UNIVERSITY OF CALIFORNIA, SAN DIEGO

DYNAMICS OF TYPE I INTERFERON AND INTERLEUKIN-6 PRODUCTION DURING ACUTE AND CHRONIC VIRAL INFECTIONS

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by

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The thesis of Lauren A. Mack is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

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Figure 2, in full, is a reprint of the material as it appears in Cell Host and Microbe 2008. E Zuniga, L Liou, L Mack, M Mendoza, and MBA Oldstone. The thesis author was the third author of this paper.

ABSTRACT OF THE THESIS

DYNAMICS OF TYPE I INTERFERON AND INTERLEUKIN-6 PRODUCTION DURING ACUTE AND CHRONIC VIRAL INFECTIONS

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Lauren A Mack

Master of Science in Biology

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Professor Elina Zuniga, Chair

Viral infections are often associated with a transient or long-lasting generalized suppression of the host immune response. In this study, we demonstrated that *in vivo* lymphocytic choriomeningitis virus (LCMV) infection in its natural rodent host resulted in a profound suppression of the unique capacity of plasmacytoid dendritic cells (pDCs) to produce Type I interferons (IFN-I). While both acute and persistent LCMV infections impaired pDC IFN-I response, only the persistent virus induced their long-lasting diversion. This immune-deficiency related to a decreased ability of the persistently infected host to mount an effective innate response to control a secondary pathogen. Importantly, the ability of pDCs to produce a variety of other cytokines, such as interleukin-6 (IL-6), was unaltered, indicating selective disruption of the IFN-I pathway. Further studies indicated that IL-6 was elevated in the serum at different times after acute

versus chronic LCMV infection and it was essential for the eventual clearance of the persistent virus. Altogether, these findings demonstrated that persistence-prone viruses can suppress pDC-IFN-I production to debilitate the host immune system but IL-6 induction remains unaltered and becomes vital to control the chronic infection.

INTRODUCTION

Virus induced-immunosuppression represents a global health problem

Virus infections are often associated with a transient or long-lasting generalized suppression of the host immune response, which has been associated with increased susceptibility to opportunistic infections. The global impact of virus-induced immunosupression is reflected in the high incidence of deadly opportunistic pathogens during human immunodeficiency virus type-1 (HIV-1) and measles virus (MV) infections in humans. Virus-induced immunosuppression has long been related to deregulation of the adaptive immune system including T cell exhaustion and deletion (1-5). Much less attention has been dedicated to investigate how an *in vivo* acute or chronic viral infection influences innate immune responses. In this regard, increasing amount of evidence associate susceptibility to opportunistic infections with a defective production of type-I interferons (IFN-I), which are important innate mediators (6-8).

pDCs are specialized IFN-I producing cells

IFN-I exerts direct, potent antiviral effects and are an essential link between innate and adaptive immunity (9,10). Although any cell can potentially produce IFN-I upon virus infection, plasmacytoid DCs (pDCs), also known as IFN-producing cells, are a unique cell-type specialized to rapidly produce prodigious amount of these innate mediators following infection by multiple

viruses (10-15). Indeed, pDCs dedicate 50% of their transcription to make IFN-I mRNA and following viral stimulation synthesize a significantly broad range of these closely related cytokines including IFN-α, IFN-β, IFN-ω, IFN-λ,and IFN-τ, in some cases without requiring viral replication(13,16). pDCs use the Toll-like receptor (TLR) system, including TLR-7 and TLR-9, which rapidly trigger IFN-I transcription through a MyD88-dependent signaling pathway (Figure 1, (17));(18-21). TLR-9 mediates recognition of CpG-rich regions in the genome of DNA viruses and bacteria including herpes simplex virus 1 and 2, murine cytomegalovirus (MCMV), and mycobaterium tuberculosis (22,23). TLR-9 also senses malaria pigment hemozoin, extending its role to host defense against parasitic infections (24). Interferon regulatory factor 7 (IRF-7) is a transcription factor constitutively expressed at high levels in pDCs and is essential for IFN-I transcription upon TLR stimulation(25,26). In contrast, IRF-5 is not required for IFN-I production but is necessary for the induction of pro-inflammatory cytokines such as interleukin (IL) 6 or IL-12, that are also secreted by pDCs upon TLR stimulation (27). It has recently been shown that IRF-7 activation and nuclear translocation in pDCs requires prolonged CpG retention in early endosomes, which appears to be critical for MyD88/IRF-7 complex formation (28,29)

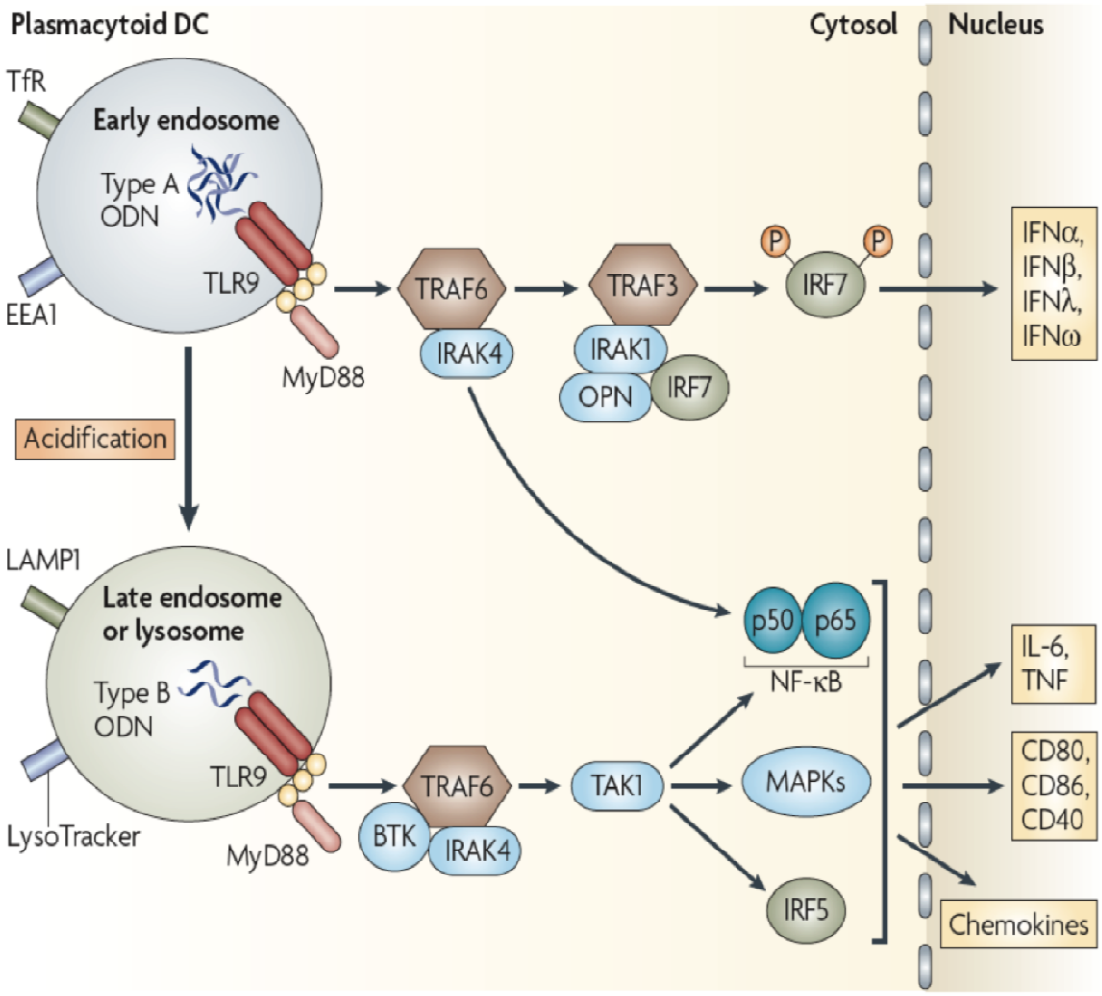

Figure 1: **TLR 9 signaling in Plasmacytoid Dendritic Cells *(Gilliet et al. Nat Rev 2008)***

pDCs produce most of the IFN-α protein within the first 24 hr following viral stimulation and then differentiate into mature DCs with enhanced antigen presenting capacity (13). In vitro studies indicated that after the first wave of IFN-I production, pDCs are refractory upon secondary stimulations to produce these cytokines (21). However, how these observations translate into an *in vivo* acute and persistent viral infection in terms of systemic IFN-I production, innate response, and susceptibility to secondary opportunistic infections is unclear.

pDCs, IFN-I and IL-6 are critical players of anti-viral responses

pDCs are considered the major source of IFN-I during several virus infections(8,10,12-14,30).Through IFN-I production, pDCs induce a cellular antiviral state and influence the functions of NK cells, conventional DCs, B cells, and T cells therefore impact innate and adaptive immune responses(17). Consistently, pDCs depletion studies have demonstrated their vital role in viral clearance during infections with MCMV, respiratory syncytial virus (RSV) and Lymphocytic choriomeningitis virus (LCMV) among other pathogens (31-34). However, the biological significance of pDC function may differ among different type of infections as indicated by the apparent dispensable role of these cells during influenza virus infection (35).

Although IFN-I is the major cytokine produced by pDCs, IL-6 is also secreted by these DCs upon TLR stimulation. IL-6 is the prototypic member of the IL-6 family (IL-6, IL-11, IL-27, LIF, oncostatin M, etc) (36). IL-6 is a multifunctional cytokine that regulates various aspects of the immune response such as antigen-specific immune responses, inflammatory reactions, cell proliferation, differentiation and survival. IL-6 production has been shown to be important in protection against acute herpes simplex virus type-1, Vesicular stomatitis virus, and Vaccinia virus in mice but its role in chronic viral infection remains unknown (37,38).

Murine LCMV infection as a model system to study host-virus interactions

LCMV is an enveloped single stranded RNA virus and a prototypic

member of the arenaviridae family which includes several human viruses such as Lassa fever, Guanarito, Junin, and Machupo (39). LCMV is a natural mouse pathogen and provides investigators with a superb model system to study virus-immune system interactions in a small animal model (40,41). LCMV can be used as a model for acute and chronic viral infection in adult mice. The Armstrong 53b (ARM) LCMV variant results in an acute infection which is cleared within 10 days by cytotoxic T lymphocytes (42). Another isolate, LCMV Clone 13 (Cl13), was derived from LCMV ARM and has only two single amino acid substitutions in the viral glycoprotein (GP) and polymerase (L), respectively(43). Despite the genetic similarity to its parental virus ARM, LCMV Cl 13 initiates abortive CD8 and CD4 T cell responses upon inoculation into mice (44-46). This results in viral persistence that lasts around 60 days in the blood and several tissues and about 200 days in brain and kidneys (43,47). The point mutation in the viral GP enables Cl 13 binding to α-dystroglycan, which is highly expressed and glycosylated in DCs, with higher affinity than ARM and allows its robust replication in DCs(48). Considering their crucial role in immune response, it is expected that Cl13 targeting of DCs would provide the virus with an advantage to subvert the host immune system. On the other hand, recent findings indicate that LCMV Cl13, but not ARM, also infect fibroreticular cells (FRC), suggesting that alteration of the FRC stromal network contributes to viral persistence during Cl13 infection (49). In addition, a second point mutation in the virus polymerase enhances Cl13 replication and facilitates its persistence (40,41,50).

It should be noted that although LCMV Cl 13 infection persists for a long

period in adult mice, the virus is eventually cleared from blood and several tissues by 2-3 months p.i. The necessary elements for the eventual clearance of LCMV Cl 13 are not completely known but CD4 T cells, CD8 T cells, B cells and antibodies were found to be required (51-54).

Aims and objectives

We used LCMV infection in mice as a model system to investigate the ability of pDCs from acutely or persistently infected hosts to produce systemic IFN-I. We evaluated the biological significance of virally-altered pDC-IFN-I production. We examined whether the pDC-IFN-I defect was at the transcriptional or post-transcriptional level and the possible mechanisms involved. We also studied the relationship between IFN-I inhibition and viral replication in pDCs. Finally, we looked at the biological role of interleukin-6 (IL-6), which secretion by pDCs is unaltered by LCMV infection, in anti-viral response and viral clearance.

RESULTS

Rapid Silencing of Systemic IFN-I Production after LCMV Infection

To understand the regulation of IFN-I production in persistent (Cl 13) and acute (ARM) infections, IFN-I levels were determined throughout the course of infections (Figure 2A, left axis). Both ARM and Cl13 infected mice showed rapid elevation of IFN-I levels in the serum by day 1 post-infection (p.i.) indicating that the mouse innate immune system was able to recognize LCMV and to initiate a potent IFN-I response. However, by day 5 p.i. serum IFN-I had returned to baseline levels and remained undetectable through the course of the infection. Therefore, even in the continuous presence of viral replication in the blood (Figure 2A right axis) and several tissues during Cl 13 infection (data not shown), the infected mice were unable to sustain elevated levels of systemic IFN-I. This observation indicated a dysregulation of the innate immune system and raised the important question of whether IFN-I could be re-induced upon secondary infections.

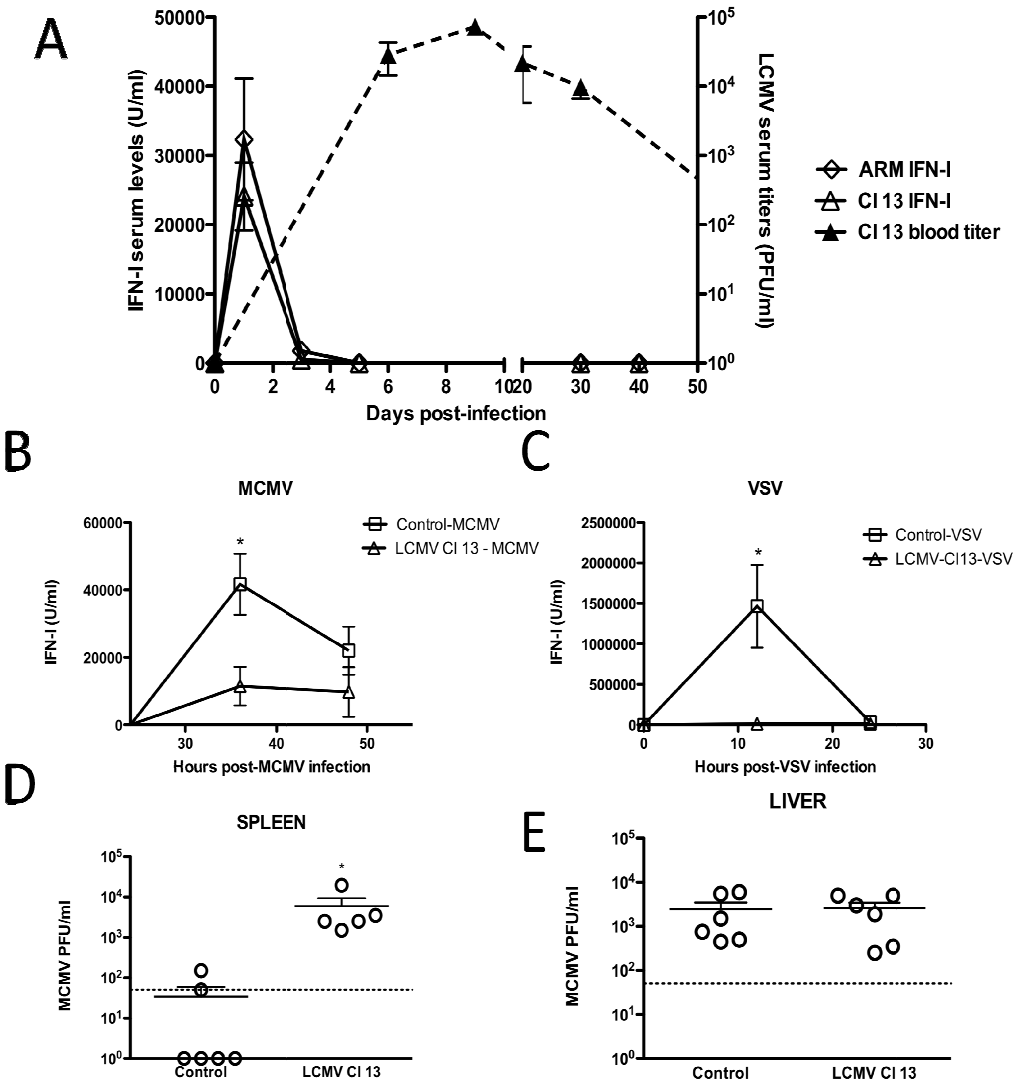

Figure 2: Systemic IFN-I Response during LCMV infection and Secondary Viral infection

(A) Mice were infected with LCMV ARM or Cl 13. Serum samples were collected before infection (day 0) and at 1, 3, 5, 30, and 40 days p.i. IFN-I activity was measured by luciferase bioassay (left y axis) and LCMV titer by plaque assay (right y axis). **(B-C)** Uninfected and LCMV Cl 13-infected mice (day 20–30 p.i.) were injected with MCMV **(B)** or with VSV **(C)**; blood samples were collected at the indicated times after secondary infection and IFN-I activity was measured by luciferase bioassay. The mean obtained from six mice per group is shown (*dual viral infection compared to single viral infection $p < 0.01$). Results are representative from one or two independent experiments. **(D-E)** Uninfected or infected Mice, with LCMV Cl 13 at day 18 to 40 p.i were injected with MCMV. Spleens **(D)** and livers **(E)** were collected at day 4 post MCMV infection and titers of MCMV were determined by plaque assay. Each symbol represents an individual mouse and dotted lines indicate the detection limit. Results are representative from two independent experiments. The mean ± SD obtained from four to six mice per group is shown (*dual viral infection compared to single viral infection $p < 0.01$).

Defective IFN-I Response During Secondary Infections

We next evaluated the impact that IFN-I silencing during LCMV Cl 13 infection could have on IFN-I production upon secondary infections. Murine cytomegalovirus (MCMV) was used as the model for secondary infection since initial IFN-I production 36 hours after MCMV infection is pDC-TLR-9 dependent (22,30,32,55). Uninfected and day 30-LCMV-Cl 13 infected mice were given MCMV and systemic levels of IFN-I determined in blood (Figure 2B). As expected, control mice infected with primary MCMV infection induced a strong IFN-I secretion in sera while a profound reduction was observed in Cl 13-infected mice receiving MCMV. To further examine the extent of IFN-I alteration during chronic LCMV infection; we tested IFN-I production in Cl 13-infected mice upon secondary infection with VSV (Figure 2C). We found that mice with a progressing Cl13 infection also failed to elicit systemic IFN-I levels upon VSV secondary infection. These data supported the idea that chronic LCMV infection caused an impaired IFN-I response upon secondary infections.

To investigate the biological impact that LCMV-IFN-I dysregulation could have on the containment of a secondary unrelated pathogen, MCMV titers were measured in of Control-MCMV and Cl 13-MCMV infected mice 4 days after MCMV infection. Because different mechanisms are responsible for resistance against MCMV in the spleen (mostly NK cells) and in the liver (mainly IFN-γ) spleens and livers were analyzed in parallel (Figure 2D and 2E, respectively).

Control mice that received a primary infection with MCMV had less than 50 PFU/ml of MCMV in spleens by 4 days p.i. In contrast; 3–4 log of infectious MCMV was detected in spleens of Cl 13-MCMV-coinfected mice (Figure 2D). No significant differences in MCMV titers were detected in the liver (Figure 2E).Interestingly when VSV was used to model a secondary infection, both Cl 13-VSV and Control-VSV infected mice were able to completely eradicate the VSV by day 4 pi (data not shown). These data suggest that the deleterious effect of LCMV infection on IFN-I response contributed to disarming the host capacity to contain early replication of an extraneous pathogen such as MCMV. However, the results obtained with VSV secondary infections emphasized that the biological impact that IFN-I inhibition may have on the early control of an opportunistic pathogen depends on the unique nature (i.e., viral properties, dose, route) of the secondary infection.

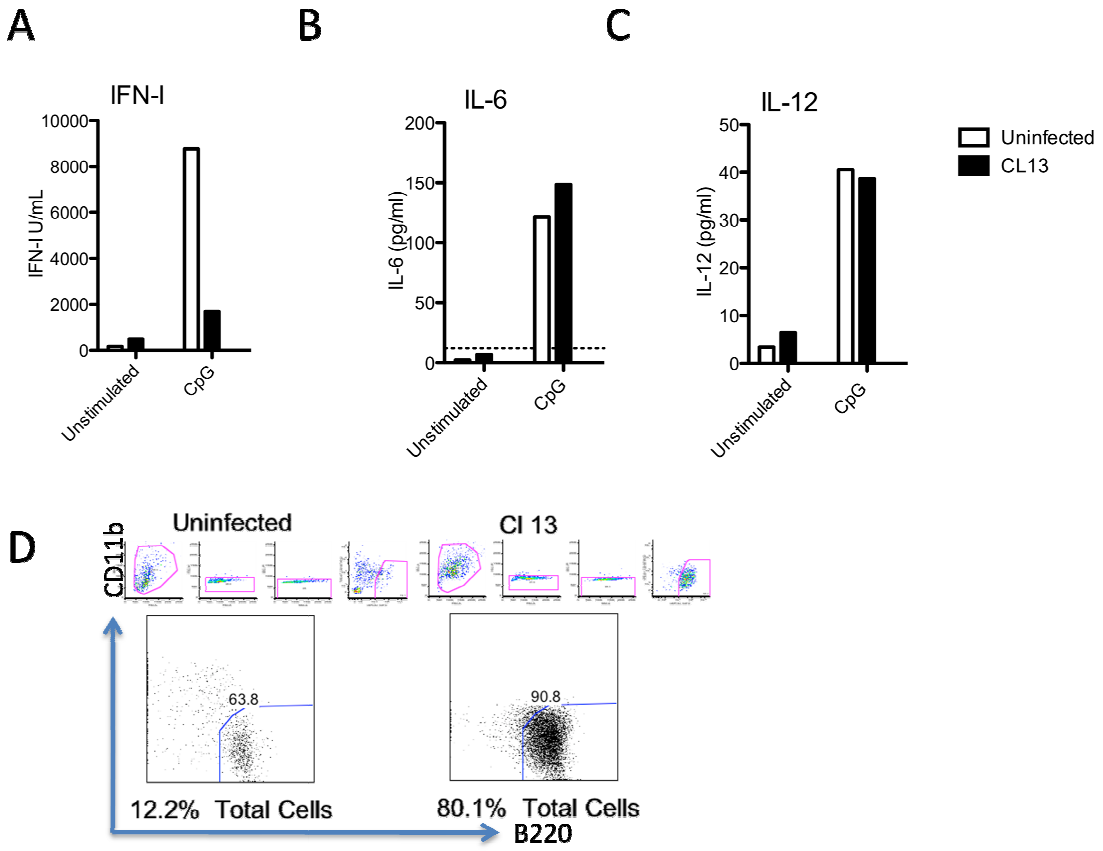

Figure 3: **Characterization of pDC-TLR response during LCMV infection**

Spleen pDCs (5 × 10^5 cells/ml) were FACS purified from uninfected (n = 2), ARM (n = 4), or Cl 13 (n = 5) infected mice at day 9 p.i. Cells were cultured with media only or CpG-1668 for 15 hr. IFN-I activity was measured in the supernatant by luciferase bioassay (A); IL-6 (B) and IL-12 (C) were measured by ELISA. Purity of pDCs was determined in (D), cells were stained with indicated markers and analyzed by FACs. These results are representative of one independent experiment; and correlates with d5 Pi data seen in Zuniga et. al 2008 (67).

PDC IFN-I production was inhibited during LCMV infection

Given that pDCs are the cell type responsible for producing IFN-I at 36h after MCMV infection(22,30,32,55), our *in vivo* data suggested that upon LCMV infection the ability of pDC to produce IFN-I could be inhibited (56). To directly test this hypothesis, pDCs (defined as $CD11c^+B220^+120G8^{high}CD11b^-NK1.1^-$

Thy1.2$^-$CD19$^-$(57)) were isolated from uninfected or Cl 13 infected mice. Cells were cultured in the presence of TLR-9 ligand (CpG) and the levels of IFN-I were determined. As shown in Figure 3A, production of IFN-I upon TLR-9 stimulation was significantly impaired in pDCs from Cl13 infected mice as compared to uninfected controls. We next analyzed the production of other cytokines (IL-6 and IL-12) by pDCs in the same culture supernatants (Figure 3B and 3C, respectively). Remarkably, in contrast to IFN-I secretion, similar levels of IL-6 and IL-12 were produced by pDCs from infected and uninfected mice. These data indicated that LCMV infection selectively abrogated pDC IFN-I production upon TLR stimulation but left unaltered their capacity to secrete other cytokines.

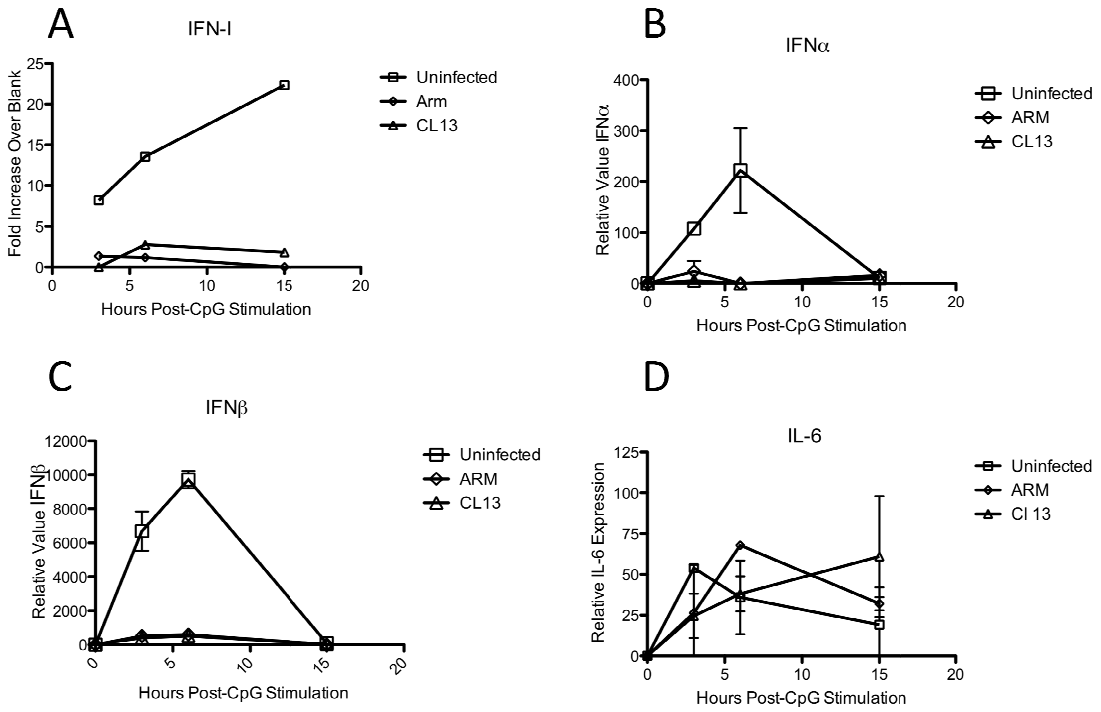

Figure 4: pDC-IFN-I inhibition is regulated at the transcriptional level

Spleen pDCs (5×10^5 cells/ml) were FACS purified from uninfected (n = 2), ARM (n = 4), or Cl 13 (n = 5) infected mice at day 5 p.i. Cells were cultured with CpG-1668 for 0, 3, 6, or 15 hr. IFN-I activity was measured in the supernatant by luciferase bioassay **(A)**;. IFN-α **(B)**, IFN-β **(C)**, IL-6 **(D)** mRNA expression was quantified by real-time RT-PCR and data are shown as average ± SD normalized to GAPDH mRNA. These results are representative of one independent experiment.

Transcriptional dysregulation of IFN-I genes in pDCs

To determine if the suppression of pDC IFN-I production was regulated at the transcriptional or post-transcriptional level, IFN-α and IFN-β mRNA expression were determined in pDCs from LCMV infected and uninfected mice before and at different times after CPG stimulation. IFN-I activity in the supernatant of the same pDC culture was determined in parallel. As expected, IFN-I activity was suppressed in pDCs from LCMV ARM and Cl 13 infected mice

at all times studied after CpG stimulation (Figure 4A). Interestingly, we observed dramatically reduced levels of IFNα and IFNβ transcripts in pDCs from Cl-13- and ARM-infected mice in comparison to uninfected controls (Figure 4B and 4C, respectively). Similar elevation in IL-6 mRNA was observed among uninfected and infected pDC, albeit the kinetic of the response could be different (Figure 4D). These data demonstrated that *in vivo* LCMV infection caused an intrinsic pDC alteration that prevented IFN-I but not IL-6 transcription upon TLR stimulation.

We next attempted to identify the underlying molecular mechanisms causing the selective inhibition of IFN-I transcription in pDCs focusing on cellular components upstream IFN-I but not IL-6 or IL-12 transcription (Figure 1).We first determined IRF-7 expression since its constitutively high levels in pDCs seem to be required for a rapid IFN-I response (58). For that, pDCs were isolated from uninfected or LCMV infected mice before and after CpG stimulation and IRF-7 expression analyzed by Real Time PCR (Figure 5A). Similar levels of IRF-7 transcripts were observed in pDCs from uninfected, ARM, and Cl 13 infected mice prior to stimulation. However, while uninfected pDCs substantially upregulated IRF-7 expression by 6h after CPG stimulation, pDCs from both ARM and Cl 13 infected mice failed to enhance IRF-7 expression.IRF-5 mRNA expression was next examined in parallel as a control transcription factor that is not involved in pDC-IFN-I response (Figure 5B). Expression of IRF-5 was slightly

higher in uninfected pDCs compared to pDCs from ARM and Cl 13-infected mice, but no differences upon CpG stimulation could be detected.

In conclusion, our data indicated that pDC-IFN-I inhibition during LCMV infection occured at the transcriptional level and related to a deficient IRF-7 up-regulation upon CpG stimulation. Because up-regulation of IRF-7 transcription is dependent on IFN-I production (59,60), it is possible that the lack of IRF-7 up-regulation in infected pDCs had been a consequence (instead of the cause) of deficient IFN-I production.

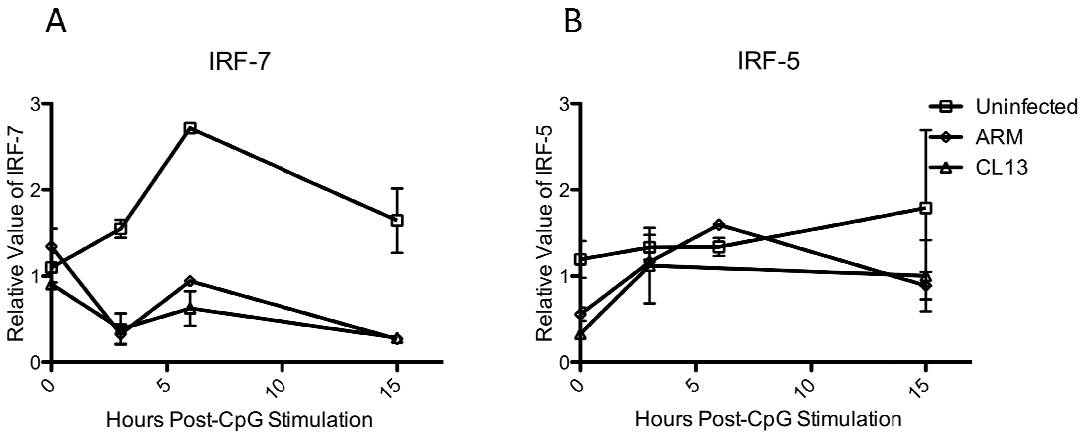

Figure 5: pDC IRF-7 and IRF-5 transcription during LCMV infection

Spleen pDCs (5×10^5 cells/ml) were FACS purified from uninfected (n = 2), ARM (n = 4), or Cl 13 (n = 5) infected mice at day 5 p.i. Cells were cultured with CpG-1668 for 0, 3, 6, or 15 hr. IRF-7 **(A)** and IRF-5 **(B)** mRNA expression was quantified by real-time RT-PCR and data is shown as average ± SD normalized to GAPDH mRNA. Results are representative of 1 independent experiment.

Dissociation between IFN-I inhibition and viral replication in pDCs

Viral interference with IFN-I biosynthesis has been associated with virus replication within the affected cells, directly altering IFN-I induction, transcription,

RNA processing, and/or translation (9). Thus, we examined if pDC-IFN-I inhibition correlated with the level of LCMV replication within pDCs. For that, we used two different technical approaches in pDCs from ARM and Cl 13 infected mice at day 5 p.i. We first determined the levels of LCMV-GP transcripts in pDCs by Real Time PCR. We also quantified the numbers of pDCs producing infectious viral particles by infectious center assay. Importantly, IFN-I secretion by pDCs upon CpG stimulation was determined in parallel. We observed that while the extend of IFN-I inhibition was similar in pDCs from ARM versus Cl13 infected mice (Figure 6A), the former ones showed substantially lower levels of virus replication as judged by LCMV-GP expression and numbers of infected pDCs (Figure 6B and 6C, respectively). pDC purity was over 95% in the aforementioned experiments (Figure 6D). These data revealed a dissociation between the degree of IFN-I inhibition and the amount of replicating virus within pDCs. It is important to note that our data did not elucidate whether virus replication in pDCs was dispensable for IFN-I inhibition or if a minimal replication threshold needed to be achieved.

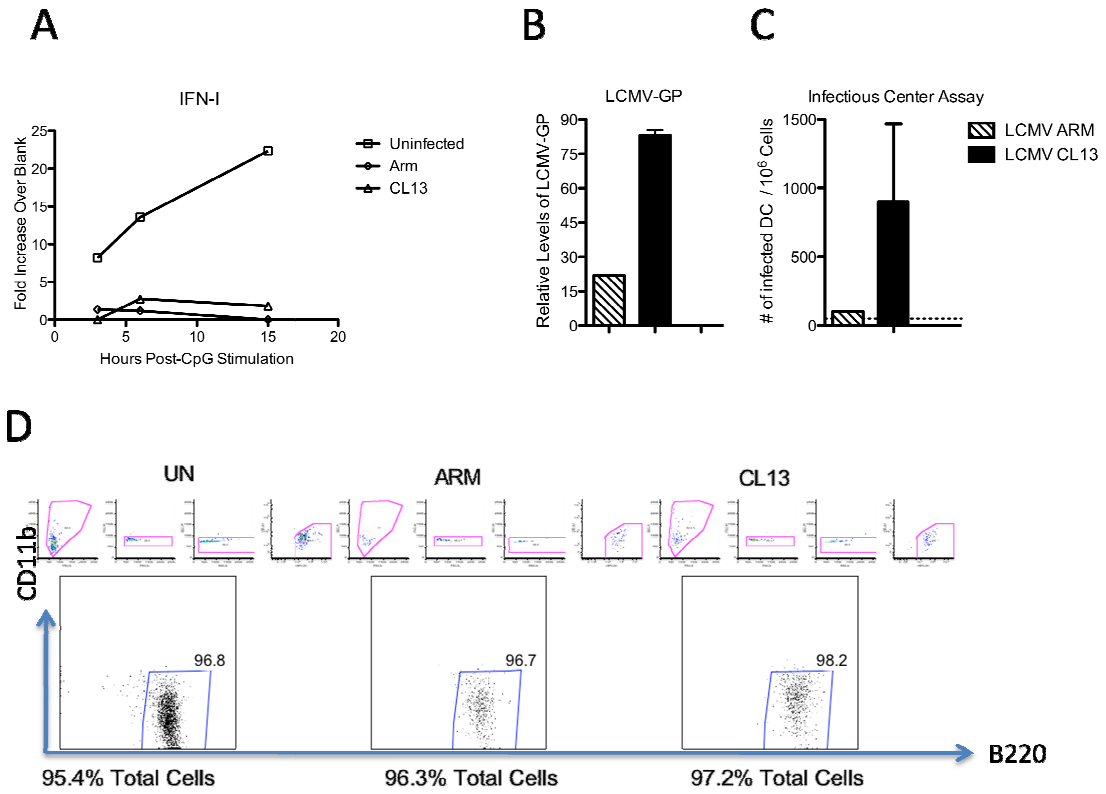

Figure 6: Dissociation between IFN-I inhibition and viral replication in pDCs

Spleen pDCs (5×10^5 cells/ml) were FACS purified from uninfected (n = 2), ARM (n = 4), or Cl 13 (n = 5) infected mice at day 5 p.i. Cells were cultured with CpG-1668 for 0, 3, 6, or 15 hr **(A)**. IFN-I activity was measured in the supernatant by luciferase bioassay. In **(B)**, LCMV-GP mRNA expression was quantified by real-time RT-PCR in freshly isolated pDCs and data are shown as average ± SD normalized to GAPDH mRNA. In **(C)**, Infectious Center Assay were performed by serial twofold dilutions of freshly isolated pDCs onto a vero cell monolayer culture followed by Plaque Assay. Purity of pDCs was determined in **(D)**, cells were stained with indicated markers and analyzed by FACs. The results are representative of one independent experiment.

IL-6 Production during Acute versus Chronic LCMV infection

As mentioned before, in contrast to pDC-IFN-I inhibition, the ability of pDCs to produce IL-6 remains unaltered during LCMV infection. Because IL-6 regulates various aspects of the immune response (61), further studies investigated systemic IL-6 production throughout the course of acute and chronic

LCMV infections (Figure 7A-B). A rapid increase of IL-6 was observed in sera from both ARM and Cl 13 infected-mice between days 1 and 5 p.i. Notably, while IL-6 levels decreased to undetectable levels by day 20 after ARM infection, elevated levels of IL-6 were observed in Cl 13 infected mice between days 20 and 40 p.i. Interestingly, the late elevation of IL-6 during Cl 13 infection coincides with substantial reduction in viremia to final clearance ~60 day p.i. Thus, our data suggest that late IL-6 elevation during chronic LCMV infection could play a role in the eventual clearance of the persistence-prone virus.

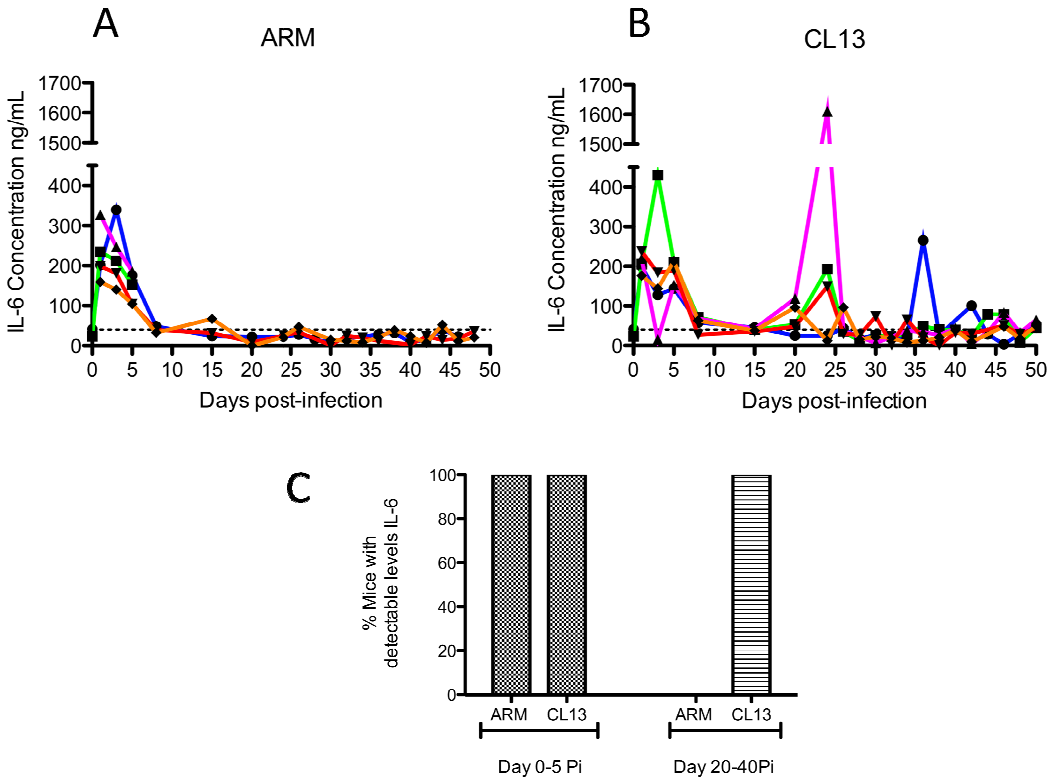

Figure 7. IL-6 Production during acute and chronic LCMV infection

Mice were infected i.v. with 2 x 10^6 PFU of either LCMV ARM **(A)** or Cl 13 **(B)**. Blood samples were taken at indicated time points P.i. and serum IL-6 levels were measured by ELISA. In **(C)**, the % ARM or Cl13 infected mice showing detectable levels of IL-6 is shown at Day 0-5 P.i. and Day 20-40P.i. Results are representative of 2 independent experiments.

Vital Role of IL-6 in the Control of Chronic LCMV infection

We next investigated the role of IL-6 in controlling acute and chronic LCMV infections. For that, we used IL-6 Knock out (KO) mice containing a neomycin insertion in the second exon of the IL-6 gene (37). Wild-type (WT) and IL-6KO mice were infected with LCMV Cl 13 and viral titers determined in blood at different times p.i.(Figure 7).While WT mice showed undetectable levels of

viremia by day 60 Pi, high titers were observed in IL-6KO mice until ~200 days p.i., which was the last time point monitored. These data demonstrated that IL-6 played a crucial role in the late clearance of Cl 13 infection. Current studies are addressing the cellular and molecular mechanism through which IL-6 exerts its function during persistent viral infection.

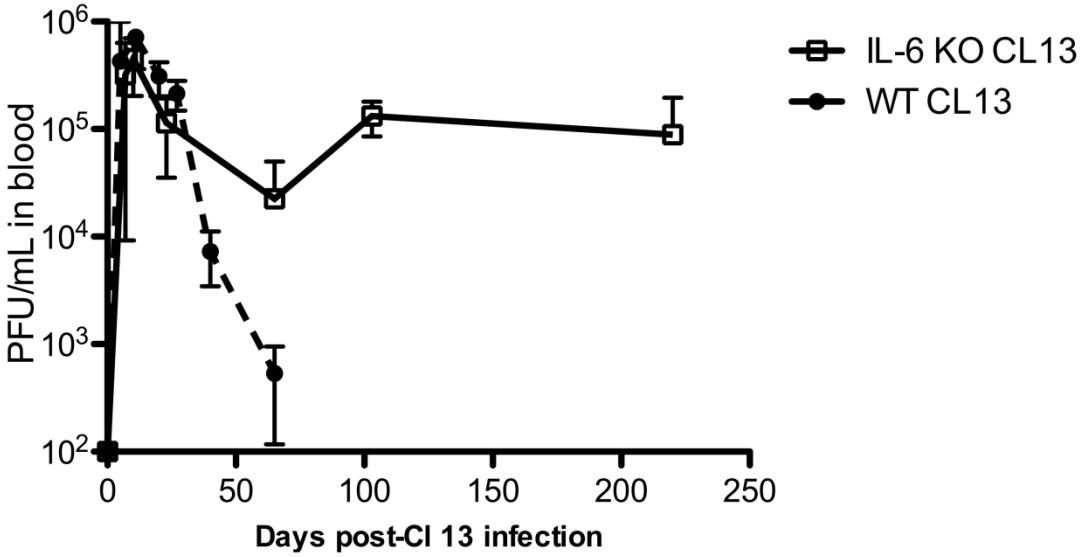

Figure 8. IL-6 deficient mice fail to clear LCMV Cl 13 from the blood.

Wildtype C57BL/6 or B6-*IL6*^{tm1Kopf} were infected i.v. with 2 x 10^6 PFU of LCMV clone 13. Blood samples were taken at multiple days post infection and viral titers measured by plaque assay. Results are representative of 2 independent experiments.

IL-6 administration was insufficient to accelerate LCMV Cl 13 clearance

Given the essential role of IL-6 in LCMV Cl 13 clearance, we next asked whetherIL-6 administration could accelerate clearance of this persistent infection. To answer this question, Cl 13 infected mice were treated with 20,000 or 200,000

units of recombinant (r) IL-6 twice a day for 7 days. Day 5 p.i. was selected as the first day of IL-6 treatment to prolong the initial production of IL-6 during Cl 13 infection, which was silenced between days 5 and 8p.i.(Figure 6B), and mice were bled at days 11, 20, 27, and 40 p.i. to monitor antiviral CD8 T cell responses and viremia (Figure 8B-D). No differences were observed in either the percentage or numbers of LCMV-GP_{33-41} or $NP-_{396-404}$ specific $CD8^{+}$ T cells detected in treated versus untreated mice (Figure 8B and data not shown). Moreover, impaired production of IFN-γ and TNF-α were observed in CD8 T cells from both treated and untreated Cl-13 infected mice upon stimulation with LCMV-GP_{33-41} peptide (Figure 8C).Finally, similar viral titers were detected in treated and untreated Cl 13 infected mice at all time points examined (Figure 8D). No differences were observed among IL-6 treatment doses in either anti-viral CD8 T responses or virus titers. These data indicated that while IL-6 was essential for natural control of LCMV Cl 13 infection it was insufficient, under the aforementioned conditions, to accelerate eradication of the persistence-prone virus.

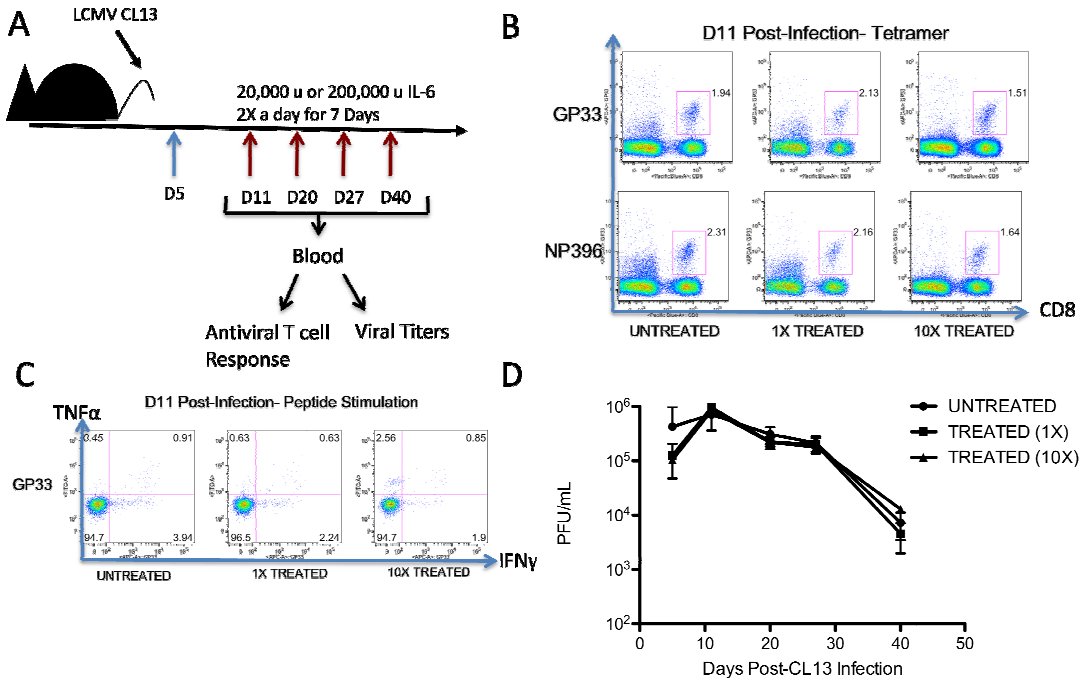

Figure 9: Il-6 Treatment during LCMV Cl 13 infection

Day 5 P.i.-LCMV Cl 13 infected mice were either untreated or treated with 20,000units/day or 200,000units/day of IL-6 for a 7 day period. In **(B)**, D11 P.i. Cells were analyzed by FACS for $CD8^{+}GP_{33\text{-}41}$Tetramer $^{+}$ or $CD8^{+}NP_{396\text{–}404}$Tetramer $^{+}$ T Cells. In **(C)**, Cells were stimulated with $GP_{33\text{-}41}$ LCMV Peptides and production of IFN-γ, TNF-α, IL-2 by CD8+ T Cells was analyzed.. Mice were bled at d5, d11, d20, d27, and d40 p.i. for viral titer determined by Plaque assay **(D)**. Results are representative from 1 independent experiment

Figure 2, in full, is a reprint of the material as it appears in Cell Host and Microbe 2008. E Zuniga, L Liou, L Mack, M Mendoza, and MBA Oldstone. The thesis author was the third author of this paper.

DISCUSSION

Virus infections initiate a race between viral growth and the host immune response that determines the outcome of the infection. Understanding the rules governing such race may help to develop improved therapies to alleviate virus associated diseases in humans. In this study, we used LCMV infection in its natural rodent host to analyze the dynamics of two elements of the immune response, namely IFN-I and IL-6, during acute and chronic viral infections *in vivo*. We showed that while LCMV infection inhibits IFN-I production by pDCs it leaves unaltered their ability to produce other cytokines like IL-6, which becomes essential and ultimately contribute to viral clearance.

Consistent with previous reports (62), we found that both acute and persistent LCMV variants induce rapid elevation of systemic IFN-I that peaks at 24 hours p.i. At present the cellular source of systemic IFN-I during LCMV infection is unclear. Although pDCs secrete IFN-I during LCMV infection (63,64), the early peak of systemic IFN-I was still detected in the absence of pDCs (65). Thus, the main source of IFN-I during LCMV infection might not be pDCs although they do produce it. Importantly we found that after its intial elevation, systemic IFN-I was rapidly silenced in both acute and chronic infection, despite high viral replication for up to 2 months during LCMV Cl 13 infection. The lack of systemic IFN-I in the presence of intense virus replication in blood and several tissues indicated a dysregulation or re-education of the host immune system.

We also investigated the biological implications of the IFN-I response

during LCMV infection by using MCMV and VSV as model opportunistic infections. MCMV is a well-characterized mouse natural pathogen (65), which is recognized by pDCs through TLR-9. As a result, a massive IFN-I response is initiated and is crucial for direct antiviral effect and the orchestration of other innate cells (66). We noted that LCMV infection compromised MCMV and VSV innate responses as indicated by the reduced levels of systemic IFN-I in dually infected mice. In the case of MCMV, IFN-I inhibition related to impaired containment of MCMV replication supporting the idea that virus dysregulation of IFN-I response could predispose to enhanced susceptibility to opportunistic infections. It should be noted that other innate immune defects during Cl 13 infection, such as poor IL-12 production upon secondary MCMV infection (67), may have coupled to the impaired IFN-I responses to determine the early MCMV spread observed.

pDCs are recognized as specialized cells with a unique potential to produce prodigious amounts of IFN-I (8,10-14). We found that *in vivo* LCMV infection alters the quality of pDC responses by disabling their distinctive function (i.e., IFN-I production) without affecting secretion of other cytokines such as IL-6 or IL12. It is interesting to note that IFN-I inhibition has been traditionally associated with viral replication within the affected cells(9). However, we observed that IFN-I is equally silenced by both ARM and Cl 13 infections despite minimal viral replication detected in pDCs during acute ARM infection. This dissociation between the degree of infection and the silencing of IFN-I suggested

that it was not the virus replication *per se* but the exposure of pDCs to the infectious environment what caused IFN-I inhibition. It is possible, as previously shown, for attenuation of T cell responses during LCMV Cl 13 infection (68-70), immunoregulatory mechanisms may dampen pDC-IFN-I response during viral infections. Although the precise mechanism of pDC-IFN-I suppression is still unknown, we demonstrated that IFN-I silencing is regulated at the transcriptional level. Notably, the primary transcription factor responsible for IFN-I induction in pDCs, IRF-7, was similarly expressed in pDCs from uninfected and LCMV infected mice, albeit its upregulation upon CpG stimulation was compromised in infected pDCs. Because IRF-7 is up-regulated in an IFN-I-dependent manner(59,60), further studies are necessary to discriminate whether the impaired IRF-7 up-regulation is cause or consequence of IFN-I inhibition. It is also possible that pDC-IFN-I inhibition results from defects on other critical steps up-stream IFN-I transcription such as IRF-7 activation, TRAF6 and TRAF3 ubiquitination, endosomal trafficking/retention, etc (28,29,71,72). Whatever this molecular defect is, it is certainly dispensable for pDC production of IL-6 and IL-12, which remained unaltered (17).

The production of IL-6 during LCMV infection was biphasic with a later elevation which coincided with the reduction of viremia after Cl 13 infection. Studies in IL-6 deficient mice uncovered a key role in the control of chronic viral infection. Identification of the cellular source/sand target/s of IL-6 may illuminate the necessary elements for controlling a persistent virus and what role IL-6 has in

this process. In recent studies the mechanism of LCMV Cl 13 clearance has been revisited (51,73-75). $CD4^+$ T cell help by way of IL-21 production has been shown to be vital for the eventual clearance of Cl 13 infection. As IL-6 is capable of inducing IL-21 under certain conditions (76), it is possible that IL-6 contributes to IL-21 elevation during chronic infection (73-75). Viral clearance has also been shown to rely on antibody responses (51). Since IL-6 can influence germinal center development and antibody production (77), lack of IL-6 may impair the formation of virus-specific antibodies and thus prevent viral clearance. Finally, IL-6 has been shown to increase survival of T cells (78,79) and may overcome pro-apoptotic signals (such as transforming growth factor-beta) that are known to occur during Cl 13 infection (80). It is possible that several critical host factors emerge together with IL-6 at late times after Cl 13 and act in conjunction to enable viral clearance. In this respect, the precise route of administration, ideal timing and necessary co-factors should be further elucidated before IL-6 could be harnessed as a therapeutic agent to accelerate clearance of chronic viral infections.

In conclusion, our results demonstrated the profound suppression of IFN-I production by pDCs during *in vivo* viral infection, could weaken the host innate response to secondary pathogens and facilitates opportunistic infections. Other elements of the host immune system, such as IL-6 were less altered during chronic infection and became crucial for its eventual eradication.

EXPERIMENTAL PROCEDURES

Viruses

The ARM53b and Cl 13 LCMV clones were grown, identified, and quantified as described (43,81,82)Smith strain of MCMV was obtained from Bruce Beutler, The Scripps Research Institute (TSRI). MCMV stocks were prepared from salivary glands of BALB/c mice given 1×10^4 PFU intraperitoneally (i.p.) (55). Homogenates of pooled salivary glands were made in sterile PBS and viral titers determined by plaque assay as described (83). New Jersey VSV strain was obtained from Dr. Robert Lamb (Northwestern University, IL) and viral titers determined on baby hamster kidney cells after 1 hr absorption at 37℃ and 48 hr of 1% agarose overlay.

Mice

C57BL/6 mice were obtained from the closed breeding colony of UCSD. *B6*.129S2-*Il6tm1Kopf*/*J* (IL-6KO) mice were obtained from *Jackson* Laboratories. Mice were bred and maintained in a closed breeding facility and mouse handling conformed to the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of UCSD. Mice at 6–8 weeks of age were infected by i.v. inoculation of LCMV (2×10^6 PFU either ARM or Cl 13), i.v. injection with VSV (5×10^7 PFU) or i.p. injection of MCMV (1×10^4 PFU).

Cell Purification

Spleens were removed and incubated with collagenase D (1 mg/ml, Roche, Indianapolis, IN) for 20 min at 37℃. Single-cell suspension of

splenocytes were enriched and purified as described previously (57). pDCs (defined as $CD11c^{+}B220^{+}120G8^{high}CD11b^{-}Thy1.2^{-}CD19^{-}NK1.1^{-}$), CD11b+ cDCs (defined as $CD11c^{+}B220^{-}CD11b^{+}CD8^{-}Thy1.2^{-}CD19^{-}NK1.1^{-}$), CD8+ cDCs (defined as $CD11c^{+}B220^{-}CD11b^{-}CD8^{+}Thy1.2^{-}CD19^{-}NK1.1^{-}$), and Macrophages (defined as $CD11c^{-}$ $CD11b^{+}$ $Thy1.2^{-}CD19^{-}NK1.1^{-}$) were separated using a FACS-Aria Sorter (Becton Dickinson, San Jose, CA).

Flow Cytometry

The following antibodies purchased from E-bioscience or BD-bioscience were used to stain blood or spleen cells: anti-IFN-γ APC, anti-TNF-α-FITC, anti-IL-2-PE, anti-CD11b-PE-CY7, anti-CD11c-APC, anti-CD19-PE or Percp-Cy5.5, anti-NK1.1-PE, anti-Thy1.2-PE, anti-B220-APC-CY7, anti-B220-FITC. Anti-120G8 monoclonal antibody was kindly provided by Giorgio Trinchieri and conjugated to Alexa 488 following manufacture instructions (Molecular Probes, Invitrogen, Carlsbad, CA). Prior to staining for Dendritic cell preparations were blocked with 3.3 µg/ml anti-mouse CD16/CD32 (Fc block, BD PharMingen) in PBS containing 1% FBS for 10 min. The Fc block was also included in all 20 min surface stains for Dendritic cells. Anti-CD8-pacific blue was purchased from Caltag. For staining with IA^{b}-$GP_{66\text{-}77}$, D^{b}-$NP_{396–404}$, D^{b}-$GP_{276–286}$ (provided by NIH Tetramer Core Facility; Atlanta, GA), or D^{b}-$GP_{33–41}$ (Beckman Coulter; Fullerton, CA) tetramers, cells were incubated for 1 hr and 15 min at room temperatureCells were acquired using the Digital LSR II flow cytometer (Becton

Dickinson, San Jose, CA). Flow cytometric data were analyzed with the FlowJo software.

Ex Vivo T Cell Stimulation

Splenocytes were stimulated with 2 μg/ml of the MHC class I-restricted LCMV $NP_{396–404}$ or $GP_{33–41}$, peptides (all >99% pure; Synpep) in the presence of 50 U/ml recombinant murine IL-2 (R&D Systems). Cells were cultured for 5 hr in the presence of brefeldin A (1 μg/ml; Sigma) and stained for surface expression of CD8, fixed, permeabilized, and stained with Abs to IFN-γ, TNF-α, and IL-2. All cultures without peptide performed in parallel show no production of cytokines.

pDC Cultures and In Vitro Stimulation

Unless otherwise indicated, pDCs were plated at 1×10^5 cells/ml in 100 μl RPMI complete medium (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 μM β-mercaptoethanol). For pDC stimulation, cells were cultured overnight in the presence of HPLC purified CpG-1668 (TCCATGACGTTCCGATGCT phosphorothioate-modified; IDT, Inc.) and supernatants then collected while cells were saved for RNA extraction

RNA Extraction and QPCR

FACs-sort purified pDCs were resuspended in RNA purified by use of the RNeasy Micro Kit (Qiagen) and reverse transcribed into cDNA. Quantification of cDNA was performed using FAST SYBR Green PCR Kit (Applied Byosistem) and Real-Time PCR Detection System (ABI). The relative RNA levels of LCMV-

GP, IFNα, IFNβ, IL-6, IRF-7 and IRF-5 were normalized against cellular glyceraldehydes 3-phosphate dehydrogenase (GAPDH) RNA.

Cytokine Measurements

IFN-I activity was measured with reference to a recombinant mouse IFN-β standard (Research Diagnostics) using a L-929 cell line transfected with an interferon-sensitive luciferase obtained from Bruce Beutler, TSRI (84). IL-6 was quantified by IL-6 ELISA kit purchased from e-bioscience.

Statistical Analysis

Statistical differences were determined by Student t test or one way analysis of variance (ANOVA) with InStat 3.0 software (GraphPad, CA.). $p < 0.05$ were considered significant.

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