-I

luciferase reporter bioassay with L-929 cells with the luciferase gene transfected

downstream of an interferon-sensitive response element [81], significant

systemic IFN-I levels were not detected until 24 hours (hr) p.i., when IFN-I levels

were at their peak (Figure 2A). As described previously, IFN-I can be produced

through several different pathways in response to viral sensing including the

endosomal TLR7/IRF7 and TLR3/IRF3 pathways associated with DCs. To

examine the individual contribution of specific proteins in these IFN-I signaling

pathways, we examined the early systemic IFN-I kinetic on various IFN-I pathway

knockout mice. For this, we measured IFN-I levels in the serum of wild type

(WT), IRF3-/-, IRF7-/-, MyD88-/-, and TLR7-/- mice throughout the first 5 days of

LCMV Cl13 infection. In comparison with WT mice, IRF3-/- mice demonstrated

no significant defect in systemic IFN-I production whereas both TLR7-/- and

MyD88-/- mice had reduced systemic IFN-I (Figure 2B). Most dramatically, IRF7-

/- mice had completely undetectable serum IFN-I. The deficiency of IRF7 also

prevented the eventual systemic clearance of LCMV Cl13 observed in WT mice.

Whereas by 140 days p.i. all WT mice had cleared Cl13, two out of three IRF7-/-
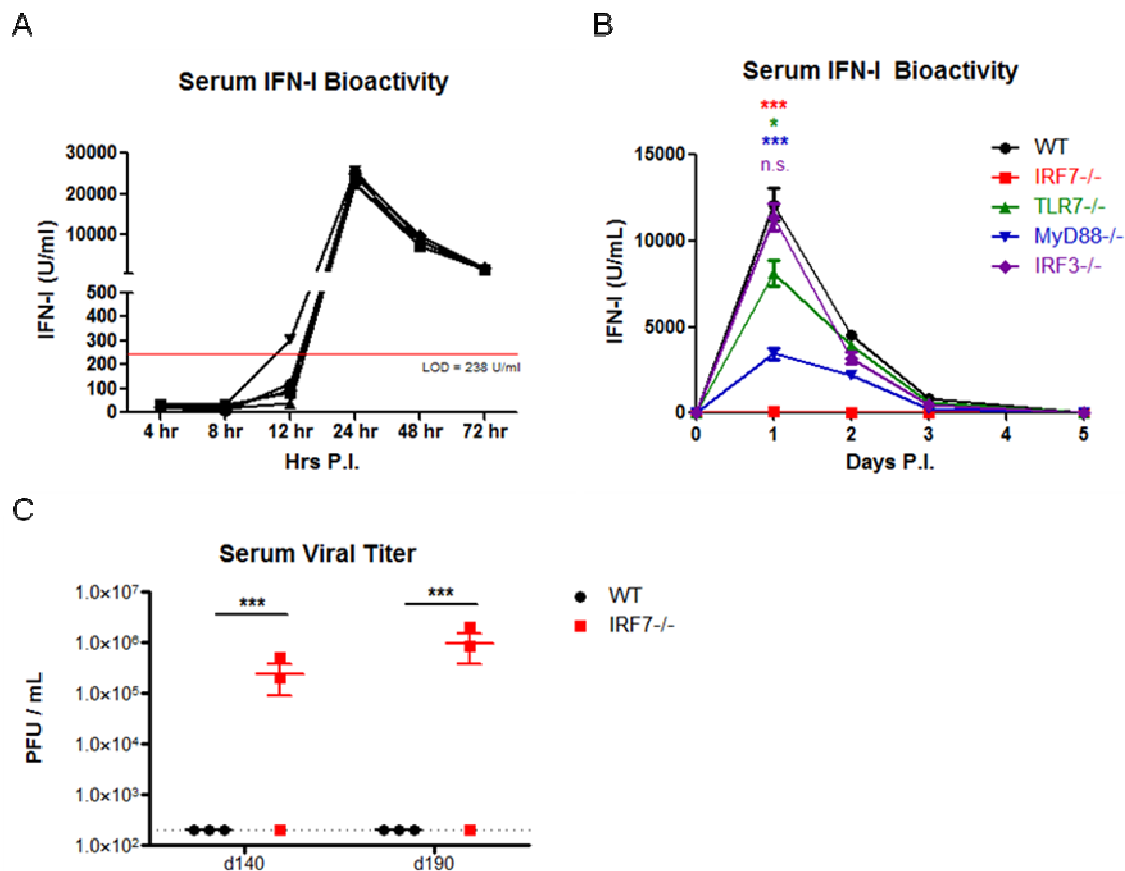
Figure 2. Characterization of Early Systemic IFN-I response and Viral Persistence in WT and IFN-I pathway Deficient Mice.

Mice were infected intravenously with $2x10^6$ pfu Cl13 LCMV and serum was extracted from blood (A) 4, 8, 12, 24, 48, and 72 hours post-infection and (B) 1, 2, 3, and 5 days post-infection. IFN-I activity levels were measured in serum by luciferase bioassay. (C) Serum viral titers were measured by plaque assay at late time points. The mean ± SEM of three to five mice is shown.

mice maintained high serum viral titers which remained at day 190 p.i. when the

mice were sacrificed (Figure 2C). These data confirm previous findings that IRF7

is the master regulator of systemic interferon production [44, 82] and it suggests

an important role for IRF7 in the eventual clearance of Cl13.

Generation of Recombinant tri-segmented GFP-LCMV Cl13

It is known that pDCs are strong producers of IFN-I [18, 71, 83] and that

Cl13 infects DCs and can directly disrupt DC function [84]. To further elucidate

the mechanism of early IFN-I production after LCMV Cl13 infection, we next

focused on which leukocyte populations were being infected and how infection

affected cell function. To do this, we utilized recombinant tri-segmented GFP

LCMV Cl13 (Cl13-GFP) reporter virus, a useful tool to study arenavirus tropism.

The virus was originally generated by engineering two plasmids to express

enhanced GFP with either LCMV *nucleoprotein* (NP) or *glycoprotein* (GP) in the

short segment [60] (Figure 3A). These plasmids were transfected into baby

hamster kidney-21 (BHK) cells along with plasmids encoding the LCMV long

segment to rescue the recombinant GFP-Cl13 LCMV (Cl13-GFP). Although this

is an artificially constructed virus which has been shown to be attenuated and

replicates slower than its wild-type counterpart, the Cl13-GFP is a useful tool

because it allows us to directly visualize and detect infected cells [60]. However,

in comparison to WT LCMV Cl13, Cl13-GFP is attenuated with a slower

replication kinetic. In order to perform *in vivo* studies, we first needed to obtain

substantially high viral titers. In light of the attenuated growth kinetics of Cl13-

GFP, our initial experiments focused on optimization of virus generation for this

recombinant virus. Typically, LCMV Cl13 is generated by infecting BHK cells at

0.1 multiplicity of infection (MOI) and collecting the supernatant at 48 hrs p.i.,

yielding titers in excess of $1x10^7$ plaque forming units (pfu)/mL [85]. However,

because the GFP-LCMV Cl13 replicates slower, standard protocols to grow the

virus resulted in low and unusable titres. By instead inoculating the BHK cells

with 0.01 MOI and collecting the supernatant 72 hrs p.i. as suggested by other

protocols [61], we found that there is a significantly increased viral titer in the

culture supernatant and that we can generate Cl13-GFP viral stock to much

higher titers (Figure 3B). The virus was further purified and concentrated by using

poly-ethylene glycol precipitation to obtain suitable titers for *in vivo* infection [86]

(Figure 3B). Using a fluorescence microscope, we were also able to image

infected BHK cells and confirm successful infection by GFP expression (Figure

3C).

Cl13-GFP Infects DCs with pDCs being the Most Infected Leukocyte Population
Examined

It is known that arenaviruses can infect macrophages and DCs and impair

IFN-I production and other innate immune responses [72, 73]. To determine

which leukocyte and DC subset was more highly infected early after LCMV

Cl13challenge, we utilized the recombinant Cl13-GFP which would express GFP
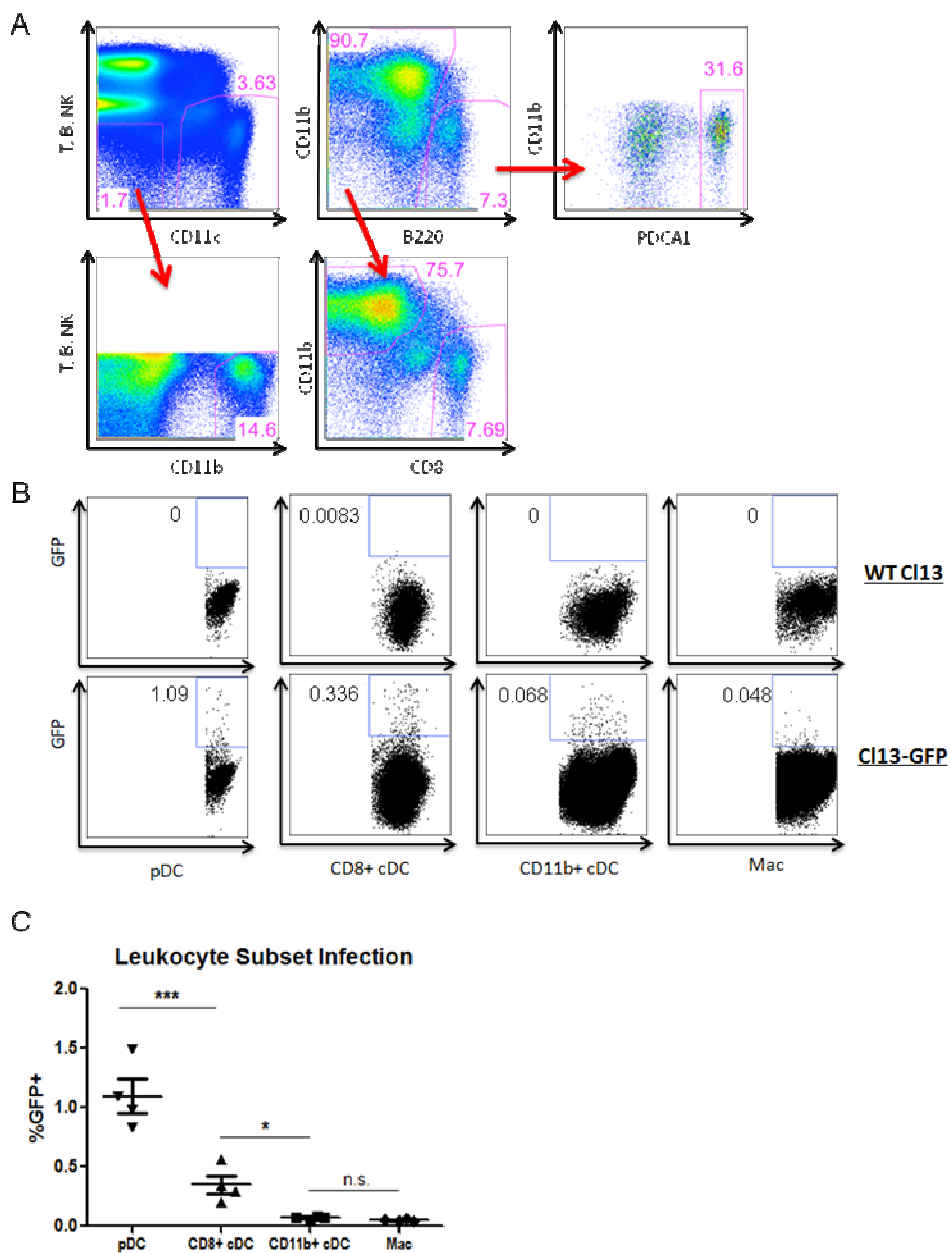
Figure 4. Cl13-GFP Infect DCs with pDCs Being the Most Infected Subset Early After Infection.

WT mice were infected with 8×10^4 pfu Cl13-GFP through tail intravenous injection. Mice were sacrificed at 24 hours post-infection and splenic mononuclear cells (SMCs) were isolated from spleens and stained. (A) FACS gating strategy used to gate SMCs into leukocyte populations (pDCs, CD11b+ cDCs, CD8+ cDCs, and macrophages). (B) Representative FACS plots for WT Cl13 or Cl13-GFP-infected (n=4) splenic leukocytes 24 hours post-infection are shown. (C) Scatter plot of %GFP+ per infected leukocyte population is shown (n=4).This experiment is representative of 7 independent experiments with 4 mice per group. *p < 0.05, **p < 0.001, ***p < 0.0001.

in infected cells. Mice were infected with Cl13-GFP and splenic mononuclear

cells (SMCs) were isolated at 24 hours p.i. Splenic DC and leukocyte subsets

were then stained and evaluated for GFP+ (infected) cells. Leukocyte subset

populations were defined as: total DC: (Lin$^-$, CD11c$^+$); CD11b$^+$ cDC: (Lin$^-$,

CD11c$^+$, CD11b$^+$, B220$^-$, CD8$^-$); CD8$^+$ cDC: (Lin$^-$, CD11c$^+$, CD11b$^-$, B220$^-$, CD8$^+$);

 pDC: (Lin$^-$, CD11c$^+$, CD11b$^-$, B220$^+$, PDCA-1$^+$); and Macrophage: (Lin$^-$, CD11c$^-$,

CD11b$^+$ (Figure 4A). Through flow cytometric analysis, pDCs demonstrated the

highest percent GFP+ cells compared to other DC populations or total

macrophages (Figure 4B, C). To confirm this finding, GFP+ total DCs were gated

on PDCA-1 versus CD11b (Figure 5A). As expected, a higher percentage of

GFP+ infected DCs were PDCA-1+ compared to CD11b+, suggesting a pDC-like

phenotype. Moreover, backgating of the GFP+ DCs showed that they expressed

PDCA-1 and B220 at higher levels than CD11b (Figure 5B), confirming that

pDCs were the main GFP-expressing splenic cell type at this early time point p.i.
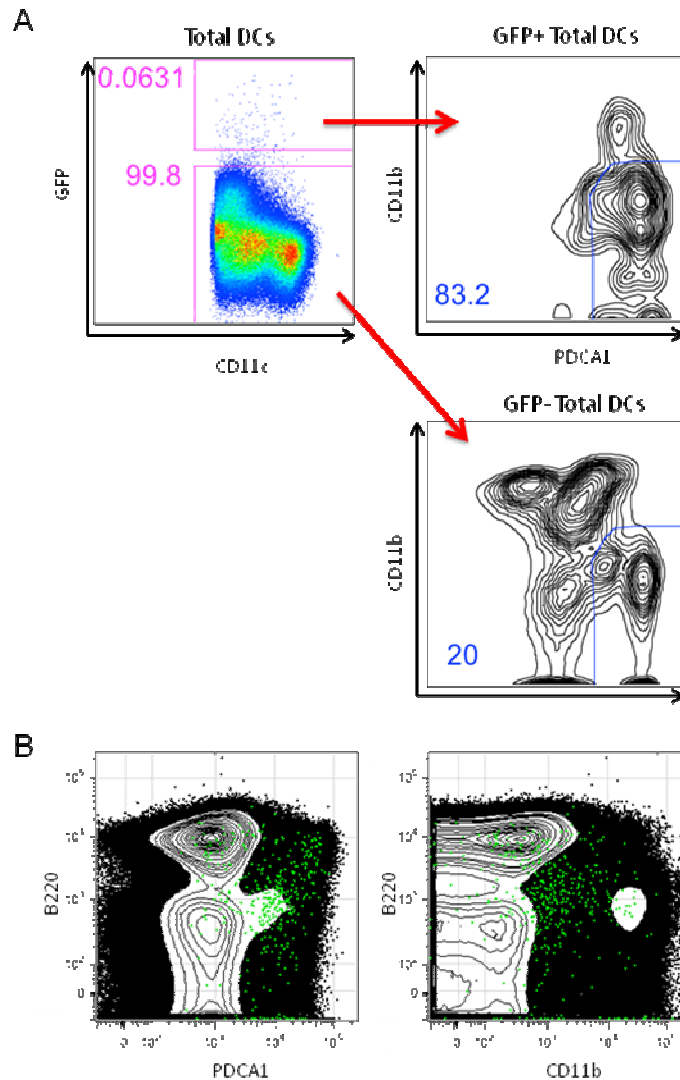
Figure 5. GFP+ DCs Have Higher Expression of PDCA1 than CD11b

SMCs from Cl13-GFP-infected mice from experiments in figure 4 were back-gated for GFP expression. (A) GFP+ and GFP- DCs were analyzed for PDCA and CD11b expression. (B) GFP+ DCs were back-gated on PDCA1 and CD11b. Data represents 7 independent experiments with 4 mice per group.

pDCs are a Highly-Infected Leukocyte Population During WT Cl13 Infection

To ensure that the data seen in Figure 4 was not specific to the engineered Cl13-GFP, we infected WT mice with WT LCMV Cl13 for 24 hours and isolated SMCs. SMCs were then FACS-purified for the different leukocyte populations and qPCR was run on these populations for LCMV *gp* and *np* mRNA transcript levels relative to *gapdh*. pDCs harbored a significantly higher level of viral *gp* and *np* transcript compared to the other populations examined (Figure 6A). Moreover, pDCs had significantly higher IFN-α and IFN-β mRNA transcript levels at 24 hours compared to other leukocyte populations (Figure 6B). These data indicate that Cl13 has increased viral tropism to pDCs compared to other leukocyte populations and that pDCs are a major IFN-I-producing subset early in infection.

pDC IFN-I Production is Dissociated from Intrinsic Viral Replication

Our previous data suggests that pDCs are not only a preferred target of Cl13, but also that they are a major IFN-I producing population early during infection. To determine whether pDC IFN-I production is dependent on intrinsic viral replication, we utilized IFNβ$^{mob/mob}$ mice which are IFNβ reporter mice that have the yellow fluorescent protein (YFP) gene inserted after the IFNβ promoter, allowing for IFNβ-producing cells to be detected by the expression of YFP. IFNβ$^{mob/mob}$ mice were infected with Cl13-GFP for 24 hrs, and infected (GFP+) and uninfected (GFP-) pDCs and cDCs were analyzed by flow cytometry for IFNβ production (YFP+). Although in cDCs, only infected (GFP+) cells seemed to

A

B
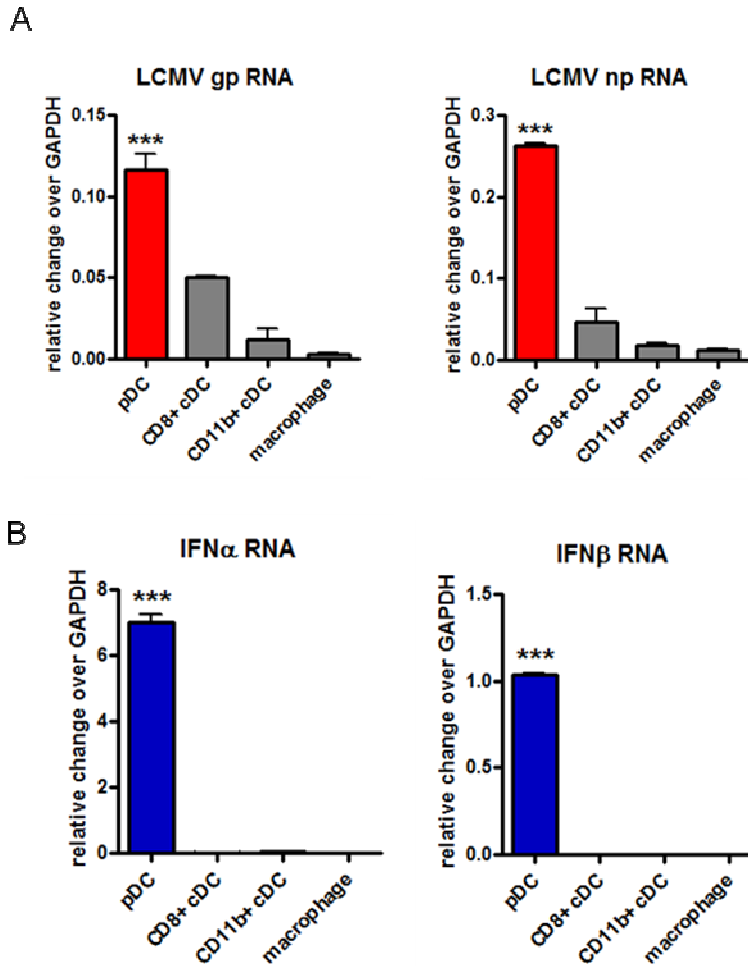
Figure 6. Wild Type Cl13 Preferentially Infected pDCs and pDCs are a Major IFN-I Producing Population Early After Infection

WT mice were infected with $2x10^6$ pfu WT LCMV Cl13 through tail intravenous injection. 24 hrs post-infection, spleens were pooled from 3-5 mice per group for FACS purification. SMCs were FACS-purified for macrophages (CD11c⁻CD11b⁺), pDCs (Thy1.2⁻CD19⁻NK1.1⁻CD11c⁺CD11b⁻B220⁺PDCA⁺), and cDCs (CD11c⁺B220⁻ CD11b⁺ and CD11c⁺B220⁻ CD8⁺). (A) Viral load was determined by qPCR for *LCMV glycoprotein* (GP) and *nucleoprotein* (NP) transcript relative to *gapdh.* (B) *IFN-α and IFN-β* mRNA transcript levels were determined by qPCR relative to *gapdh.* Data represents 3 independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.
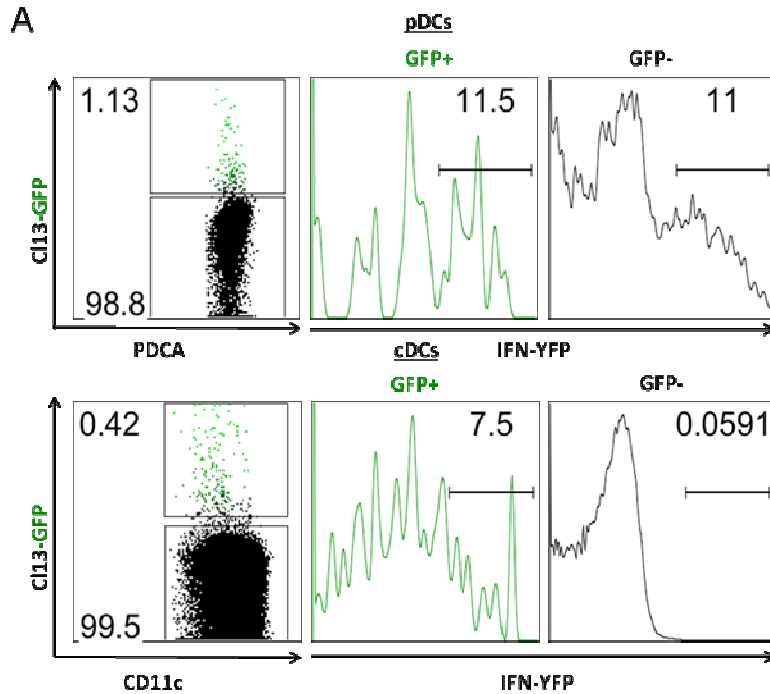
Figure 7. pDC IFN-I Production is Dissociated from Intrinsic Viral Replication

IFNβ$^{mob/mob}$ mice were infected with 4.5x10^5 Cl13-GFP through tail intravenous injection. (A) Mice were sacrificed at 24 hours post-infection and splenic mononuclear cells (SMCs) were isolated from spleens and YFP signal was analyzed by flow cytometry in infected (GFP+) and uninfected (GFP-) PDCA-enriched pDCs and cDCs. Representative dot plots are shown. Results are representative of 3 independent experiments with 3-5 mice per group.

make IFNβ (YFP+), in pDCs, a similar proportion of infected and uninfected cells were positive for IFNβ production (Figure 7A). This suggests that pDC early IFN-I production is dissociated from intrinsic viral replication. Also, because infected pDCs are capable of producing IFN-I, the data suggests that although Cl13 may inhibit pDC IFN-I production, it does not completely ablate it.

Bone Marrow-Derived pDCs are more Readily Infected than Bone Marrow-Derived cDCs

To confirm Cl13 tropism in pDCs and cDCs in bone marrow-dervied (BM) DCs, we extracted bone marrow from WT mice and cultured the bone marrow for 7 days in the presence of Fms-related tyrosine kinase 3 ligand (Flt3L) for 7-8 days to generate a mixed population of cDCs and pDCs [87]. We infected the BM-DCs *in-vitro* with 3 MOI Cl13 for 24 or 48 hrs, or left uninfected as a control, and stained them with an antibody against LCMV NP. The cells were then analyzed by flow cytometry for LCMV NP+ pDCs and cDCs. pDCs harbored significantly more LCMV-NP than cDCs (Figure 8A), confirming the Cl13 pDC tropism seen in our previous *in-vivo* studies.
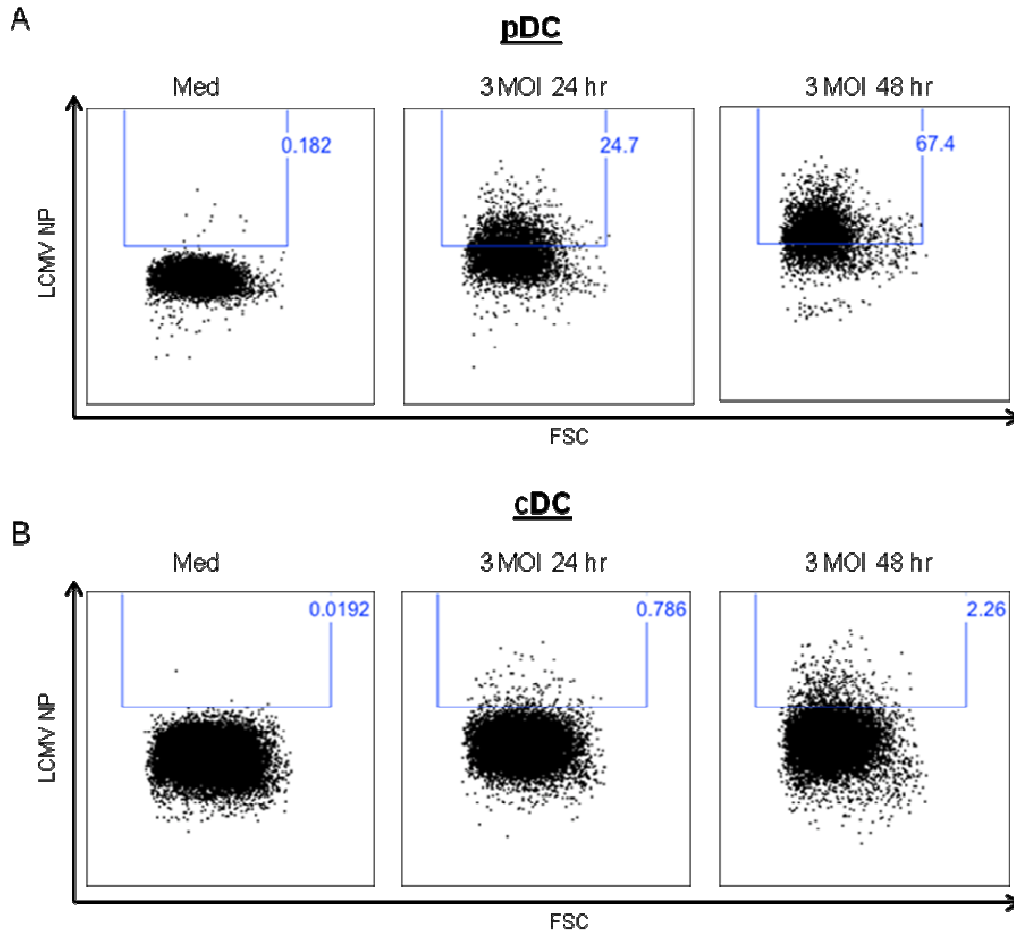
Figure 8. BM-derived pDCs are more Readily Infected than BM-dervied cDCs.

BM-derived DCs (~60% cDC ~25% pDC) were generated by extracting bone marrow and culturing bone marrow cells with Flt3L for 8 days. DCs were infected *in-vitro* at 3 MOI with Cl13 for 24 hrs or 48 hrs and stained intracellularly with cDC/pDC markers along with an antibody against LCMV NP. Percentage LCMV NP+ pDCs (A) and cDCs (B) are shown. Data represents 3 independent experiments.

<u>Discussion</u>

The initial interactions between a pathogen and its host can dramatically influence the outcome of an infection. In the first part of this study, we wished to determine the early events of LCMV Cl13 infection and IFN-I production. Our data demonstrates that several members of the IFN-I pathway, including TLR7, MyD88, and to a greater extent IRF7, play important roles in early systemic IFN-I response to LCMV Cl13, as well as a potential and previously undescribed role for IRF7 in LCMV viral clearance. Furthermore, this study demonstrates that pDCs, which rely exclusively on the TLR7/IRF7-mediated signaling pathway for IFN-I production, are preferential early targets of LCMV Cl13, but that this intrinsic viral infection is dissociated from pDC IFN-I production.

Several studies have shown that IRF7 is crucial for a systemic IFN-I response *in*-vivo [44, 69]. However, it has not yet been shown to be important for viral clearance. Studies in Arm infections suggest that IRF7 controls early viral replication but is not crucial for clearance of an acute Arm infection [69]. However, our data suggests that IRF7 is important for the eventual clearance of persistent Cl13. Whether this is due to the importance of IRF7 and IRF7-mediated IFN-I early in the course of infection to prime different immune responses or due to a later response during chronic infection is unclear. Interestingly, our data also demonstrates that MyD88 deficiency leads to decreased systemic IFN-I when compared with the decrease IFN-I observed with TLR7 deficiency. This suggests a previously uncharacterized TLR7 independent, MyD88 and IRF7 dependent pathway that contributes significantly to systemic

IFN-I upon LCMV infection. Future studies determining what this pathway is, whether it is involved in pDC IFN-I signaling, and whether this pathway plays a role in other viral settings will provide valuable insight into a potentially novel and exciting new mechanism of IFN-I production.

We also observed that pDCs were a readily infected leukocyte population. Because Cl13 uses α-DG for viral entry and splenic DCs express higher levels of α-DG compared to other leukocytes [2], and pDCs have higher binding affinity to LCMV-GP [71], it is reasonable to hypothesize that pDCs would be more readily infected. From an evolutionary perspective, targeting pDCs to disrupt their cytokine production may give the virus an advantage which allows it to persist. It is also hypothesized that because pDCs are in circulation, infection of pDCs allows the virus to travel throughout the host and provide access to other cell types including non-leukocyte targets which do not migrate. In this regard, pDCs can be thought of as an initial vehicle for the virus. It has been shown that strains of LCMV which bind strongly to α-DG are associated with increased persistence [2]. Moreover, Cl13, but not Arm, infects the majority of splenic DCs by day 15-20 and that Cl13 but not arm infection impairs DC function *in vivo* [57]. Perhaps Cl13's enhanced ability to target DCs, especially pDCs, gives the virus an early replication advantage which allows it to establish persistence. Furthermore, despite several studies describing direct inhibition of IFN-I by LCMV viral proteins, our observations suggest that direct infection does not fully inhibit pDC IFN-I production early in infection. Interestingly, cDCs required viral infection to produce IFN-I, although the majority of cDCs were unable to produce IFN-I early

after infection. In contrast, both infected and uninfected pDCs produced IFN-I

early after infection, suggesting that pDCs can respond to LCMV without being

directly infected and that infection does not completely, if at all, silence pDC IFN-I

within 24 hrs p.i. Nonetheless, previous studies described pDC IFN-I exhaustion

at later stages of LCMV Cl13 infection [58]. This later silencing of pDCs may be a

result of timing and direct infection may contribute to pDC IFN-I exhaustion at

later time points. Thus more specific analysis on potential mechanisms

contributing to the eventual DC functional defects must be examined.

Our observations increase our understanding of the early host-virus

interactions which may lead to viral persistence. However, our observations also

lead to further questions. Future experiments addressing the mechanisms of a

TLR7 independent, MyD88 and IRF7 dependent IFN-I signaling pathway in viral

infection may shed light onto the contribution of this pathway during pathogen

challenge as well as potential cell types utilizing this pathway for IFN-I

production. Furthermore, it remains unclear why pDCs do not require intrinsic

viral replication to produce IFN-I, especially when considering TLR7 is localized

in endosomal compartments within the cell. Perhaps during Cl13 infection *in-vivo*

pDCs may use IFNAR signaling to produce IFN-I or other less well-characterized

mechanisms such as exosome transfer of viral RNA [88]. Exactly how this is

achieved is currently unknown and requires further study. In addition to

enhancing our knowledge of early events of a chronic viral infection, our

observations, as well as the future studies suggested by our findings, may also

provide key insight into the mechanisms of eventual IFN-I silencing during

chronic phases of viral infection.

Chapter 2: Mechanisms of Chronic DC Functional Defects

Introduction

Innate immune dysfunction has been observed in various chronic viral diseases and may contribute to viral persistence and poor immune responses to secondary pathogens. Studies have shown that chronic viral infections lead to various innate dysfunctions including pDC defects [58, 83]. pDCs from chronic hepatitis B patients have drastically impaired IFN-α production compared to pDCs from healthy donors upon TLR stimulation [83]. Similarly, LCMV-infected mice also have impaired pDC IFN-I response to secondary challenge and have compromised control of secondary infections [58]. pDCs isolated from a LCMV chronically-infected mouse are functionally exhausted in the production of IFN-I but interestingly maintain the ability to produce pro-inflammatory cytokines after stimulation [58], suggesting a selective IFN-I inhibition in pDCs that has also been observed during HIV infection [59]. As studies have suggested the important role IFN-I can play in controlling chronic viral infection [89], elucidating the mechanisms which lead to pDC IFN-I functional exhaustion may provide novel therapeutic strategies for the treatment of chronic viral infections.

Continuous antigenic stimulation is an important mechanism which leads to immune defects during many chronic viral infections. For example, persistent HIV, HCV, and LCMV infections all lead to the upregulation of the co-inhibitory molecule PD-I on virus-specific CD8 T cells, leading to functional anergy [9]. In fact, blockade of PD-I enhances the anti-viral response and lowers viral load in

LCMV and simian immunodeficiency virus (SIV) [90, 91]. However, whether chronic stimulation leads to functional defects of innate immune cells like pDCs remains to be determined. Furthermore, mechanisms contributing to pDC exhaustion as a result of continuous antigenic stimulation, such as whether continuous signaling affects only mature pDCs or whether the defect is at the level of pDC development remains unclear.

In this part of the study, we examine continuous antigenic stimulation on mature pDCs *in-vitro* as a mechanism of the IFN-I-specific functional defect seen *in-vivo.* Moreover, we explore TLR stimulation on pDC progenitors *in-vitro* as an alternative means of inducing pDC developmental and functional defects during chronic viral infections. We hypothesize that pDCs stimulated *in-vitro* will mimic *ex-vivo* pDCs from Cl13-infected mice in the ablation of IFN-I but maintenance of pro-inflammatory cytokine response to secondary stimulation. We also hypothesize that this continuous stimulation affects pDC function at the level of development. We observe that repeated stimulation alone is capable of exhausting pDCs for IFN-I production and that stimulation during development leads to not only reduced pDC differentiation, but also exhaustion of pDCs for IFN-I and TNFα.

Results

Repeated TLR Stimulation in-vitro Leads to Exhaustion of BM-pDC IFN-I

During chronic viral infections, pDCs are exposed to constant viral presence, and because they sense many of these viruses through TLR7, one hypothesis is that continuous TLR signaling causes functional exhaustion in mature pDCs. To better understand the mechanism of DC exhaustion, we attempted to create an *in-vitro* system using repeated TLR stimulation to mimic an *in-vivo* infection. A mixed population of cDCs and pDCs was generated by extracting and culturing bone marrow (BM) with Fms-related tyrosine kinase 3 ligand (Flt3L) for 7-8 days [71] (Figure 10A) and pDCs were FACS-purified. To simulate Cl13 stimulation, pDCs were then stimulated with loxoribine (a TLR7 agonist) for 15 hours (hrs), (primary stimulation) then washed and stimulated again with loxoribine, CpG (a TLR9 agonist), or media alone for another 15 hrs (secondary simulation). CpG or media alone primary stimulation were also included as controls. pDCs produced copious amounts of IFN-I after primary stimulation with either loxoribine or CpG. However, they failed to produce significant IFN-I or TNF-α after a secondary stimulation with either agonist. Although this data suggests our *in*-vitro exhaustion system does not match the phenotype seen in *in*-vivo data, the results support the hypothesis that antigenic stimulation causes functional exhaustion in DCs.
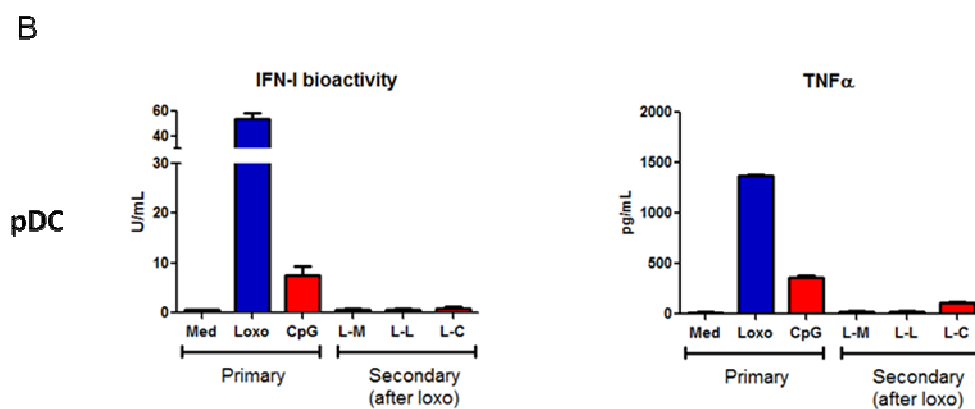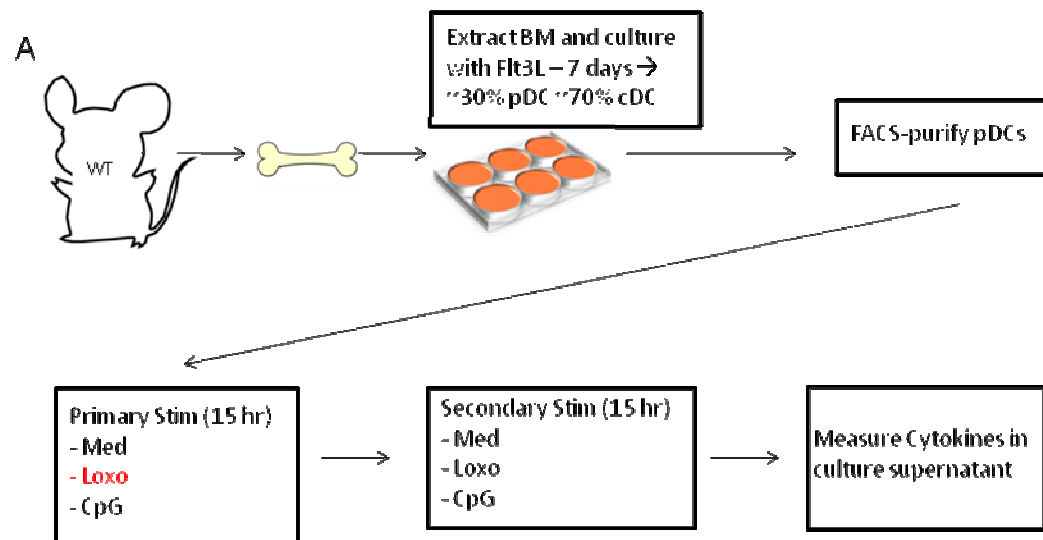
Figure 9. Repeated TLR Stimulation *in-vitro* Leads to Exhaustion of BM-pDC IFN-I and TNF-α

BM-derived DCs were FACS purified to obtain over 97% pure BM-pDCs. (A) pDCs were stimulated for 15 hours with media alone (med), 100 µM Loxoribine (loxo), or 0.1 µM CpG 1668. Loxoribine-stimulated pDCs were washed and then stimulated again (secondary) with media alone, loxoribine, or CpG. (B) Supernatant IFN-I activity was measured by luciferase bioassay and TNF-α levels were measured by ELISA. Data represents 2 independent experiments.

In-vitro TLR7 and TLR9 Stimulation of BM-pDC Down-Regulates E2-2 Expression

E2-2 is a transcription factor specifically expressed in pDCs and not in most other leukocytes, and has been demonstrated to be crucial for pDC development and pDC-mediated IFN-I production [92]. Previous data from the lab demonstrates that pDC E2-2 is down-regulated during the persistent phase of infection, suggesting this as a mechanism of pDC IFN-I inhibition, although how viral persistence down-regulates pDC E2-2 is unclear. To study the effect of TLR signaling on the pDC transcriptional profile, FACS-purified BM-pDCs were stimulated *in-vitro* with loxoribine and CpG for 6 hrs and E2-2 expression was evaluated by qPCR. E2-2 was down-regulated compared to pDCs before stimulation, demonstrating that TLR7 and TLR9 signaling alone can down-regulate E2-2 expression (Figure 12). As expected no E2-2 was observed in cDCs in any stimulation condition.
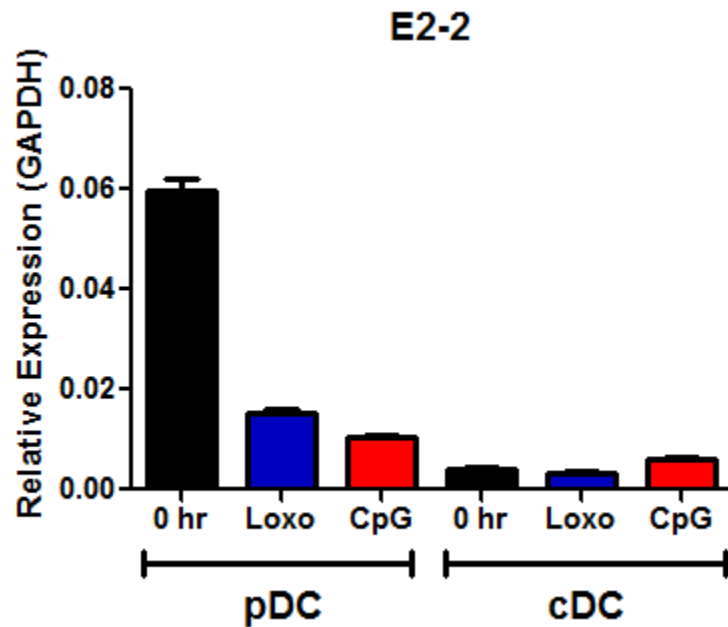
Figure 10. *In-vitro* **TLR7 and TLR9 Stimulation of BM-pDC Down-Regulates E2-2 Expression**

BM-DCs were FACS-purified for pDCs and cDCs and stimulated for 6 hrs with 100 µM loxoribine (loxo) or 1 µM CpG. E2-2 transcript expression was measured by qPCR relative to GAPDH. Data represents 2 independent experiments.

Stimulation of pDC Progenitors Leads to Cellular and Functional Defects

Studies have suggested that LCMV Cl13 infection promotes pDC developmental defects, reducing the number of DC progenitor cells in BM as well as inducing a more cDC-like phenotype [87]. As pDCs have a relatively short life span of ~3.5 days [93], new pDCs develop and emerge regularly in the presence of antigen during chronic viral infection, yet they seemingly emerge functionally and selectively silenced for IFN-I production. In light of these findings, another hypothesis to explain the functional defect of pDCs during chronic Cl13 is that stimulation of pDC progenitors leads to developmental and functional defects. To test this, we isolated and cultured BM with Flt3L for 8 days and treated the culture with 100 uM of TLR7 agonist loxoribine starting at day 0 and then every other day for 2 or 5 days, when the loxoribine was washed out, and cells were cultured till day 8 in Flt3L only. With loxoribine treatment for 5 days, we observed a reduction in total numbers of cells post-culture (Figure 13A) whereas we do not observe this reduction with only 2 days of treatment. Moreover, there were reduced proportions of CD11b+ B220- cDCs and B220+CD11b+PDCA1+ pDCs and development of a CD11b+B220+ intermediate population (Figure 13B). Interestingly, 5 days of loxoribine treatment led to a significant reduction in cell viability as measured by propidium iodide (PI) staining (Figure 13B). To test the function of treated DCs, cells were stimulated with loxoribine or CpG for 6 hrs, in the presence of brefeldin-A for the last 4 hrs, and analyzed by flow cytometry Although pDCs from untreated cultures exhibited strong pro-inflammatory TNF-α and IL-12 cytokine production, pDCs from 2 day treatment were partially
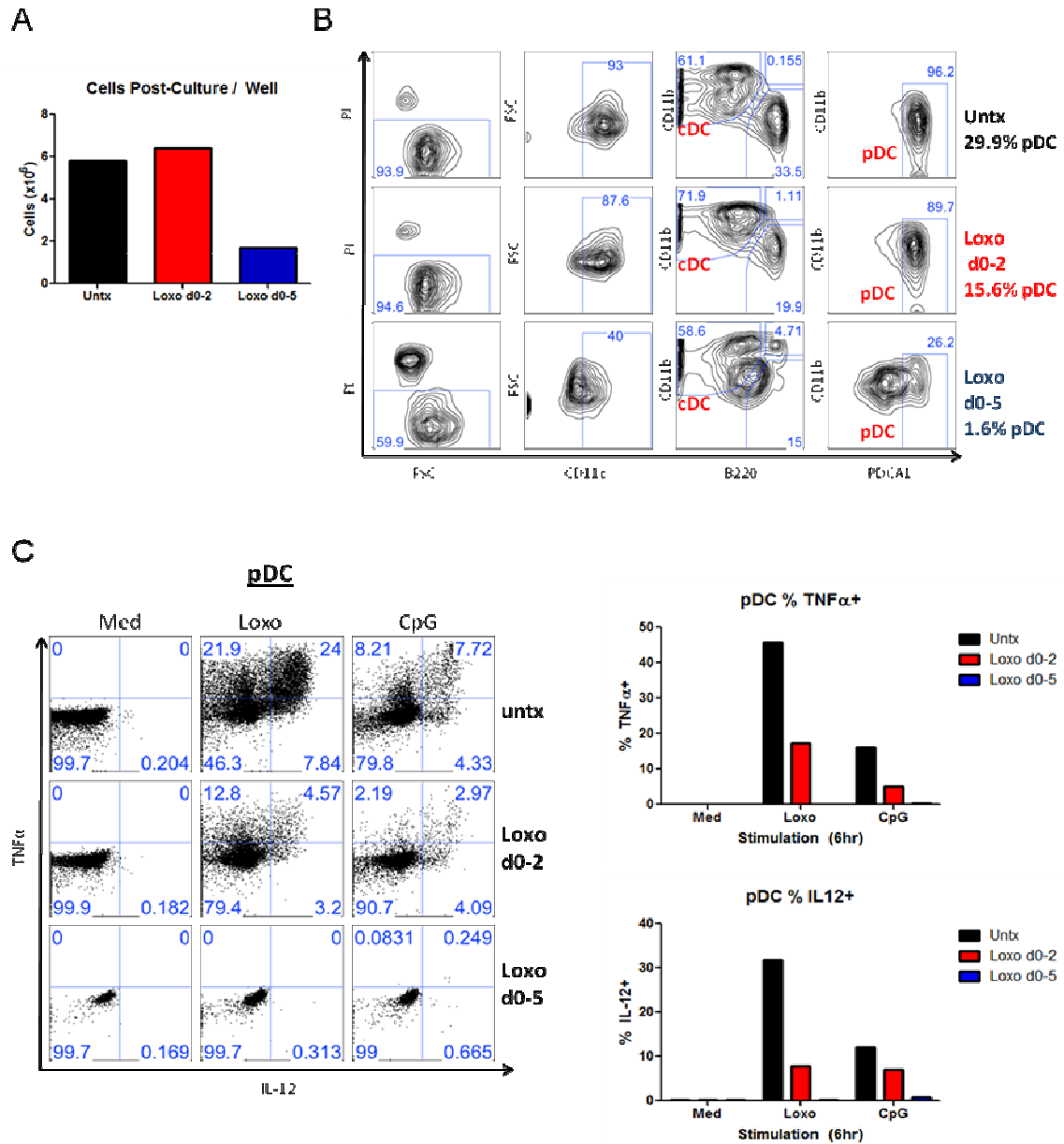
Figure 11. *In-vitro* **Stimulation of BM-pDC Progenitors Leads to Developmental and Functional Defects**

BM was cultured for 8 days as previously described either untreated (untx), treated every 2 days with loxoribine until day 2 (loxo d0-2) or day 5 when cells were washed and media replaced (loxo d0-5). (A) Cell number per culture well is shown. (B) Viability (PI-) and differentiation of BM-DCs Cells and (C) pDC cytokine secretion after loxoribine or CpG stimulation at day 8 for 6 hrs with BFA added after 2 hours were analyzed by flow cytometry. (D) pDCs were FACS-purified and stimulated for 15 hrs with med, loxoribine, or CpG. Culture supernatant IFN-I bioactivity was measured.

Figure 11 Continued

D

pDC IFN-I Bioactivity
(14,000 cells / well)

pDC IFN-I Bioactivity
(100,000 cells/well)
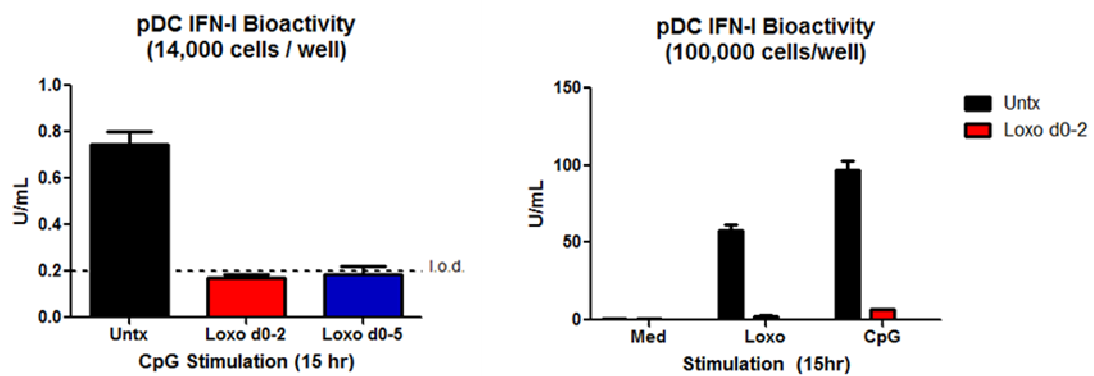
exhausted, and pDCs from 5 day treatment were completely exhausted for TNF-α and IL-12 (Figure 13C). Moreover, pDCs from each group were FACS-purified and despite a strong IFN-I response in supernatant of untreated pDCs after 15 hrs of additional stimulation, there was no IFN-I detected in the 2 or 5 day treated cells (Figure 13D), demonstrating that pDC development in an immuno-stimulatory environment leads to exhaustion of IFN-I.

Discussion

Chronic viral infections cause innate immune exhaustion which facilitates viral persistence and can compromise the host's immune system. In the second part of the study, we wished to determine what mechanisms contribute to DC functional exhaustion during the persistent phase of infection. Our data demonstrates that continuous TLR7 stimulation can lead to functional exhaustion in mature pDCs, possibly by down-regulation of E2-2 thus altering the pDC-specific transcriptional profile. Furthermore, this study demonstrates that persistent stimulation early during DC development leads to developmental defects and dysfunction, suggesting that pDC dysfunction in chronic viral infection may be due to viral effects at both the level of pDC development and on mature pDCs.

Although *ex-vivo* splenic pDCs from persistent Cl13-infected mice exhibit selective exhaustion of IFN-I but maintained pro-inflammatory cytokines [58], mature bone-marrow derived pDCs stimulated repeatedly *in-vitro* were exhausted for all cytokines. Although our goal was to create an *in-vitro* BM-pDC

exhaustion system, selective IFN-I exhaustion was not achieved. Perhaps this is due to dosing or timing that can be altered to achieve the desired phenotype. Nonetheless, although the phenotype seen *in-vitro* does not perfectly match the *in-vivo* data, our system may still provide a useful tool to study general exhaustion mechanisms.

Moreover, TLR7 and TLR9 stimulation led to down-regulation of E2-2. Previous studies have demonstrated that E2-2 controls the pDC gene expression program including TLR7, TLR9, and IRF7 [92]. Thus, our data implies stimulation-mediated down-regulation of E2-2 could lead to decreased capability to produce IFN-I. Recent studies have also reported that IFN-α treatment during pDC development down-regulates E2-2 expression and represses them for further IFN-I production after TLR stimulation [94]. Taken together with our data, the relative contributions of TLR7 signaling alone or IFNAR signaling to E2-2 down-regulation is unknown. This question can be addressed by loxoribine stimulation of IFNAR-/- pDCs because they would only respond to the loxoribine and not to the IFN-I produced in the culture. Such studies can elucidate the roles of IFNAR and TLR7 signaling in E2-2 down-regulation during infection.

We also determined that stimulation of pDC progenitors can lead to developmental and functional defects. Not only did early culture stimulation lead to reduced pDC numbers and proportions, there was an augmentation of a CD11b+B220+ intermediate population, expressing markers for both cDCs and pDCs. The functional characteristics of this intermediate population have yet to be fully determined. Moreover, we observed reduced ability for loxoribine-treated

culture pDCs to respond to further stimulation by IFN-I but maintain some ability to produce pro-inflammatory cytokines. Previous studies have shown that bone marrow pDCs have plasticity and can differentiate into cells with cDC-like phenotype and function upon not only Cl13 infection but also dsRNA (Poly(I:C)) injection [87]. This suggests that the pDC to cDC differentiation is mediated by IFN-I. Although we observe a differentiation of DC progenitors into a pDC/cDC intermediate population, our data supports previous findings that stimulation of developing DCs leads to altered differentiation [87]. Future studies will address the mechanisms contributing to the altered DC differentiation and the functional consequences of these developmental defects.

Our observations increase our understanding of the mechanisms which lead to innate immune exhaustion in DCs during chronic stage of viral persistence. We demonstrate that repeated TLR stimulation alone can cause silencing of cytokine production in mature pDCs and that development in an immuno-stimulatory environment leads to DC developmental and cytokine functional defects. Moreover, our data suggests that pDC IFN-I exhaustion may also be due to down-regulation of E2-2 after TLR7 stimulation. However, the mechanisms contributing to this exhaustion remain to be fully elucidated. Lastly, although we observed developmental defects in BM-pDCs when cultured with loxoribine, it is unknown how the CD11b+B220+ intermediate population functions. Perhaps this intermediate population has different antigen presentation capability compared to untreated cDCs or pDCs, which may be addressed with future experiments examining levels of MHCII and CD86 as well as *in-vitro*

antigen presentation experiments. Further studies addressing the specific

kinetics of pDC developmental impairment as well as transcriptional profiles of

these altered pDCs will provide exciting new insight into mechanisms of TLR7

signaling during pDC development and function. In summary, it is likely that both

continuous antigenic stimulation and developmental defects caused by pDC

progenitor stimulation contribute to pDC ablation and functional defects.

CONCLUSION

Numerous persistent viruses including HIV, HCV, and LCMV ablate DC

numbers and modulate their function to produce cytokines and present and

stimulate T cells [47]. However, the responsible molecular and viral factors

involved in these immune evasion strategies remain poorly defined. Although

these viruses are highly distinct, clearly they have all evolved mechanisms to

subvert DCs, suggesting that DCs play an important role in orchestrating a strong

immune reaction and that they are an attractive target for viruses to manipulate.

Unfortunately, it is extremely difficult to test if DC dysfunction leads to

persistence because reversal of virus-induced modulation of DCs can only be

achieved through therapy. One overarching question in the field is whether direct

infection or indirect viral presence promotes or inhibits DC function—specifically

for IFN-I production. Mechanisms for both direct functional inhibition and general

immune-stimulation-mediated exhaustion have been implicated in HIV, HCV, and

LCMV [48, 53, 59, 72].

Our data suggests that early during infection, viral presence promotes

IFN-I production as seen by the strong peak of IFN-I at 24 hrs post-infection.

cDCs, but not pDCs, seemed to require direct infection for IFN-I production. This

is interesting because pDCs primarily recognizes LCMV through endosomal

TLR7, which would requires intrinsic viral entry. One question that arises is how

uninfected pDCs mediate IFN-I production. Uninfected pDCs may be using

IFNAR signaling in response to systemic IFN-I produced by initially infected cells

or they may be producing IFN-I mediated by exosome transfer of viral RNA by

infected cells as seen in HCV infection [88]. Nonetheless, our studies indicate that the majority of infected pDCs are not producing IFN-I, suggesting direct viral inhibition of the IFN-I pathway may contribute to DC dysfunction early in infection. As LCMV Cl13 viral proteins have been shown to directly target members of the RIG-I and TLR3 pathways [72, 95], the interaction of LCMV Cl13 proteins with members of the TLR7-mediated IFN-I signaling pathway in pDCs remains to be described. Taken together these data suggests that early in infection, viral presence promotes IFN-I production by DCs and that at early time points, direct viral inhibition does not lead to complete IFN-I inhibition.

On the other hand, our data suggests that during the chronic phase of infection, viral persistence inhibits and exhausts DC IFN-I production. Although direct infection may contribute to the IFN-I exhaustion, our findings imply that the chronic antigenic environment of viral persistence is a significant contributor for DC exhaustion. We determined that repeated TLR stimulation alone is enough to inhibit further mature pDC IFN-I production. HIV and Cl13 infection also lead to decreased pDC numbers and pDC IFN-I dysfunction [47, 58]. Furthermore, pDCs have a half-life of approximately 3 days and thus pDCs isolated from persistently infected mice developed in the context of antigenic stimulation [93]. It is then reasonable to hypothesize that viral persistence leads to pDC developmental defects. We demonstrate that *in-vitro* treatment with a TLR7 agonist during pDC development causes decreased pDC numbers and inability to produce cytokine upon further stimulation. It should be noted that *in-vivo*, there is no detectable systemic IFN-I during viral persistence, but in our *in-vitro* culture, TLR7

stimulation may have promoted the production of IFN-I. Studies have shown that IFN-I negatively regulates pDC numbers *in-vivo* [96]. Therefore, we cannot rule out continuous IFN-I signaling in the cultures as a major mechanism for decreased pDC numbers and dysfunction. Thus, it is likely that the pDC defect we observed is caused by either continuous TLR7 or autocrine and paracrine signaling through IFNAR and future experiments to delineate the contribution of IFN-I signaling through IFNAR or other TLR7 signaling mechanisms are warranted. Taken together, viral persistence causes continuous TLR stimulation and developmental defects, which lead to decreased pDC numbers and exhaustion of IFN-I production *in-vitro*.

In summary, our data suggests that early during infection, viral presence promotes innate activation and robust IFN-I production. pDCs are early targets of Cl13 but are also early responders which do not require intrinsic viral replication for IFN-I production and control intrinsic infection using TLR7 signaling. During the chronic stage of infection however, the relationship between virus and host immunity shifts and viral persistence causes chronic innate antigenic stimulation. This chronic stimulation not only exhausts DCs for IFN-I production but also causes developmental and functional defects in developing pDCs.

Future Directions

Although this study aids our understanding of DC-LCMV interactions, it also opens questions which may be addressed with future experiments. One question is to elucidate the unknown TLR7- independent MyD88 and IRF7-

dependent pathway of IFN-I. It is also known that Cl13 directly inhibits IFN-I in DCs, but the factors involved are less clear. We could use techniques such as generating tagged recombinant viruses which we can use to infect DCs and isolate specific virus-DC interacting proteins through proteomic methods.

Moreover, previous *in-vivo* data shows that pDCs from Cl13-infected mice are exhausted for IFN-I production but still maintain ability to produce pro-inflammatory cytokines. This work establishes a foundation for the development of an *in-vitro* pDC exhaustion system. Although repeat stimulation of BM-pDCs exhausted them for IFN-I production, they were also exhausted for TNFα. If an *in-vitro* exhaustion system can be generated to produce a phenotype that matches the *in-vivo* data, the *in-vitro* system can be used for mechanistic studies. For example, TLR7 antagonists can be tested *in-vitro* to see if blockade of TLR7 allows for maintenance of function during repeat stimulation. In addition, global gene expression analysis by microarray on the *in-vitro* exhausted pDCs can be done to validate exhausted pDC-specific expression profiles. Candidate genes which are up-regulated or down-regulated in exhausted pDCs can be knocked-down or overexpressed, respectively, to determine if gene silencing or rescue can restore normal pDC function. Therapeutically, blockade of TLR signaling during pDC development during infection may lead to increased pDC anti-viral activity which may improve disease outcome.

To conclude, our data adds new insight on the mechanisms which cause DC functional exhaustion. Altogether, it seems that direct infection and chronic stimulation both contribute to DC functional modulation during persistent viral

infections. Perhaps by therapeutically blocking binding receptors early during Cl13 infection, we can prevent direct infection-mediated pDC functional inhibition. Moreover, perhaps by blocking TLR stimulation during the chronic phase of infection, we can allow pDCs to recover or develop normally and perhaps orchestrate a secondary robust anti-viral response. More studies are required to elucidate what factors mediate pDC functional exhaustion, which may shed light on reversing pDC dysfunction and allow us to better understand and treat chronic viral infections.

MATERIALS AND METHODS

Cells

BHK-21 cells were obtained from the ATCC and were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 5% tryptose phosphate broth. L-929 cells used for IFN-I bioassay were stably transfected with an interferon-sensitive luciferase construct [81] and maintined in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin.

Mice

WT C57Bl/6, WT CD45.1, IRF7$^{-/-}$, IRF3$^{-/-}$, MyD88$^{-/-}$, IFNβ$^{mob/mob}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *TLR7*$^{-/-}$ mice were described previously [40]. Mice were bred and maintained in a closed breeding facility, and mouse handling complied with the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of the University of California San Diego. Six- to ten-week-old mice were infected intravenously with 2×10^6 pfu WT LCMV Cl13 or either 8×10^4 or 4.5×10^5 pfu Cl13-GFP, as indicated.

Virus Generation

WT Cl13 LCMV was propagated by inoculating BHK cells with 0.1 MOI WT Cl13 LCMV for 1 hour, followed by incubation for 48 hours at 37°C with virus-free

media. The supernatant was collected and viral titer determined by plaque assay.

Recombinant GFP-Cl13 LCMV was generously provided by Dr. Juan Carlos de

la Torre at The Scripps Research Institute and generated as previously described

[60]. The virus was then propagated by inoculating BHK cells with 0.01 MOI for 1

hour, followed by incubation for 72 hours at 37°C with media. Viral titers were

determined by plaque assay on BHK cells after 1 hr absorption at 37°C and 6

days of agarose overlay. Viral purification was performed by overnight precipitate

with polyethylene glycol in 4°C. The precipitate w as ultracentrifuged through a

renegraphin-60 gradient and the purified virus was collected and tittered as

described previously.

Cell Purification

Bone marrow-derived DCs were FACS purified using a BD ARIA (BD

Biosciences, San Jose, CA) for pDCs (CD11c$^+$CD11b$^-$B220$^+$PDCA$^+$) and

CD11b$^+$ cDCs (CD11c$^+$B220$^-$CD11b$^+$). Purity for all cell types was >94%.

Flow Cytometry

Antibodies used were either purchased from Biolegend, BD Pharmingen

or eBioscience (San Diego, CA) to stain cells CD11b-PE-CY7, anti-CD11c-APC,

anti-CD19-PB, anti-NK1.1-PB, anti-Thy1.1- PB, anti-B220-APC-CY7, anti-

PDCA1-FITC, anti-PDCA1-PE, anti-CD8-PercpCy5.5. Anti-LCMV-NP (clone 113)

was a gift from M. Oldstone (TSRI). Surface stains were done on ice for 20 min.

Intraceullar stains were done by fixing the cells with 1% formaldehyde for at least

10 minutes and permeabilizing cells with saponin. Cells were acquired using the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed with the FlowJo software (Treestar, Inc., Ashland, OR).

Cytokine Measurements

Type I IFN activity was measured by a bioassay with reference to a recombinant mouse IFN-b standard using an L-929 cell line transfected with an interferon-sensitive luciferase construct [81]. TNF-α and IL-6 levels were determined by ELISA kits (eBioscience,San Diego, CA). To measure intracellular cytokines by flow cytometry, cells were stimulated for 6 hrs in complete media with brefeldin-A added for the last 4 hr at 37℃ pr ior to staining.

DC Cultures and In-Vitro Stimulation

To generate bone marrow-derived DCs, total bone marrow was extracted from the hind leg femurs and tibias of indicated mice. Total bone marrow was then cultured for 7-8 days at 2×10^6 cells/mL in 5 mL/well in 6-well plates. Total bone marrow was cultured in RPMI complete medium (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 15 mM β-mercaptoethanol) supplemented with Fms-related tyrosine kinase 3 ligand (Flt3-L). Half the media was changed at day 5 with fresh addition of Flt3L and β-mercaptoethanol. For pDC stimulation, cells were cultured for 15 hrs in the presence of CpG-1668 (TCCATGACGTTCCGATGCT phosphorothioate-

modified; IDT, Inc.) at 0.1 µM or Loxoribine (Invivogen, San Diego, CA) at 100
µM and supernatants collected.

Quantitative PCR

Total RNA was extracted from cells using RNeasy Mini kit (QIAGEN)
digested with DNase I (RNase-free DNase Set, QIAGEN) and reverse
transcribed into cDNA using the MMLV reverse transcriptase system (Promega,
Madison, WI). Quantification of cDNA was performed using SYBR Green PCR
Kit (Applied Byosistem) and Real-Time PCR Detection System (ABI). The
relative RNA levels of all genes examined were normalized against cellular
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Primer
sequences are as follows: *gapdh* Fwd   5'-TCCCACTCTTCCACCTTCGA-3',
gapdh Rev AGTTGGGAT AGGGCCTCTCTT-3', *lcmv-gp* Fwd 5'-
CATTCACCTGGACTTTGTCAGACTC-3', *lcmv-gp* Rev 5'-
GCAACTGCTGTGTTCCCGAAA-3', *lcmv-np* Fwd 5'-GCATTGTCTGGCTGTA-3'
lcmv-np Rev 5'-CAATGACGTTGTACAA-3', *ifn-α* Fwd 5'-
TATGTCCTCACAGCCAGCAG-3', *ifn*-α Rev 5'- TTCTGCAATGACCTC CATCA-
3', *ifn-β* Fwd 5'-AGATGTCCTCAACTGCTCTC-3', *ifn-β* Rev 5'-AGAT
TCACTACCAGTCCCAG-3', *il-6* For 5'-ACAGAAGGAGTGGCTAAGGA-3', *il-6*
Rev 5'- CGCACTAGGTTTGCCGAGTA-3', *e2-2* For 5'- AGGGGGAAAATGAGG
TGCTCGGT-3', *e2-2* Rev 5'- TGGCCTCCAGTTCCCCAGGAC-3'.

Statistical Analysis

Statistical differences were determined by one-way or two-way analysis of variance (ANOVA) or Student's t test using the InStat 3.0 software (Graphpad, La Jolla, CA).

REFERENCES

1. Mathers, C.D. and D. Loncar, *Projections of global mortality and burden of disease from 2002 to 2030.* PLoS Med, 2006. **3**(11): p. e442.

2. Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K.P. Campbell, J.C. de La Torre, and M.B. Oldstone, *Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells.* J Exp Med, 2000. **192**(9): p. 1249-60.

3. Tan, I.L. and J.C. McArthur, *HIV-associated neurological disorders: a guide to pharmacotherapy.* CNS Drugs, 2012. **26**(2): p. 123-34.

4. Sheth, A.N., K.N. Althoff, and J.T. Brooks, *Influenza susceptibility, severity, and shedding in HIV-infected adults: a review of the literature.* Clin Infect Dis, 2011. **52**(2): p. 219-27.

5. Luetkemeyer, A.F., D.V. Havlir, and J.S. Currier, *Complications of HIV disease and antiretroviral therapy.* Top Antivir Med, 2012. **20**(2): p. 48-60.

6. Aghemo, A. and M. Colombo, *Hepatocellular carcinoma in chronic hepatitis C: from bench to bedside.* Semin Immunopathol, 2012.

7. Dubrow, R., M.J. Silverberg, L.S. Park, K. Crothers, and A.C. Justice, *HIV infection, aging, and immune function: implications for cancer risk and prevention.* Curr Opin Oncol, 2012. **24**(5): p. 506-16.

8. Fevrier, M., K. Dorgham, and A. Rebollo, *CD4+ T cell depletion in human immunodeficiency virus (HIV) infection: role of apoptosis.* Viruses, 2011. **3**(5): p. 586-612.

9. Porichis, F. and D.E. Kaufmann, *Role of PD-1 in HIV pathogenesis and as target for therapy.* Curr HIV/AIDS Rep, 2012. **9**(1): p. 81-90.

10. Hazenberg, M.D., S.A. Otto, B.H. van Benthem, M.T. Roos, R.A. Coutinho, J.M. Lange, D. Hamann, M. Prins, and F. Miedema, *Persistent immune activation in HIV-1 infection is associated with progression to AIDS.* AIDS, 2003. **17**(13): p. 1881-8.

11. Novak, N., S. Koch, J.P. Allam, and T. Bieber, *Dendritic cells: bridging innate and adaptive immunity in atopic dermatitis.* J Allergy Clin Immunol, 2010. **125**(1): p. 50-9.

12. Lopez-Bravo, M. and C. Ardavin, *In vivo induction of immune responses to pathogens by conventional dendritic cells.* Immunity, 2008. **29**(3): p. 343-51.

13. Iwasaki, A., *The importance of CD11b+ dendritic cells in CD4+ T cell activation in vivo: with help from interleukin 1.* J Exp Med, 2003. **198**(2): p. 185-90.

14. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-52.

15. Shortman, K. and W.R. Heath, *The CD8+ dendritic cell subset.* Immunol Rev, 2010. **234**(1): p. 18-31.

16. Tversky, J.R., T.V. Le, A.P. Bieneman, K.L. Chichester, R.G. Hamilton, and J.T. Schroeder, *Human blood dendritic cells from allergic subjects have impaired capacity to produce interferon-alpha via Toll-like receptor 9.* Clin Exp Allergy, 2008. **38**(5): p. 781-8.

17. Asselin-Paturel, C., G. Brizard, J.J. Pin, F. Briere, and G. Trinchieri, *Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody.* J Immunol, 2003. **171**(12): p. 6466-77.

18. Reizis, B., A. Bunin, H.S. Ghosh, K.L. Lewis, and V. Sisirak, *Plasmacytoid dendritic cells: recent progress and open questions.* Annu Rev Immunol, 2011. **29**: p. 163-83.

19. von Glehn, F., L.M. Santos, and K.E. Balashov, *Plasmacytoid dendritic cells and immunotherapy in multiple sclerosis.* Immunotherapy, 2012. **4**(10): p. 1053-61.

20. McKenna, K., A.S. Beignon, and N. Bhardwaj, *Plasmacytoid dendritic cells: linking innate and adaptive immunity.* J Virol, 2005. **79**(1): p. 17-27.

21. Ito, T., Y.H. Wang, and Y.J. Liu, *Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9.* Springer Semin Immunopathol, 2005. **26**(3): p. 221-9.

22. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. **21**: p. 335-76.

23. Gauzzi, M.C., M. Del Corno, and S. Gessani, *Dissecting TLR3 signalling in dendritic cells.* Immunobiology, 2010. **215**(9-10): p. 713-23.

24. Leung, D.W. and G.K. Amarasinghe, *Structural insights into RNA recognition and activation of RIG-I-like receptors.* Curr Opin Struct Biol, 2012. **22**(3): p. 297-303.

25. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.

26. Sadler, A.J. and B.R. Williams, *Interferon-inducible antiviral effectors.* Nat Rev Immunol, 2008. **8**(7): p. 559-68.

27. Antonelli, G., *Biological basis for a proper clinical application of alpha interferons.* New Microbiol, 2008. **31**(3): p. 305-18.

28. Horisberger, M.A., *Interferons, Mx genes, and resistance to influenza virus.* Am J Respir Crit Care Med, 1995. **152**(4 Pt 2): p. S67-71.

29. Mondini, M., S. Costa, S. Sponza, F. Gugliesi, M. Gariglio, and S. Landolfo, *The interferon-inducible HIN-200 gene family in apoptosis and inflammation: implication for autoimmunity.* Autoimmunity, 2010. **43**(3): p. 226-31.

30. Cheng, Y., N.J. King, and A.M. Kesson, *Major histocompatibility complex class I (MHC-I) induction by West Nile virus: involvement of 2 signaling pathways in MHC-I up-regulation.* J Infect Dis, 2004. **189**(4): p. 658-68.

31. Nguyen, K.B., T.P. Salazar-Mather, M.Y. Dalod, J.B. Van Deusen, X.Q. Wei, F.Y. Liew, M.A. Caligiuri, J.E. Durbin, and C.A. Biron, *Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection.* J Immunol, 2002. **169**(8): p. 4279-87.

32. Hervas-Stubbs, S., J.L. Perez-Gracia, A. Rouzaut, M.F. Sanmamed, A. Le Bon, and I. Melero, *Direct effects of type I interferons on cells of the immune system.* Clin Cancer Res, 2011. **17**(9): p. 2619-27.

33. Santodonato, L., G. D'Agostino, R. Nisini, S. Mariotti, D.M. Monque, M. Spada, L. Lattanzi, M.P. Perrone, M. Andreotti, F. Belardelli, and M. Ferrantini, *Monocyte-derived dendritic cells generated after a short-term culture with IFN-alpha and granulocyte-macrophage colony-stimulating factor stimulate a potent Epstein-Barr virus-specific CD8+ T cell response.* J Immunol, 2003. **170**(10): p. 5195-202.

34. Sampson, L.L., J. Heuser, and E.J. Brown, *Cytokine regulation of complement receptor-mediated ingestion by mouse peritoneal macrophages. M-CSF and IL-4 activate phagocytosis by a common*

mechanism requiring autostimulation by IFN-beta. J Immunol, 1991. **146**(3): p. 1005-13.

35. Fink, K., K.S. Lang, N. Manjarrez-Orduno, T. Junt, B.M. Senn, M. Holdener, S. Akira, R.M. Zinkernagel, and H. Hengartner, *Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses.* Eur J Immunol, 2006. **36**(8): p. 2094-105.

36. Brinkmann, V., T. Geiger, S. Alkan, and C.H. Heusser, *Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells.* J Exp Med, 1993. **178**(5): p. 1655-63.

37. Marrack, P., J. Kappler, and T. Mitchell, *Type I interferons keep activated T cells alive.* J Exp Med, 1999. **189**(3): p. 521-30.

38. Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke, and S. Vucikuja, *CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion.* J Immunol, 2006. **176**(8): p. 4525-9.

39. Agarwal, P., A. Raghavan, S.L. Nandiwada, J.M. Curtsinger, P.R. Bohjanen, D.L. Mueller, and M.F. Mescher, *Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory.* J Immunol, 2009. **183**(3): p. 1695-704.

40. Lund, J.M., L. Alexopoulou, A. Sato, M. Karow, N.C. Adams, N.W. Gale, A. Iwasaki, and R.A. Flavell, *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5598-603.

41. Zhou, H., J. Zhao, and S. Perlman, *Autocrine interferon priming in macrophages but not dendritic cells results in enhanced cytokine and chemokine production after coronavirus infection.* MBio, 2010. **1**(4).

42. Gautier, G., M. Humbert, F. Deauvieau, M. Scuiller, J. Hiscott, E.E. Bates, G. Trinchieri, C. Caux, and P. Garrone, *A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells.* J Exp Med, 2005. **201**(9): p. 1435-46.

43. Loo, Y.M. and M. Gale, Jr., *Immune signaling by RIG-I-like receptors.* Immunity, 2011. **34**(5): p. 680-92.

44. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi, *IRF-7 is the master regulator of type-I interferon-dependent immune responses.* Nature, 2005. **434**(7034): p. 772-7.

45. Higgs, R. and C.A. Jefferies, *Targeting IRFs by ubiquitination: regulating antiviral responses.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 453-8.

46. Toubi, E. and Y. Shoenfeld, *Toll-like receptors and their role in the development of autoimmune diseases.* Autoimmunity, 2004. **37**(3): p. 183-8.

47. Liu, B., A.M. Woltman, H.L. Janssen, and A. Boonstra, *Modulation of dendritic cell function by persistent viruses.* J Leukoc Biol, 2009. **85**(2): p. 205-14.

48. Donaghy, H., B. Gazzard, F. Gotch, and S. Patterson, *Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1.* Blood, 2003. **101**(11): p. 4505-11.

49. Feldman, S., D. Stein, S. Amrute, T. Denny, Z. Garcia, P. Kloser, Y. Sun, N. Megjugorac, and P. Fitzgerald-Bocarsly, *Decreased interferon-alpha production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors.* Clin Immunol, 2001. **101**(2): p. 201-10.

50. Soumelis, V., I. Scott, F. Gheyas, D. Bouhour, G. Cozon, L. Cotte, L. Huang, J.A. Levy, and Y.J. Liu, *Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients.* Blood, 2001. **98**(4): p. 906-12.

51. Anthony, D.D., N.L. Yonkers, A.B. Post, R. Asaad, F.P. Heinzel, M.M. Lederman, P.V. Lehmann, and H. Valdez, *Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection.* J Immunol, 2004. **172**(8): p. 4907-16.

52. Kanto, T., M. Inoue, H. Miyatake, A. Sato, M. Sakakibara, T. Yakushijin, C. Oki, I. Itose, N. Hiramatsu, T. Takehara, A. Kasahara, and N. Hayashi, *Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection.* J Infect Dis, 2004. **190**(11): p. 1919-26.

53. Averill, L., W.M. Lee, and N.J. Karandikar, *Differential dysfunction in dendritic cell subsets during chronic HCV infection.* Clin Immunol, 2007. **123**(1): p. 40-9.

54. Kasprowicz, V., J. Schulze Zur Wiesch, T. Kuntzen, B.E. Nolan, S. Longworth, A. Berical, J. Blum, C. McMahon, L.L. Reyor, N. Elias, W.W. Kwok, B.G. McGovern, G. Freeman, R.T. Chung, P. Klenerman, L. Lewis-

Ximenez, B.D. Walker, T.M. Allen, A.Y. Kim, and G.M. Lauer, *High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome.* J Virol, 2008. **82**(6): p. 3154-60.

55.   Tinoco, R., V. Alcalde, Y. Yang, K. Sauer, and E.I. Zuniga, *Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo.* Immunity, 2009. **31**(1): p. 145-57.

56.   Garba, M.L., C.D. Pilcher, A.L. Bingham, J. Eron, and J.A. Frelinger, *HIV antigens can induce TGF-beta(1)-producing immunoregulatory CD8+ T cells.* J Immunol, 2002. **168**(5): p. 2247-54.

57.   Sevilla, N., S. Kunz, D. McGavern, and M.B. Oldstone, *Infection of dendritic cells by lymphocytic choriomeningitis virus.* Curr Top Microbiol Immunol, 2003. **276**: p. 125-44.

58.   Zuniga, E.I., L.-Y. Liou, L. Mack, M. Mendoza, and M.B.A. Oldstone, *Persistent Virus Infection Inhibits Type I Interferon Production by Plasmacytoid Dendritic Cells to Facilitate Opportunistic Infections.* Cell host & microbe, 2008. **4**(4): p. 374-386.

59.   Martinson, J.A., A. Roman-Gonzalez, A.R. Tenorio, C.J. Montoya, C.N. Gichinga, M.T. Rugeles, M. Tomai, A.M. Krieg, S. Ghanekar, L.L. Baum, and A.L. Landay, *Dendritic cells from HIV-1 infected individuals are less responsive to toll-like receptor (TLR) ligands.* Cell Immunol, 2007. **250**(1-2): p. 75-84.

60.   Emonet, S.F., L. Garidou, D.B. McGavern, and J.C. de la Torre, *Generation of recombinant lymphocytic choriomeningitis viruses with trisegmented genomes stably expressing two additional genes of interest.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3473-8.

61.   Welsh, R.M. and M.O. Seedhom, *Lymphocytic choriomeningitis virus (LCMV): propagation, quantitation, and storage.* Curr Protoc Microbiol, 2008. **Chapter 15**: p. Unit 15A 1.

62.   Nunberg, J.H. and J. York, *The curious case of arenavirus entry, and its inhibition.* Viruses, 2012. **4**(1): p. 83-101.

63.   Byrne, J.A. and M.B. Oldstone, *Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus. VI. Migration and activity in vivo in acute and persistent infection.* J Immunol, 1986. **136**(2): p. 698-704.

64.   Borrow, P., C.F. Evans, and M.B. Oldstone, *Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression.* J Virol, 1995. **69**(2): p. 1059-70.

65.   Wherry, E.J., J.N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed, *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol, 2003. **77**(8): p. 4911-27.

66.   Ahmed, R., A. Salmi, L.D. Butler, J.M. Chiller, and M.B. Oldstone, *Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence.* J Exp Med, 1984. **160**(2): p. 521-40.

67.   Bergthaler, A., L. Flatz, A.N. Hegazy, S. Johnson, E. Horvath, M. Lohning, and D.D. Pinschewer, *Viral replicative capacity is the primary determinant of lymphocytic choriomeningitis virus persistence and immunosuppression.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21641-6.

68.   Sullivan, B.M., S.F. Emonet, M.J. Welch, A.M. Lee, K.P. Campbell, J.C. de la Torre, and M.B. Oldstone, *Point mutation in the glycoprotein of lymphocytic choriomeningitis virus is necessary for receptor binding, dendritic cell infection, and long-term persistence.* Proc Natl Acad Sci U S A, 2011. **108**(7): p. 2969-74.

69.   Zhou, S., A.M. Cerny, K.A. Fitzgerald, E.A. Kurt-Jones, and R.W. Finberg, *Role of interferon regulatory factor 7 in T cell responses during acute lymphocytic choriomeningitis virus infection.* J Virol, 2012. **86**(20): p. 11254-65.

70.   Trinchieri, G., *Type I interferon: friend or foe?* J Exp Med, 2010. **207**(10): p. 2053-63.

71.   Macal, M., G.M. Lewis, S. Kunz, R. Flavell, J.A. Harker, and E.I. Zuniga, *Plasmacytoid dendritic cells are productively infected and activated through TLR-7 early after arenavirus infection.* Cell Host Microbe, 2012. **11**(6): p. 617-30.

72.   Borrow, P., L. Martinez-Sobrido, and J.C. de la Torre, *Inhibition of the type I interferon antiviral response during arenavirus infection.* Viruses, 2010. **2**(11): p. 2443-80.

73. Martinez-Sobrido, L., E.I. Zuniga, D. Rosario, A. Garcia-Sastre, and J.C. de la Torre, *Inhibition of the type I interferon response by the nucleoprotein of the prototypic arenavirus lymphocytic choriomeningitis virus.* J Virol, 2006. **80**(18): p. 9192-9.

74. Yuan, W. and R.M. Krug, *Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein.* EMBO J, 2001. **20**(3): p. 362-71.

75. Talon, J., C.M. Horvath, R. Polley, C.F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre, *Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein.* J Virol, 2000. **74**(17): p. 7989-96.

76. Donelan, N.R., C.F. Basler, and A. Garcia-Sastre, *A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice.* J Virol, 2003. **77**(24): p. 13257-66.

77. Kumthip, K., P. Chusri, N. Jilg, L. Zhao, D.N. Fusco, H. Zhao, K. Goto, D. Cheng, E.A. Schaefer, L. Zhang, C. Pantip, S. Thongsawat, A. O'Brien, L.F. Peng, N. Maneekarn, R.T. Chung, and W. Lin, *Hepatitis C virus NS5A disrupts STAT1 phosphorylation and suppresses type I interferon signaling.* J Virol, 2012. **86**(16): p. 8581-91.

78. Zhang, T., R.T. Lin, Y. Li, S.D. Douglas, C. Maxcey, C. Ho, J.P. Lai, Y.J. Wang, Q. Wan, and W.Z. Ho, *Hepatitis C virus inhibits intracellular interferon alpha expression in human hepatic cell lines.* Hepatology, 2005. **42**(4): p. 819-27.

79. Foy, E., K. Li, R. Sumpter, Jr., Y.M. Loo, C.L. Johnson, C. Wang, P.M. Fish, M. Yoneyama, T. Fujita, S.M. Lemon, and M. Gale, Jr., *Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2986-91.

80. Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp, *Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus.* Nature, 2005. **437**(7062): p. 1167-72.

81. Jiang, Z., P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos, M. Freudenberg, and B. Beutler, *CD14 is required for MyD88-independent LPS signaling.* Nat Immunol, 2005. **6**(6): p. 565-70.

82. Chang, J., R.J. Lindsay, S. Kulkarni, J.D. Lifson, M. Carrington, and M. Altfeld, *Polymorphisms in interferon regulatory factor 7 reduce interferon-alpha responses of plasmacytoid dendritic cells to HIV-1.* AIDS, 2011. **25**(5): p. 715-7.

83. Martinet, J., T. Dufeu-Duchesne, J. Bruder Costa, S. Larrat, A. Marlu, V. Leroy, J. Plumas, and C. Aspord, *Altered Functions of Plasmacytoid Dendritic Cells and Reduced Cytolytic Activity of Natural Killer Cells in Patients With Chronic HBV Infection.* Gastroenterology, 2012.

84. Ng, C.T., B.M. Sullivan, and M.B. Oldstone, *The role of dendritic cells in viral persistence.* Curr Opin Virol, 2011. **1**(3): p. 160-6.

85. de la Torre, J.C. and M.B. Oldstone, *Selective disruption of growth hormone transcription machinery by viral infection.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9939-43.

86. Lewis, G.D. and T.G. Metcalf, *Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples.* Appl Environ Microbiol, 1988. **54**(8): p. 1983-8.

87. Zuniga, E.I., D.B. McGavern, J.L. Pruneda-Paz, C. Teng, and M.B. Oldstone, *Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection.* Nat Immunol, 2004. **5**(12): p. 1227-34.

88. Dreux, M., U. Garaigorta, B. Boyd, E. Decembre, J. Chung, C. Whitten-Bauer, S. Wieland, and F.V. Chisari, *Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity.* Cell Host Microbe, 2012. **12**(4): p. 558-70.

89. Wang, Y., M. Swiecki, M. Cella, G. Alber, R.D. Schreiber, S. Gilfillan, and M. Colonna, *Timing and magnitude of type I interferon responses by distinct sensors impact CD8 T cell exhaustion and chronic viral infection.* Cell Host Microbe, 2012. **11**(6): p. 631-42.

90. Wherry, E.J., S.J. Ha, S.M. Kaech, W.N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J.N. Blattman, D.L. Barber, and R. Ahmed, *Molecular signature of CD8+ T cell exhaustion during chronic viral infection.* Immunity, 2007. **27**(4): p. 670-84.

91. Dyavar Shetty, R., V. Velu, K. Titanji, S.E. Bosinger, G.J. Freeman, G. Silvestri, and R.R. Amara, *PD-1 blockade during chronic SIV infection*

reduces hyperimmune activation and microbial translocation in rhesus macaques. J Clin Invest, 2012. **122**(5): p. 1712-6.

92.   Reizis, B., *Regulation of plasmacytoid dendritic cell development.* Curr Opin Immunol, 2010. **22**(2): p. 206-11.

93.   Liu, K., C. Waskow, X. Liu, K. Yao, J. Hoh, and M. Nussenzweig, *Origin of dendritic cells in peripheral lymphoid organs of mice.* Nat Immunol, 2007. **8**(6): p. 578-83.

94.   Li, H.S., A. Gelbard, G.J. Martinez, E. Esashi, H. Zhang, H. Nguyen-Jackson, Y.J. Liu, W.W. Overwijk, and S.S. Watowich, *Cell-intrinsic role for IFN-alpha-STAT1 signals in regulating murine Peyer patch plasmacytoid dendritic cells and conditioning an inflammatory response.* Blood, 2011. **118**(14): p. 3879-89.

95.   Zhou, S., A.M. Cerny, A. Zacharia, K.A. Fitzgerald, E.A. Kurt-Jones, and R.W. Finberg, *Induction and inhibition of type I interferon responses by distinct components of lymphocytic choriomeningitis virus.* J Virol, 2010. **84**(18): p. 9452-62.

96.   Swiecki, M., Y. Wang, W. Vermi, S. Gilfillan, R.D. Schreiber, and M. Colonna, *Type I interferon negatively controls plasmacytoid dendritic cell numbers in vivo.* J Exp Med, 2011. **208**(12): p. 2367-74.