Competitive Regulation of Splenic DC Subsets During Acute Infection:

MCMV-induced loss occurs independently of IFN-I and can be reversed by virus-sensitive, licensed NK cells

William Thomas Nash
Bothell, WA

B.S. in Biology, Gonzaga University, 2006

A Thesis presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology, Immunology, and Cancer Biology

University of Virginia
May 2016

_____

_____

_____

_____

_____

DEDICATION

I dedicate this thesis to my parents and grandparents who have encouraged fostered my creativity, curiosity, problem solving, and perseverance/stubbornness. I believe a healthy balance of these qualities are present in all who obtain success and owe a great deal of my completion of this dissertation to their past guidance and present support. I love you all dearly and thank you for all you've done for me. In particular, I would like to make a special note of my appreciation for my Grandma, Lena Versaggi, who passed away in 2009 just before I started my life as a graduate student. I always cherished our time together and am proud to have learned the art of the crossword from you. To this day, I often take one with me when I eat lunch to allow my mind a small break from scientific puzzles. Che duci, Grandma.

ACNOWLEDGEMENTS

First and foremost, I must acknowledge the Dr. Michael Brown lab – past and present. Those who came before me laid a groundwork for innovative thinking and scientific excellence that I am proud to carry forward. Those who have worked with me have embraced this and made it easy to sustain the tradition of rigorous thought while keeping science enjoyable. These qualities are clearly valued by my mentor, Dr. Michael Brown, and I cannot thank him enough for his patience, encouragement, understanding, and questioning. He has created an environment that fosters clear, open thought and scientific rigor while also encouraging the growth and well-being of the individuals. I truly appreciate his guidance and will continue to strive to "say what I mean and mean what I say,".

My thesis advisory committee deserves tremendous credit as well. Drs. Sally (Sarah) Parsons, Tom Braciale, Dan Engel, and Young Hahn have continued to ask the difficult questions and shaped me to be able to ask them myself. I greatly appreciate their tremendous patience with me and the pride they expressed in what I have now accomplished. I hope I can continue to develop and make them proud.

I would also like to acknowledge the individuals who have contributed to the lab and its environment during my graduate career. Drs. Virginia Carroll, Mike Stadnisky, Hairong Wei, and Pinar Pezuk, Ebony Coats, Jeffrey Teoh, Awndre Gamache, Alyssa Gillespie, Jessica Prince, and the many undergraduates who have shared their time with us over the years. The lab would not be the same without each of your influences. In particular, I would like to acknowledge Mike Stadnisky for his early contributions that defined the initial questions from which my project would grow. His discussions, guidance, and continued interest in my work are things I will always value immensely. Also, the camaraderie I have experienced with Jeff Teoh and Awndre Gamache during our time in lab is something I cherish immensely. Our ability to simultaneously push and support each other has always made me proud. You have both made my time here more productive and enjoyable and it will be a sad day when the three of us must part ways.

Credit is also due to Alyssa Gillespie and Jessica Prince. Over the years they have provide invaluable support in the forms of technical and administrative assistance as well as just plain fun. I will always fondly remember our Secret Santa gatherings. On that note, I would like to make special mention of two undergraduate students I have worked with closely. Susan Bond worked with me on some of the early IFN-I studies and provided me with one of my favorite Secret Santa gifts during her time in the lab. Jack Cronk has worked on and off in the lab nearly as long as I have been here and has developed into an excellent young scientist. These two truly embraced the lab culture and it has been a pleasure working with them.

Throughout this experience, friends and family have been a constant source of sanity and support. My colleagues and classmates especially deserve credit as we have been through much together. There are too many names to mention and I cannot bring myself to only write a few, but I cherish all of you who have been a part of my journey and memories. Thank you.

Additionally, the support staff in the Carter Immunology Center, Microbiology Department/BIMS program, and UVA core facilities have greatly facilitated the completion of this work and my growth as a scientist. Without you, none of this would likely be possible.

In terms of the experiments presented in this dissertation, acknowledgment must be made to those who have contributed reagents to the project. I would like to make special mention of Dr. Melissa Swiecki of the Colonna lab who did not hesitate to assist a young grad student and provided us with the pDC-depleting antibody. Also, Dr. John Lukens was kind enough to meet with me to discuss my questions about cell death mechanisms and graciously gave us the RIP3-KO mice.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Tables

Figures

ABSTRACT

Dendritic cells (DC) and natural killer (NK) cells are critical components of the innate immune response to infection. Conditions marked by the absence of these cells result in rampant viral infections and immune pathologies. As first-line sensors and effectors, they are prime targets of immune-evasion by a multitude of viruses, including cytomegalovirus (CMV), measles virus (MV), lymphocytic choriomeningitis virus(LCMV), herpes simplex virus (HSV), Rauscher leukemia virus (RLV), Epstein-Barr virus (EBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) In particular, murine CMV is known to induce a drastic loss of DC in the spleen during acute infection. In mice, the MHC class I haplotype H-2^k is historically associated with improved MCMV control. Mice of this genetic background exhibit reduced levels of viral replication compared to other strains. Our lab identified the H-$2D^k$ molecule (D^k) as an essential factor in this model of genetic viral control. Subsequently, we also uncovered a crucial role for NK cells expressing the inhibitory receptor Ly49G2 in mediating this D^k-dependent control. D^k is a cognate ligand for Ly49G2 and is capable of educating NK cells expressing this inhibitory receptor – licensing them for enhanced sensitivity to activation signals and heightened effector functions (i.e. cytokine production and cytolysis). Ly49G2$^+$ NK cells (G2+ NK) are capable of specifically detecting MCMV infection, but the mechanism of recognition is still the subject of ongoing work. However, these cells are critical to viral control since specific depletion of this subset results in virus levels comparable to mice lacking efficient MCMV control. Using this model, we have explored the ability of MCMV to dysregulate DC dynamics during infection and the ability of these virus-responsive, licensed NK cells to counter this process. Type I IFN (IFN-I) has been regarded as a prime candidate for inducing dendritic cell toxicity during acute MCMV infection. However, upon rigorously exploring this phenomenon, we observed the majority of DC loss during MCMV infection occurred independently of IFN-I signaling. Interestingly, sensitivity to IFN-I-induced loss appears to vary between different subsets of splenic DC. We have also further explored the IFN-independent mechanism of DC loss, investigating IL-6, soluble serum factors, cell death pathways, and cell trafficking. To date, we have not been able to conclusively determine the source of DC attrition, but work is ongoing. Whatever the mechanism, it is clear that virus-responsive NK cells play a critical role in reversing the DC loss. Mice that control MCMV via G2+ NK rapidly recover their DC populations and display enhanced DC numbers following recovery. This effect is dependent on G2+ NK and their ability to efficiently recognize MCMV infection. Hence, we have investigated, for the first time, mechanisms of MCMV-induced DC loss in an MHC-dependent model of viral control and the role of MHC class I-licensed NK cells in countering this form of immunosuppression.

ABBREVIATIONS

Ab – antibody
Ag – antigen
APC – antigen presenting cell; can also stand for the fluorophore allophycocyanin
APC-Cy7 – allophycocyanin-cyanin 7 tandem fluorophore
BATF3 -- Basic leucine zipper transcriptional factor ATF-like 3
B6 – C57Bl/6 inbred mouse strain
BM – bone marrow
Degrees C – degrees Celsius
CCL – C-C motif chemokine; CCR ligand
CCR – C-C motif chemokine receptor
CD – cluster of differentiation
cDC – conventional dendritic cells
Cg – congenic
CLEC9A – C-type lectin domain family 9 member A
CpG – unmethylated cytosine-phosphate-guanine motif; used to generally refer to reagents containing these motifs
CpG-A – CpG oligodeoxynucleotide type A
CpG-B – CpG oligodeoxynucleotide type B
DC – dendritic cells
d – day(s)
D^b – H-2D murine MHC class I molecule; b haplotype
D^k – H-2D murine MHC class I molecule; k haplotype
DN – double negative
dpi – day(s) post infection
ESAM – endothelial cell-selective adhesion molecule
F1 – filial generation 1
F2 – filial generation 2
FMO – fluorescence minus one; fluorescent staining panel minus one mAb to determine background signal
G2 – Ly49G2 inhibitory NK receptor
G2+ NK – Ly49G2-expressing NK cells
Gy – gray; unit of radiation
H – Ly49H activating NK receptor
H+ NK – Ly49H-expressing NK cells
h – hour(s)
hpi – hour(s) post infection
IFN – interferon
IFN-I – type I interferon; interferon α/β
IFNAR – interferon α/β receptor; type I interferon receptor
IL – interleukin
IMQ – imiquimod treatment

i.p. – intraperitoneally
i.v. – intravenously
KO – knockout
L – C57L inbred mouse strain
LPS – lipopolysaccharide
LTα/β – lymphotoxin alpha/beta
Ly49G2 – lymphocyte antigen 49G2
Ly49H – lymphocyte antigen 49H
M – MA/My inbred mouse strain
mAb – monoclonal antibody
MACS – magnetic-activated cell separation
MCMV – murine cytomegalovirus
MHC – major histocompatibility complex
mIgG – murine immunoglobulin; gamma class (non-specific Ab isotype control)
mPDCA – murine plasmacytoid dendritic cell antigen; also known as bone marrow stromal cell antigen (BST2)
NK – natural killer/natural killer cells
NKC – natural killer gene complex
NOTCH2 – neurogenic locus notch homolog protein 2
PAMP – pathogen-associated molecular pattern
PBS – phosphate buffered saline
pDC – plasmacytoid dendritic cells
PI – propidium iodide
pIC – abbreviated shorthand for poly I:C
poly I:C – polyinosinic:polycytidylic acid
PRR – pattern recognition receptor
rIgG – rat immunoglobulin; gamma class (non-specific Ab isotype control)
RBC – red blood cell(s)
RIP3 – receptor-interacting protein kinase 3
SG – salivary gland
TCP – tissue culture passage
Tg – transgenic
Tg-D^k – mouse strain transgenically expressing H-$2D^k$
Non-Tg – littermates of transgenic mice lacking expression of transgene
TLR – toll-like receptor
VCL – vehicle treatment
wk – week(s)
WT – wild type
ZBTB46 – zinc finger and Broad-complex, Tramtrack, Bric-a-brac/Poxvirus effector domain containing protein 46
z-VAD-fmk - carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone

INTRODUCTION

Preface

While their importance (or even their existence) wasn't immediately accepted, dendritic cells (DC) are now acknowledged as the preeminent antigen presenting cell (APC) of the immune system. In the 1970s, Ralph Steinman and Zanvil Cohn published a series of papers identifying and rigorously describing this novel cell type among adherent mouse splenocytes (1–5). They coined these cells "dendritic", due to their extensive, branching processes A particularly important function ascribed to dendritic cells (DC) is the potent and specialized ability to prime T cell responses (6, 7). However, other members of the field remained speculative toward the existence of this new cell type, unconvinced that it was a distinct lineage separate from macrophages. Hence, the DC field has been rife with controversy and confusion since its inception.

This is, in part, due to the rarity of the population described by Steinman and the fact that the DC adhered to glass, like the macrophages so many scientists thought were responsible for T cell priming. Skeptics did not initially accept that the dendritic cell was a unique population due to its incredibly low frequency and overlapping characteristics with other myeloid cell types. It seemed counterintuitive to some that such a small cell population would be so immunologically important, tasked with a function like T cell priming (8–10).

Interestingly, around the same time Steinman was discovering DC, another research team was pursuing a rare, briefly glass-adherent (1-4 h in culture) "accessory" cell (or A cell). This A cell was necessary for stimulating mouse spleen cells to produce antibodies against sheep red blood cells (RBC) in culture (9, 11). They eventually determined a three-part interaction was required, involving two non-adherent populations (T & B cells) and one minor, transiently adherent population (DC) (12). Later, a DC specific antibody generated by the Steinman group was used to show that A cell function was eliminated when DC were removed from the reactions, strongly indicating that these cell were identical (13–15). Interestingly, these studies from the Rowley lab were also some of the first indications that DC could be targets of natural killer (NK) cell recognition, a concept that is still under investigation today.

Currently, DC and their functions are still a subject of intense research and debate. The work by the Steinman and Rowley groups laid a strong basis for the DC field, but many of the finer points of DC biology are just now becoming appreciated. Recent advances in the field have finally defined DC as their own lineage and will be invaluable in translating our knowledge of these cells into practical use.

Dendritic cell lineage and nomenclature

Delineating DC subsets has proven a long, controversial process for the field. All DC develop from bone marrow precursors, but, unlike other immune cells, can develop from both the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) (16, 17). This quality has led to some of the inherent confusion pervasive to the DC field. Since some DC express the T cell surface marker CD8α while others express the myeloid marker CD11b, DC were originally divided into "lymphoid" and "myeloid" subtypes. This is now regarded as misleading since both CLP and CMP can give rise to $CD8^+$ and $CD8^-$ DC populations (18). DC differentiation potential is now known to be governed by expression of fms-like tyrosine kinase 3 (flt3), such that any progenitors expressing it retain some capacity for developing into DC (17). This helps explain some of the confusion, since both CLP and CMP can express flt3.

While both CLP and CMP have the potential to produce DC, *in vivo* contributions to the DC compartment appear skewed toward the CMP. Fate mapping studies have estimated that 90% of DC present in lymphoid organs (e.g. the spleen) derive from CMP (18, 19). Regardless, during early stages of differentiation, DC lineage development is more dependent on a flt3-enforced signaling program than CLP or CMP origins; hence distinction at the level of early progenitor source may be unnecessary. Differentiation from early $flt3^+$ precursors progresses to granulocyte-macrophage progenitors (GMP) followed by monocyte DC progenitors (MDP) and then the common DC progenitor (CDP). It is at the CDP level that true DC commitment has taken place, as these exclusively generate all recognized DC subtypes (16, 17).

After this stage comes the first bifurcation in DC lineages. CDP in the bone marrow develop into either plasmacytoid DC (pDC) or pre-DC (16). These populations then exit bone marrow and enter circulation to patrol the blood and seed tissues. Once pre-DC enter a tissue, they further develop into resident conventional DC (cDC). The cDC population is further comprised of distinct subsets, but studying cDC populations has presented a significant challenge since distinct tissues seems to have differing compositions of cDC subsets, with little uniformity or exclusivity in surface marker patterns. A recent advance in the field that has greatly aided the identification and study of defined cDC populations in tissues was the identification of the transcription factor ZBTB46 as a unique marker of cDC and their immediate precursors (pre-DC). This has allowed for the unambiguous identification of cDC populations throughout the body and the development of genetic tools to label or deplete these populations (20, 21).

cDC subsets

To date, cDC have been most extensively studied in lymphoid tissue, e.g. the lymph nodes and spleen. Spleen and lymph nodes have three easily discernable resident cDC subsets, but originally only two were appreciated. As mentioned above, these were defined by expression of CD8α and CD11b. These markers are still used today for defining cDC subsets in lymphoid tissue, but the terms lymphoid DC and myeloid DC no longer apply. The CD11b subset can now be broken down further into $CD4^+$ and $CD4^-$

cells. Hence, a current convention has been to define these DC subsets as CD8, CD4, and DN DC.

Along with defined patterns of surface marker expression, these three populations exhibit differences in transcription factor dependence and functional specialization. CD8α^+ DC (CD8 DC) are, so far, the most well characterized. These cells rely on the transcription factor BATF3 for steady state development (16). Mice knocked-out for the *Batf3* gene specifically lack the CD8 DC lineage of DC. Using this model, these cells have been identified as superior stimulators of CD8 T cell responses in response to viral infection or challenge with immunogenic cancer cells (22). This is, in large part, due to their specialization for cross-presentation. CD8 DC express an array of surface receptors involved in the uptake of dead and dying cells and extracellular debris (e.g. CD205, CD36, CLEC9A) (16). While material endocytosed by DC will usually enter an endocytic processing pathway for display on MHC class II, CD8 DC can shuttle this material out of phagosomes and into the cytosol for processing and display on MHC class I. This cross-presentation capacity is essential for priming CD8 T cells to antigens that would otherwise be sequestered inside of diseased or damaged cells.

CD4 DC development is now known to be reliant on lymphotoxin (LT) signaling and the NOTCH2 transcription factor (23). Mice with the *Notch2* gene knocked out have a profound deficiency in their CD4 splenic DC, but retain CD8 and DN subsets. Interestingly, these mice were also less efficient at priming CD4 T cell responses,

indicating a functional specialization of this subset for generating helper T cell responses (23). This *Notch2*-dependent population is also important for producing IL-23 and orchestrating the immune response to attaching and effacing bacteria (24).

While a specific transcription factor regulating the DN DC subset has not yet been identified, the *Notch2*-KO mice may help provide insight into their function. We do know that these cells are also separated by surface expression of the endothelial cell-selective adhesion molecule (ESAM). CD4 DC express high ESAM levels while DN DC are low for ESAM expression. Spleen DC that are $CD11b^{+}$ $CD4^{-}$ $CD8^{-}$ $ESAM^{lo}$ have exhibited greater responsiveness to toll-like receptor 9 (TLR9) stimulation, producing higher levels of IL-12 and tumor necrosis factor α (TNFα) than their $ESAM^{hi}$ counterparts (23). Hence, these cells appear more specialized for cytokine secretion than *in vivo* T cell priming, but further characterization and validation of these phenotypes are certainly required.

The delineation of these subsets is an interesting and ongoing area of work in the field. While a picture of their functional specializations is beginning to emerge, it will be additionally important to define the stimuli that influence these cells. Means of specifically activating, inhibiting, or depleting specific subsets of cDC will prove invaluable for properly tuned treatments of complex pathologies. For example, if the DN subset is indeed a superior producer of cytokines, it could contribute to pathology in sepsis-like syndromes. One study has actually shown that this DN population increases

during the cecal ligation and puncture model of sepsis and that these cells produce aberrant levels of IL-10 (25).

DC in immunity

The importance of DC in immunity is clearly illustrated by models like the *Batf3*- and *Notch2*- deficient mice, which have severely impaired resistances to virus and cancer cell challenge or intestinal bacterial infection (respectively) (22, 24). Beyond their ability to stimulate T cells, though, DC also have an important role as sentinels of the immune system (16, 26). DC subsets are specialized to sample and interrogate their environment through continuous uptake of extracellular material and expression of pattern recognition receptors (PRR) for detecting foreign material common to invading pathogens.

DC subsets are divergent in their PRR expression, which further enforces their functional specializations. pDC, which are hard wired for type I interferon (IFN-I) production in response to viruses, are well known for expressing toll-like receptors (TLR) 7 and 9 for the detection of single stranded RNA and unmethylated CpG DNA (respectively) – elements common in viral infections. CD8 DC do not express TLR7, but are the only subset to express TLR3 at high levels for the detection of double stranded RNA. CD4 DC express all TLR molecules except TLR3 and DN DC appear to have some level of expression of all TLR molecules (27, 28). Triggering of these or other PRR results

in DC maturation and a shift from sentinel to stimulator. DC cease their continuous uptake of material, secrete cytokines (e.g. IFN-I and IL-12 and 18), increase antigen processing and MHC loading pathways, and alter their cytokine receptor expression (16, 29, 30). The upregulation of the C-C motif chemokine receptor CCR7 allows DC to follow gradients of the chemokines CCL19 and 21 for migration into T cell zones of lymphoid tissue, where they can perform their role as stimulators.

While the process of DC migration into lymphoid tissue is well characterized, less attention has been paid to DC migration following T cell priming. For a long time, it was thought that DC could not or did not exit lymphoid tissue after migration. However, there are now studies that indicate DC may recirculate from lymph nodes after initial T cell priming (30–34). However, the mechanisms of this and how it impacts systemic immunity are still under investigation. An additional question is if lymphoid resident DC retain this recirculation potential. Since these cells develop in the sites of immune priming and do not need to migrate through lymph, their trafficking potential is unknown.

DC interactions with natural killer cells

Along with their ability to stimulate T cells, DC are also known to engage in intimate cross-talk with natural killer (NK) cells (35–39). In response to PRR triggering, DC produce IFN-I, IL-12, IL-18, and IL-15, all of which influence the NK response and, in

turn, DC maturation. IL-15 is critical since NK cells fail to acquire full maturity or functionality without it (40, 41). However, IL-15 by itself is not a potent activator of NK cells. The critical mechanism for NK activation via IL-15 is trans-presentation by DC. NK cells express the low affinity receptor chain for IL-15 (IL15Rβ) while DC express the high affinity chain (IL15Rα). Upon production of IL-15 by DC, they pick it up on their surface via their high affinity receptor and present it to NK cells. This produces efficient, concentrated IL-15 signaling in the NK at the interaction synapse and, interestingly, brings the DC and NK into direct contact for other potential feedback interactions. In the absence of this mechanism, NK do not mature properly during homeostasis and do not attain full functional capacity during infection (40, 41). This interaction is a very interesting point in the initiation of immune responses as there is direct contact between the DC and NK. This could be the source of immense bi-directional feedback on the two cells, with potential to shape their subsequent behavior. Interestingly, NK can acquire molecules from the surface of DC by a process termed trogocytosis, or cell eating (42). Through interactions with DC, NK cells become surface-MHC II^+, despite no internal production of transcript. IL-15 likely has a role in this as a strong mediator of cell-cell contact. Surprisingly, though, this MHC II on NK cells leads to abortive CD4 T cell priming since the NK cells do not acquire enough co-stimulatory molecules during the process (42). This could indicate that NK-DC interactions during early immune responses shape the ensuing T cell response.

In addition to this IL-15 interaction, TLR signaling stimulates DC to produce IFN-I, IL-12, and IL-18. These also signal to NK cells and reinforce their effector potential by promoting proliferation, cytotoxicity, and cytokine secretion (e.g. IFNγ and TNFα). IFNγ and TNFα, in turn, act back on DC to promote maturation into potent APC, increasing antigen processing and display, expression of co-stimulatory molecules, and migration (35, 38).

While these mechanisms are well established in the field, there are likely additional instances of NK-DC communication that we do not yet appreciate. As two cornerstones of the innate immune response, it is critical that we understand how NK and DC influence each other in the context of infection and inflammation.

Virally-induced DC suppression

Due to their essential roles as sentinels and immune stimulators, it is not surprising that a variety of viruses have evolved strategies to target DC functions. These mechanisms can manifest via virally induced proteins or direct DC infection and include: TLR inhibition (VV); disruption of antigen processing (adenoviruses, herpesviruses, HIV); inhibition of costimulatory signaling (Ebola, Lassa fever, HSV, HIV); and inhibition of cytokine secretion (HCV, HIV, measles, dengue)(43). In addition immune-suppression has been seen in the face of high levels of IFN-I driven by persistent viral infection (LCMV, measles)(43). In these instances, suppression took the form of long term

inhibition of bone marrow DC precursors, making them unresponsive to flt3 signaling and resulting in greatly reduced DC numbers (43). While IFN-I cytokines are generally protective, especially during the early stages of infection, the concept of suppressive IFN-I has gained traction over the years. For example, two reports have now shown that blocking IFN-I signaling improves clearance of persistent LCMV infection (44, 45).

The potentially suppressive effects of chronic IFN-I are clear, based on the evidence above. However, a question remaining in the field is if IFN-I can have acute suppressive effects. A 2007 study by Robbins et al. (46) observed loss of splenic DC subsets during the first 3 days of MCMV infection. MCMV is a strong inducer of acute IFN-I and the DC loss they observed correlated with levels of pDC IFNα production. This has led to the hypothesis that high levels of virally-induced, acute IFN-I can lead to DC attrition and suppression. This is an important concept and merits rigorous study. Knowledge of DC regulation during infection and the suppressive vs protective roles of IFN-I will inform clinical work on many levels. To this end, MCMV is an excellent model for studying the effects of IFN-I on DC; the main advantages being the ability to study this phenomenon in the context of a natural mouse pathogen that induces high levels of acute IFN-I.

Type I interferon signaling

Despite being comprised of multiple subtypes (14 IFNα subtypes, a single IFNβ subtype, and a variety of other single subtypes variably expressed across species), all IFN-I proteins share the same receptor. Binding the IFNAR1/IFNAR2 heterodimer activates the JAK1 and TYK2 protein kinases. These in turn phosphorylate STAT1 and STAT2 which form heterodimers and traffic to the nucleus. The STAT1 and STAT2 heterodimer binds IRF9 to form the IFN-stimulated gene factor 3 (ISGF3) complex which goes on to recognize and bind IFN-stimulated response elements (ISREs) in promoter sequences. This triggers the expression of the hundreds of downstream IFN-stimulated genes (ISGs) (47).

Aside from their initial function of interfering with viral infection, IFN-I is also known to have a variety of effects on immune cells (both positive and negative). The ultimate outcome of IFN-I sensing is governed by the cell type and context in which signaling takes place. Hence, while hundreds of genes have the potential for IFN-I-regulation, the actual pattern of gene expression will vary between cell types. This has been clearly illustrated in a study comparing the profiles of transcript modultion for NK and DC in response to IFN-I (48).

Beneficial immune cell effects that have been attributed to IFN-I include enhancing NK cell lytic capacity, NK blastogenesis, differentiation of DC from monocytes, increased antigen presentation and costimulatory functions of DC, DC migration, production of IL-15 and other cytokines, increased DC cross-presentation, and direct stimulation of T

and B cells (49–52). However, studies on IFN-I signaling have also tied these cytokines to tissue damage (53–56), aggressive leukemia (57, 58), and T cell apoptosis (59–61). The seemingly paradoxical roles of IFN-I are illustrated in reports throughout the literature. While the two LCMV studies mentioned above showed IFN-I inhibition reversed immune suppression and enhanced chronic LCMV clearance (44, 45), additional studies showed IFN-I protection of LCMV-specific CD8+ T cells from NK-cell cytotoxicity (62, 63). A similar protective effect was also seen for NK cells. IFN-I signaling allowed specifically-responding NK cell populations to expand by reducing their expression of activating ligands, protecting them from fratricide (64).

Early responses to murine cytomegalovirus

The primary targets of MCMV during the establishment of infection are splenic stromal cells, many of which are CD169-expressing marginal zone metallophilic macrophages (65, 66). Upon infection, these cells respond by producing an initial wave of IFN-I. This cytokine expression peaks between 8-10 hpi and decreases by 24 hpi (66). Interestingly, in MCMV infection, this first wave of IFN-I requires B cells and lymphotoxin α/β (LTα/β) signaling. Stromal macrophages express the LTα/β receptor and respond to the B cell-derived LTα/β, resulting in production of IFN-I. This then establishes the initial antiviral state in the surrounding environment, limiting viral replication in infected cells and decreasing the permissibility of neighboring cells to infection (67).

Interestingly, this first wave of IFN-I appears to be important for establishing a viral set point of sorts as immunocompetent mice do not exhibit significant increases in viral load between 32 and 120 hours post infection (hpi) (68). This effect is reliant, in part, on IFN-I-dependent stimulation of IFNγ production by NK cells. The effect appears to be the result of a general, cytokine-mediated NK activation rather than triggering of any specific receptors. NK depletion removes this protective effect and allows for increased MCMV outgrowth at 32 hpi.

While the initial IFN-I wave disappears 24 hpi, a second phase of IFN-I production occurs at 36-48 hpi, this time in response to MCMV completing its first replication cycle (~30 h *in vivo*)(66). At this point, IFN-I secretion is no longer dependent on the stromal cells, but rather derives largely from pDC sensing virus through TLR9, with some contribution from cDC and other cells (50, 69). DC sensing and responding to infection through PRR generate large amounts of inflammatory cytokines during this phase, including IFN-I, IL-6, IL-15 and IL-12/IL-18 (38, 70). This wave of cytokine production contributes to another round of NK activation, marked by enhancements in cytotoxicity and proliferation along with production of cytokines. At this point, NK cells that can specifically respond to virus play a large role in limiting the infection. Generally, if mice lack an NK population capable of specific MCMV-recognition, virus levels remain elevated and an environment of immune suppression is established (38).

Specific NK responses to MCMV

The first genetic resistance factor identified for MCMV turned out to be an NK cell activating receptor, Ly49H (71). This activating receptor directly recognizes the virally encoded protein m157, which is an MHC class I mimic displayed on the surface of infected cells (72, 73). So far, B6 mice are the only strain identified with expression of this protective Ly49H, but mice can be made resistant to MCMV infection by transferring $Ly49H^+$ NK cells or through transgenic expression of the Ly49H protein.

Another model of robust NK-mediated viral control has been previously developed by our lab. In contrast to Ly49H, resistance in this system is dependent on MHC class I (H-2 in mice) genotypes. Historically, mice of the $H\text{-}2^k$ haplotype have exhibited increased resistance to MCMV compared to other backgrounds (74). Work performed by our lab refined the genetic locus to a region encompassing the $H\text{-}2D^k$ (D^k) molecule and then showed D^k itself to be the resistance factor (75–77). Transgenic expression of D^k on a susceptible background transfers the resistance effect (78). Although an MHC class I molecule was identified as the resistance factor, virus control is mediated through NK cells expressing the Ly49G2 inhibitory receptor (77). Depletion of this subset using mAb specific for Ly49G2 (either 4D11 or AT8) abrogates the protective effect of D^k (77). Since D^k is a cognate ligand of Ly49G2, the two interact during development of the NK cells and during homeostasis. Hence, when NK inhibitory receptors are engaged in this fashion, they become educated and increase their effector output upon stimulation of their activating receptors (79–81). Therefore, the hypothesis is that education on D^k

generates functionally potent Ly49G2^{+} NK cells, capable of recognizing alterations in D^{k} expression during viral infection. This recognition event would then allow activation to proceed and unleash the effector activity of the Ly49G2^{+} NK.

While these two models of NK-mediated resistance differ greatly in their mechanism and complexity, the end result is an ability to rapidly detect and limit MCMV infection in an NK-dependent fashion. An interesting question that arises is how these specifically responding NK cell subsets affect surrounding cells, like DC, and the ensuing immune response.

Eye toward the future - vaccinology

It is clear that DC, IFN-I, and NK cells are intimately connected. Their dynamic interactions orchestrate the initial response to viral infection and set the stage for downstream immunity. Hence, the more we know about these interactions and the mechanisms that regulate them, the better equipped we will be for generating clinical interventions to effectively and safely exploit them. Looking back at the first DC publication from Steinman, "Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution" (1) and comparing it to one of his final publications, "Classical Flt3L-dependent dendritic cells control immunity to protein vaccine" (82) really highlights the progression and current state of the field. Advances in our understanding of the DC lineage have allowed us to move forward and

ask more detailed questions about their role in immunity. A focus one vaccine technology has come to the forefront in recent years and much work with DC is trending that direction. This is true for NK cells as well and special attention is being given to the potential of NK-DC crosstalk for enhancing vaccine technology (83–89).

Significance

Dendritic cell (DC) regulation has profound impacts on health and disease. Their intimate relationship with T cells makes them important targets for both dampening autoimmunity and triggering protective immunity. In order to fully realize the therapeutic potential of this rare cell population, it is imperative that we better understand the mechanisms regulating its development, survival, activation, and function. With this in mind, the original work discussed in the body of this dissertation had two main goals. The fact that DC are lost from the spleen during murine cytomegalovirus (MCMV) infection provided us with an excellent model for studying DC attrition with the goal of better defining elements negatively regulating their numbers. As a corollary to DC loss, we also observed rapid reconstitution of DC in mice that mount a licensed NK cell response to MCMV. This presented us with the opportunity to investigate elements involved in DC recovery and accumulation. The second main goal was, therefore, to determine if the NK cell itself was providing signals that positively regulate DC numbers. This work advances our knowledge of DC regulation in general, providing insight into the circumstances surrounding their loss,

preservation, and expansion. The basis provided here can be built on in future studies to generate a clearer picture of DC behavior and further their clinical utility.

Materials & methods

Mice

Characteristics of mouse strains important to the studies in this dissertation are summarized in Table 1. C57L-derived R2, R7, and R12 MHC-congenic strains were previously generated and described (77). Briefly, C57L and MA/My inbred strains were crossed using a speed congenic strategy to generate MHC-congenic strains. Mice were selected for H-2^k alleles from MA/My on the C57L background. Mice congenic for the H-2^k interval were then further intercrossed to refine the genetic locus of interest, generating intra-H-2^k recombinant (R) strains on the C57L background. The relevant recombinant strains used here are C57L.M-*H2*^{k}(R2), C57L.M-*H2*^{k}(R7), and C57L.M-*H2*^{k}(R12). Here, MCMV-susceptible C57L (L) and H-2^k-recombinant 2 (R2) are collectively referred to as D^b as these mice express this class I allele rather than the protective D^k. MCMV-resistant R7 and R12 are, conversely, collectively referred to as D^k. C57L.Tg3-D^k were generated and described previously (78). C57Bl/6 mice (B6) were purchased from The Jackson Laboratory and maintained in house at University of Virginia. IFNAR-KO mice (B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax) were obtained from the Mutant Mouse Resource & Research Centers (MMRRC). CD45.1 mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were purchased from The Jackson Laboratory. B6.Cg-*Nkc*^{c57l} (referred to as *NKC*^{l}) congenic mice were generated by introgressing a C57L natural killer gene complex (NKC) into the B6 background using a speed congenic approach described previously (77, 90). The C57L NKC lacks *Ly49h* gene expression and, therefore, Ly49H-dependent

Table 1. Mouse strains relevant to present studies and licensed NK cell MCMV resistance. Cg-*Nkc*^{m} mice are L background animals congenic for the NKC region of the MA/My genome. Likewise, Cg-*Nkc*^{l} represents mice congenic for the NKC from the C57L genome. Abbreviations are as follows: B6 (C57Bl/6), L (C57L), M (MA/My), NKC (natural killer gene complex), Cg (congenic), Tg (transgenic). For resistant strains, elements important for resistance are represented in bold.

Strain	MHC-I	NKC	CMV phenotype
C57Bl/6 (B6)	K^b; D^b	B6 (**Ly49H+**)	**Resistant**
MA/My (M)	K^k; **D^k**	M (**Ly49G2+**)	**Resistant**
C57L (L)	K^b; D^b	L (Ly49G2+)	Susceptible
R2	K^k; D^b	L (Ly49G2+)	Susceptible
R2.Cg-*Nkc*^{m}	K^k; D^b	M (Ly49G2+)	Susceptible
R7	K^k; **D^k**	L (**Ly49G2+**)	**Resistant**
R7.Cg-*Nkc*^{m}	K^k; **D^k**	M (**Ly49G2+**)	**Resistant**
R12	K^b; **D^k**	L (**Ly49G2+**)	**Resistant**
L.Tg-D^k	K^b; D^b; **D^k**	L (**Ly49G2+**)	**Resistant**
B6.*Nkc*^{l}	K^b; D^b	L (Ly49G2+)	Susceptible
B6.D^k.Cg-*Nkc*^{l}	K^b; D^b; **D^k**	L (**Ly49G2+**)	**Resistant**
B6.Tg-D^k	K^b; D^b; D^k	B6 (**Ly49H+**)	**Resistant**

MCMV resistance. B6.Tg-D^k and B6.Tg-D^k.Cg- *NKC*^{l} were generated using the same speed congenic approach. *NKC*^{l} and IFNAR-KO mice were crossed to generate litters of *NKC*^{l} mice that were either homozygous, heterozygous, or lacking the *Ifnar1*^{tm1Agt} mutation. RIP3-KO mice (B6-*rip3*$^{-/-}$ *casp8*$^{flox/flox}$) were a kind gift from Dr. John Lukens (91–93). No cre recombinase expression was present in our experiments so these mice are functionally deficient for RIP3 with intact caspase 8 activity. Mice were bred and maintained under SPF conditions at the University of Virginia. All protocols were approved by the IACUC.

Virus stock preparation

Stock virus (Smith strain, VR-1399) was obtained from ATCC. NIH 3T3 cells were infected with stock virus (MOI 0.01) and incubated for 5 d. Culture supernatant was collected and clarified by centrifugation at 2000 xg for 10 minutes (4 degrees C). This tissue culture passage 1 stock (TCP1) was used to infect female BALB/c weanlings ($5x10^4$ PFU). Salivary glands (SG)were harvested from the BALB/c mice 19 dpi. SG were dounce homogenized to release virus followed by centrifugation (2000 xg, 10 minutes, 4 degrees C). Supernatant was collected from between the pellet and upper fatty layer and stored in liquid nitrogen or at -80 degrees C. The *in vivo* salivary gland passaging was performed three more times to generate high titer salivary gland passage 4 (SGP4) working stock. This was then used for experimental infections.

To titer virus stocks, NIH 3T3 and M2-10B4 cells were used in separate assays. Cells were seeded into 24- or 6-well plates followed by the addition of multiple dilutions of virus stock spanning 10^{-1} to 10^{-7}. Virus dilutions were added to the cells and allowed to infect in low volume for 1-2 h. A methylcellulose overlay was then added to the wells and the plates were incubated for 5 d at 37 degrees C. After 5 d, the supernatant was removed and cells were fixed in 1% paraformaldehyde for 1-2 h. Cells were then stained with methylene blue and plaques counted under a microscope. Working stocks were tittered at least twice.

Infection and treatments

Mice were i.p. infected with salivary gland passaged MCMV as indicated by figure legends. Antibody treatments are summarized in Table 2 and were performed as follows. For immunodepletion of pDC, mice were i.p. injected 2x with 250 μg mAb 927 (kindly provided by Marco Colonna) 48 and 24 h prior to infection. For immunodepletion of G2+ NK cells, mice were i.p. injected with 200 μg mAb 4D11 (hybridoma kindly provided by Wayne Yokoyama) 48 h prior to infection. For blocking type I IFN receptor, mice were i.p. injected with 1 mg mAb MAR1-5A3 (Leinco Technologies, Inc.) before infection and 500 μg every 24 h thereafter. For blocking Ly49H receptors, mice were i.p. injected with 200 μg mAb 3D10 (hybridoma also provided by Wayne Yokoyama) 24 h prior to infection. For IL-6 blockade, mice were i.p. injected with 300 μg mAb MP5-20F3 (Bio X Cell) ~18 h before infection with 2 boosts during infection at 1 and 2 dpi. For NK depletion, mice were i.p. injected with 200 μg mAb PK136 (in

Table 2. Antibodies used for *in vivo* treatments

mAb Clone	Specificity	Used for
3D10	Ly49H	Blockade
4D11	Ly49G2	Depletion
927	mPDCA	Depletion
MAR1-5A3	IFNAR	Blockade
MP5-20F3	IL-6	Blockade
PK136	NK1.1	Depletion
N418	CD11c	*in vivo* labeling

house hybridoma) 48 h prior to infection. For *in vivo* CD11c labeling, mice were i.v. injected at 2 dpi with ~10 μg mAb N418 conjugated to PE (BioLegend) for either 24 h or 30 minutes. For TLR stimulations, mice were i.p. injected with 200 μg poly I:C (Amersham Biosciences) or imiquimod (Ivivogen), or i.v. injected with 75 μg CpG-A or CpG-B (Invivogen; ODN 2216 and 1668 respectively). Dosages were based on literature searches for biologically effective *in vivo* treatments performed in mice (CpG-A (94); CpG-B (95); imiquimod (96, 97); poly I:C (98, 99)). Vehicle treated mice received 200 μl of a 3:1 mix of endotoxin free water and PBS i.p.

Bone Marrow Chimeric mice

Bone marrow (BM) transplantations with L.Tg-D^k mice were performed as described previously (100). For IFNAR-KO chimeras, B6 mice were irradiated 2x with 5.5 Gy (11 Gy total) 2 h apart prior to i.v. injection of ~4.5 x 10^6 donor BM cells (CD45.1 IFNAR-WT, CD45.2 IFNAR-KO, or 1:1 mix). Recipient mice were maintained on sulfate drinking water for 3 wk following irradiation. Peripheral blood analysis was performed at 4 wk to verify chimerism. After 8 wk, mice were 3D10-blocked to impede Ly49H-mediated MCMV resistance followed by 3 d infection and analysis of splenocytes.

Serum Transfer

Donor B6 mice were 3D10 treated to block Ly49H-mediated NK resistance 24 h prior to infection with 5x10^4 PFU MCMV or mock infection with PBS. At ~40 hpi, blood was collected from the donor mice via post-mortem cardiac puncture and allowed to clot at 4

degrees C for 5 h. Serum was isolated from blood by centrifugation at 11,000 xg for 8 minutes. Serum fractions were pooled within groups (CMV and PBS) and the total volume transferred into naïve, untreated recipient B6 mice (~250 μl/mouse).

Tissue processing for single cell suspensions

Single cell suspensions for DC analysis were performed essentially as described previously with some slight variations (101). Spleens were collected from mice and placed into tubes containing Clicks media which had been pre-weighed. The difference in mass of tube+media (pre-harvest) and tube+media+spleen (post-harvest) was calculated to determine the total mass of the spleen ("spleen mass"). For some experiments, pieces of the spleen were removed for either tissue sectioning or DNA isolation. If any portions of the spleen were removed, the remaining portion to be prepared for flow staining was re-weighed to obtain the "prep mass". Samples were transferred into labeled 6-well plates and the spleens were disrupted by grinding the capsule against the bottom of the well with the back-end of a 5-ml syringe plunger. Disrupted spleen tissues were digested with collagenase D (Roche) for either 30 minutes at 37°C or for 1 h on ice and processed into single cell suspensions by grinding tissue through 70 μm Falcon cell strainers. Suspensions were centrifuged at 400 RCF for 4 minutes at 4-7°C and the pellet resuspended in 1ml RBC lysis buffer per spleen (made in house; 90ml 0.16M NH_4Cl + 10ml 0.17M Tris) for 8-10 minutes. Following incubation, the buffer was diluted with 1ml Clicks media per 1ml buffer and the cells pelleted again (400 RCF, 4min, 4-7°C). The RBC-free pellet was resuspended in 5 ml sorting buffer (SB)

per spleen and filtered through nylon mesh 3x to remove any debris from RBC lysis. Other organs were harvested and processed in essentially the same fashion. Samples were counted using a hemocytometer, generally using a 1:25 or 1:50 dilution in trypsin, and the number of cells in the prep were determined. These values were used to determine the number of total splenocytes as follows:

cells/ml = (hemocytometer count/# squares counted) x dilution factor x 10^4

cells/sample = (count/# squares) x d.f. x 10^4 x 5ml

cells/g tissue = "cells/sample"/"prep mass"

cells/spleen; total splenocytes = "cells/g tissue" x "spleen mass"

Flow cytometry

Cells were Fc-blocked with mAb 2.4G2 and then surface stained with mAbs purchased from eBiosciences, BioLegend, BD Pharmingen, and Miltenyi. This included: 2G9 (MHC II), 53-6.7 (CD8), MAR1-5A3 (IFNAR1), 145-2C11 (CD3), 6D5 (CD19), P84 (SIRPα), N418 (CD11c), HL3 (CD11c), 129c1 (mPDCA/BST2), M1/70 (CD11b), GK1.5 (CD4), RA3-6B2 (B220), 53-2.1 (Thy1.2), A20 (CD45.1), 104 (CD45.2), and 30-F11 (pan-CD45). LIVE/DEAD® Viability Dye (Invitrogen) was used for dead cell exclusion. Samples were run on a BD Canto II with Diva acquisition software followed by analysis in FlowJo. For calculating DC numbers, we used counts obtained from our flow cytometry gating. The number of events from the final gate of interest (i.e. CD8 DC) was divided by the number of events in the "live splenocyte" gate to obtain the frequency of the population of interest within live cells. This was then multiplied by the total number of splenocytes

(i.e. "cells/spleen") to obtain the total number of cells of interest that were present in the spleen. An example follows:

("event count CD8 DC"/"event count live cells") x 100 = frequency CD8 DC in total cells

"cells/spleen" x (CD8 DC frequency/100) = total number of CD8 DC/spleen

MACS cell sorting

Magnetic columns and kits for cell sorting were purchased from Miltenyi. Positive selection of DC was performed by incubating splenocytes with magnetic beads conjugated to an anti-CD11c mAb and passing the solution through an automacs apparatus, according to the manufacturer's instructions.

DC culture

CD11c-sorted DC were pelleted and re-suspended in culture media (DMEM + 10% FCS, 1% HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.05 M β-mercaptoethanol). DC were seeded into a 24-well plate at $3x10^5$ cells/well. Cells were incubated at 37 degrees for 5 h in 1 ml media containing either poly I:C (50 μg/ml) or MCMV (10 MOI). Following incubation, cells were surface stained and analyzed by flow cytometry.

PCR for virus levels

Small snips of organ tissue were incubated overnight with PK and cell lysis buffer. The following day, genomic DNA was isolated from the tissue snips using a Gentraprep kit

according to the manufacturer's instructions. Quantitative real-time PCR was performed with the genomic DNA as described previously (102) to determine the ratio of viral genomes (IE1 amplicons) to cellular genomes (β-actin amplicons) within the tissue.

IFNα ELISA

Blood was collected via tail bleed or post-mortem cardiac puncture. Serum was isolated from blood and IFNα quantitation performed with VeriKine Mouse IFN Alpha ELISA kits (PBL) according to the manufacturer's instructions.

Statistical analysis

Significance was determined using GraphPad Prism software. Student's *t*-test was used for data sets containing two groups. For more than two groups ANOVA was performed followed by the Holm-Šidák post hoc test unless otherwise noted. Significance is represented as follows: *ns* = not significant, * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). Error bars denote SD.

CHAPTER II – Impact of IFN-I on splenic cDC subset loss

Introduction

Dysregulation of dendritic cells (DC) is a common evasion strategy seen in a variety of viral infections. One mechanism of such interference is outright loss of dendritic cell populations at key sites of immune cell priming, e.g. the spleen. Investigators have seen this phenotype with TLR3, 4, or 9 stimulations as well as viral infections, like MCMV and LCMV (46, 98, 103–106). While the exact cause of DC loss is still unclear, toxicity from overproduction of type I interferons (IFNα/β; IFN-I) has been touted as a likely candidate. This was first suggested using a congenic model of MCMV susceptibility/resistance (46). BALB/c mice are highly susceptible to acute MCMV infection and produce large amounts of IFN-I 36 hours post infection (hpi). The source of IFN-I at this early time point was traced to plasmacytoid DC (pDC). However, when the B6 natural killer gene complex (NKC) was introgressed onto the BALB/c background, mice were able to control MCMV titers far more efficiently and their pDC apparently produced far less IFN-I as a result.

This benefit was due to expression of the B6-derived activating receptor, Ly49H, on NK cells – endowing them with the ability to recognize and respond to infected cells. In Ly49H-dependent MCMV resistance, Ly49H$^+$ NK cells (H+ NK) specifically recognize expression of the MCMV-encoded antigen m157 on infected cells (71). Since Ly49H is an activating receptor, this direct recognition strongly stimulates NK cells and elimination of the infected cell proceeds.

In conjunction with the resulting Ly49H-mediated virus control and reduced inflammation, it was observed that cDC populations were essentially preserved. The authors also observed DC loss in resistant mice upon administration of physiological amounts of exogenous IFN-I during MCMV infection, although loss in this setting was still not as severe as that seen in the susceptible counterparts. These data led to the postulate that heightened IFN-I production was driving DC loss.

Since then, this idea has been further investigated in two additional reports with slightly conflicting results (48, 98). In one of the studies, poly I:C-driven DC loss was dependent on IFNAR expression. Further analysis suggested that loss could be prevented by simultaneously knocking out multiple BH3 pro-apoptotic molecules, but no single knockout could rescue DC numbers (98). This led them to conclude that IFN-I signaling was driving DC toward death via a coordinated increase in apoptotic signals from BH3 family molecules. In the other report, mRNA profiling of DC with or without IFNAR expression following MCMV infection showed that cells with IFNAR upregulated both pro- and anti-apoptotic pathways, but downregulated gene expression associated with active cell cycle. The authors also observed a reduction in DC precursors following MCMV infection in mice with an intact IFNAR response. Hence they proposed that DC loss may not be due to direct IFN toxicity, but rather to an inability to repopulate the cells after turnover (48). There is, however, another study that conflicts with this analysis (107). This work showed that, while DC turnover is indeed increased in response to IFN-I stimulation, production of DC from IFNAR-KO bone marrow was less efficient than $IFNAR^{+}$ bone marrow. Therefore, it may not be expected that precursors without IFNAR

expression would be able to efficiently restore the DC compartment. Here we further explore the impact of IFNAR signaling on DC populations during MCMV infection.

Results

MCMV infection induces loss of splenic DC in C57L-background mice

Due to the apparent benefit of H+ NK cells on MCMV control and DC maintenance, we postulated that another model of NK-mediated viral control would display similar DC preservation. We explored this using a genetic system previously developed in our lab. In this system, virus control is dependent on MHC-I expression. Mice expressing the MHC-I molecule H-2D^k (D^k) exhibit superior MCMV resistance compared to those expressing a H-2D^b (D^b) allele (76–78). The MHC-I-dependent MCMV resistance is also dependent on NK cells; particularly, NK cells expressing the inhibitory receptor Ly49G2 (G2+ NK). Depleting NK cells generally or G2+ NK cells specifically abrogates the protective effect of D^k. G2 receptors specifically recognize and bind D^k as a cognate ligand (108), and we have previously shown that G2+ NK cells are licensed as a result of this interaction (109). G2+ NK cells that have been educated by D^k during development produce greater amounts of IFNγ in response to activating receptor triggering compared to NK cells from the same environment that lack G2 expression, i.e. the cells are licensed. Presumably, D^k-mediated licensing primes G2 NK cells to specifically recognize and kill MCMV-infected cells. While we have not yet fully worked out the mechanism

underlying G2-recognition, we envision that infection results in a disruption of G2 binding to D^kat the surface of infected cells, leading to activating receptor triggering and aggressive attack by G2+ NK.

Previously, we showed splenic DC numbers declined by 2 days after MCMV infection in both D^k (MCMV-resistant) and D^b (MCMV-susceptible) C57L-derived mouse strains (109). However, attempts to replicate this timing did not exhibit reliable DC loss at 2 days post infection (dpi). Although we did not detect DC loss at d 2, extending the infection to 3 d revealed reduced numbers of splenic DC in the spleens of D^b mice (Figure 1A). Interestingly, the CD8 DC subset appeared most strongly affected, compared to CD11b DC. An extended time course analysis in resistant D^k mice also showed that these mice exhibit significant DC attrition between 54 and 72 hours post infection (hpi), again primarily due to CD8 DC loss (Figure 1B).

We were puzzled by the differences in loss severity and timing between our previous and current results and speculated that the potency of our virus stocks may have differed. Thus we acquired new MCMV stock virus from ATCC (VR-1399) and generated fresh, high titer, salivary gland stocks serially passaged 4 times through female BALB/c weanlings. The titer of this new stock was rigorously quantified by performing multiple plaque assays.

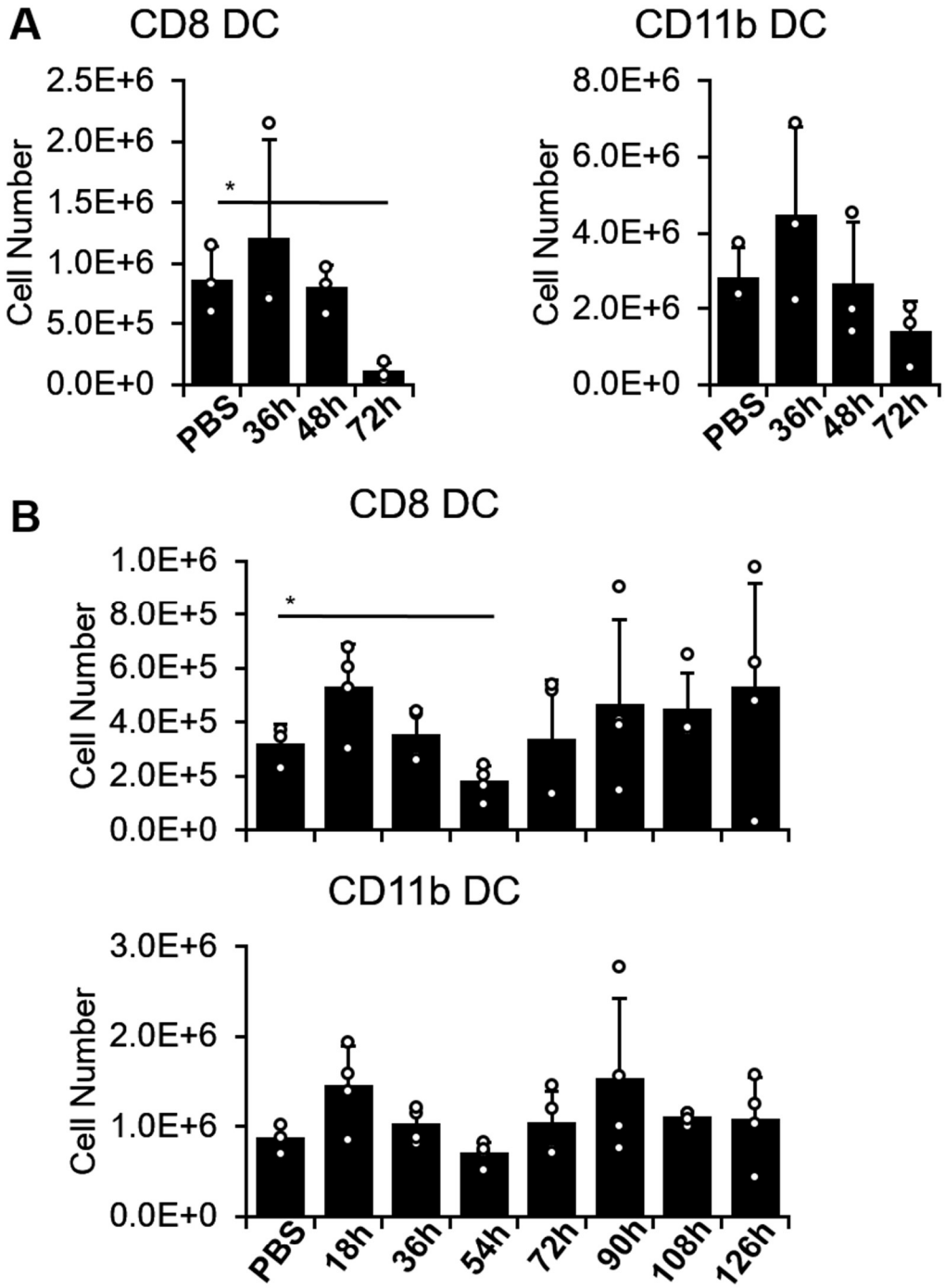


Figure 1. *DC numbers decrease 3 days post MCMV.* **(A)** D^b mice (R2) were infected with $2x10^4$ PFU MCMV for 36, 48, or 72 h. Splenocytes were analyzed by flow cytometry for DC populations. **(B)** D^k mice (R7 and R12) were infected with $1x10^4$ PFU MCMV for 18, 36, 54, 72, 90, 108, and 126 h. Splenocytes were analyzed by flow cytometry for DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

Next, we performed a biological titration by infecting D^k mice with multiple doses and assessing DC retention at 3 dpi. The degree of loss appeared to be somewhat dose dependent, with an intermediate dose of $5x10^4$ PFU producing reliable loss of DC subsets in D^k mice (Figure 2). These results informed additional experiments with D^k and D^b mice. Intermediate dose MCMV infection ($5x10^4$ PFU) resulted in a loss of cDC by 3 dpi in both D^k and D^b spleens (Figure 3). Interestingly, though, in these experiments the degree of loss in D^k mice was still less profound than that observed in D^b animals, which is in slight contrast to our previous findings (109). These results indicated that G2+ NK cells could actually provide some level of early protection to DC. Regardless, significant loss was detected in both strains, which is an apparent departure from observations made in mice with H+ NK-mediated MCMV control. Mice with H+ NK cells exhibit near complete protection of their splenic DC (46, 106, 110). Another interesting observation from this study is that loss of DN DC was minimal compared to CD4 and CD8 DC subsets, a distinction that has not yet been appreciated in the field.

Ly49H prevents DC loss in B6-background mice

We wanted to investigate the patterns of splenic DC loss in resistant and susceptible B6 strains for comparison to our resistant and susceptible mice. Hence, B6 mice were infected with $5x10^4$ PFU of our new MCMV stock for 3 d. As noted above, B6 mice can be made susceptible to MCMV infection by interfering with Ly49H-mediated recognition of infected cells. Thus we compared Ly49H-neutralized B6 mice, that had been treated with the Ly49H-blocking mAb (3D10), to either isotype treated or

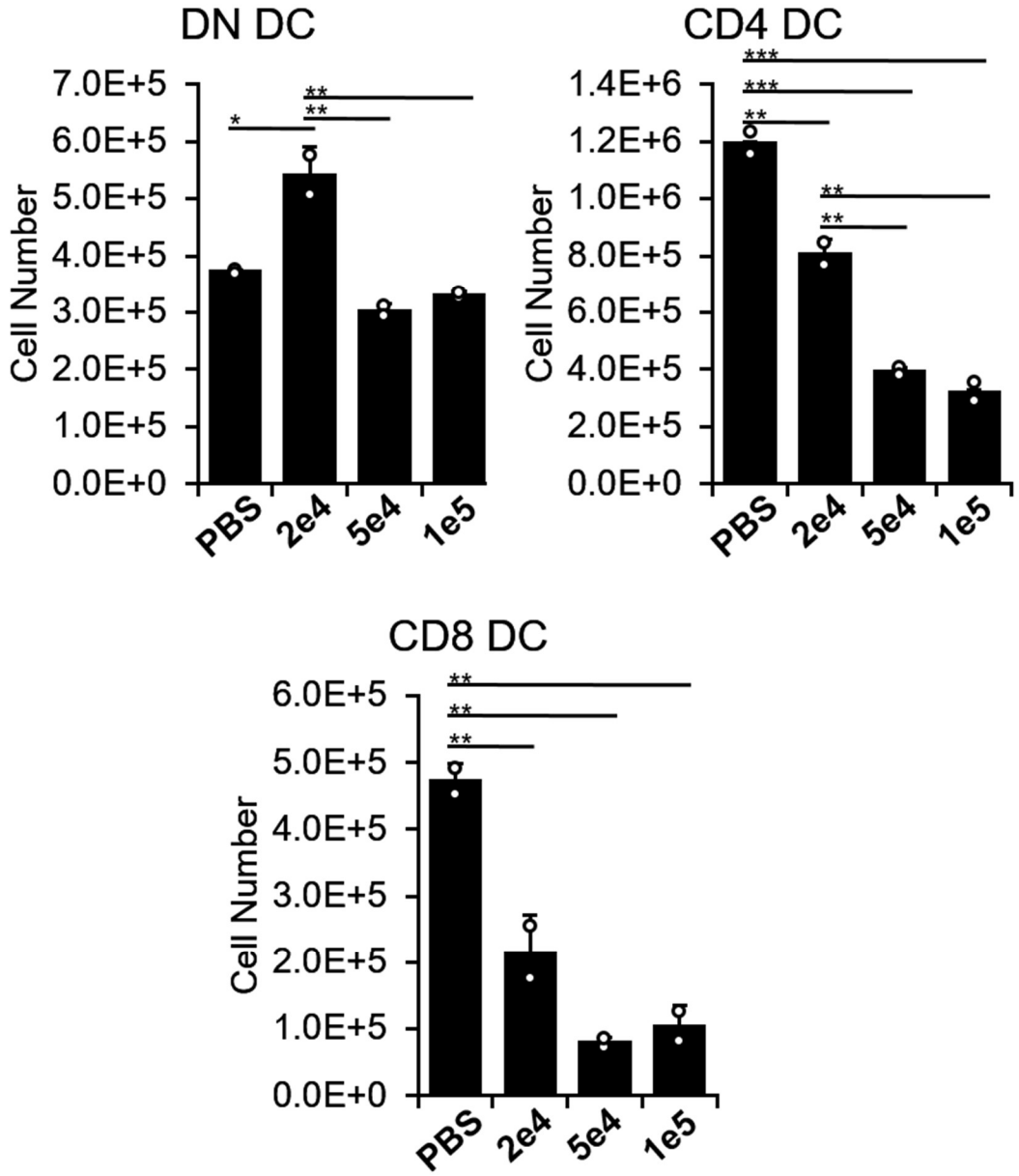


Figure 2. *Loss of DC numbers is dose sensitive.* D^k mice (R7) were infected with $2x10^4$, $5x10^4$, or $1x10^5$ PFU MCMV for 3 d. Splenocytes were analyzed by flow cytometry for DC subsets. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

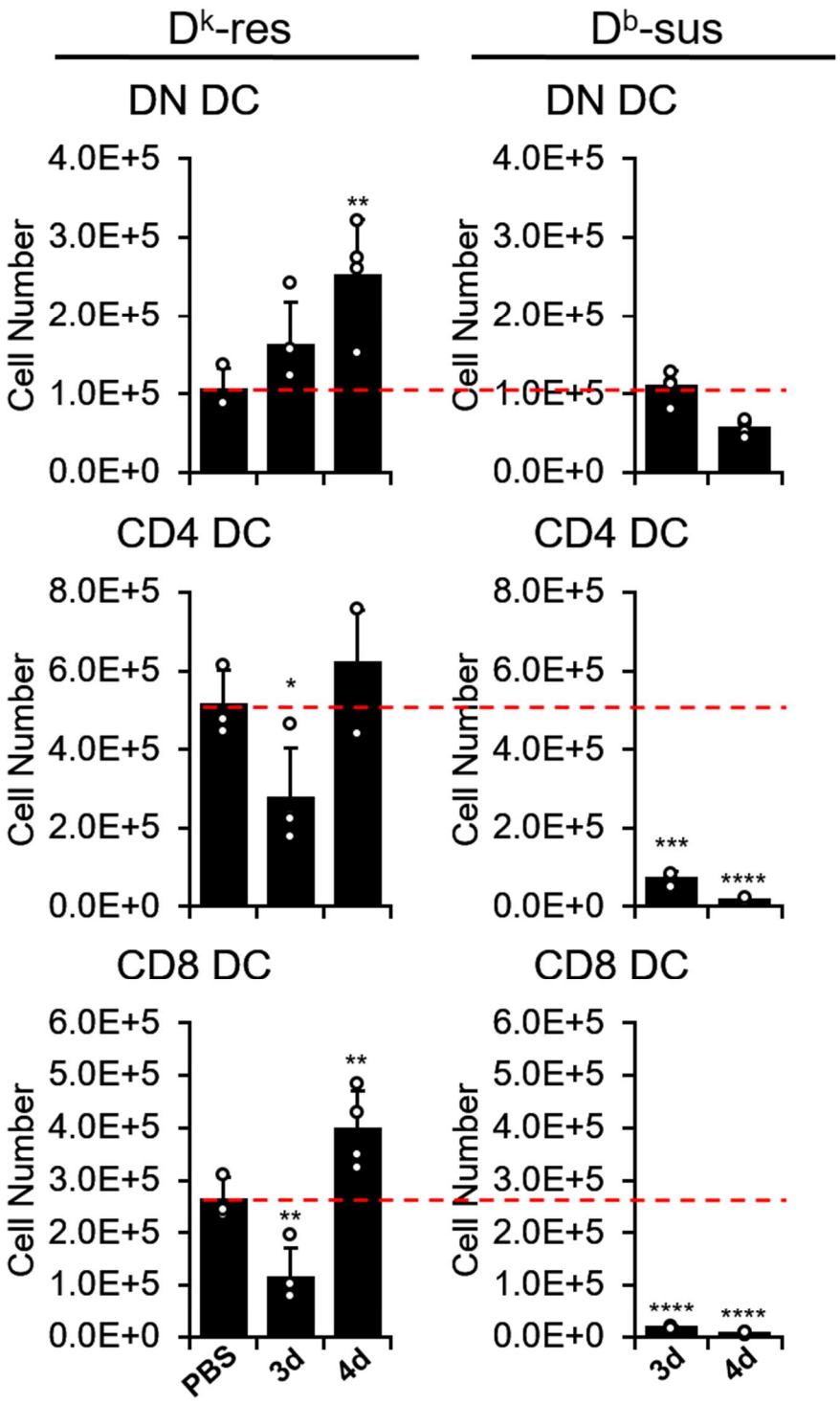


Figure 3. *DC loss is more severe in susceptible D^b mice.* D^k (R7) and D^b (R2) mice were infected with $5x10^4$ PFU MCMV for 3 d. Splenocytes were analyzed by flow cytometry for DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ and represents difference from PBS group (Dunnett's test). Error bars represent SD. Red lines indicate numbers from mock infected.

uninfected B6. In addition, we analyzed DC loss in B6 mice congenic for the C57L natural killer gene complex (NKC). Since Ly49H is encoded by the B6 NKC, replacing this region with the C57L NKC abrogates Ly49H expression on NK cells in this B6.Cg-*NKC*^{c57l} strain (hereafter NKC^{l}).

We observed that B6 mice made susceptible to MCMV exhibited significant loss of DC populations but, as expected, there was no significant loss in isotype treated mice compared to uninfected B6 (Figure 4). This is in contrast to what was observed with D^{k} mice after infection with the same virus stock, virus dose, and time point (Figure 3), highlighting the divergence between these models of MCMV control. Interestingly, the 3D10 and NKC^{l} mice exhibited significant loss of DN DC in the B6 background and the mIgG control mice exhibited significant loss of CD4 DC despite protection of CD8 and DN subsets. The implications here are that additional background genetic factors may influence some DC subsets during MCMV infection. While CD4 DC and CD8 DC subsets appear universally sensitive to MCMV-induced suppression, CD4 DC may ultimately be the most sensitive. On the other hand, DN DC sensitivity to loss may exhibit more variation between strains. Overall, though, the data suggest DC loss is a common occurrence in MCMV infection that occurs across multiple mouse strains.

IFN-I is elevated in serum of C57L-strain mice

Given the divergence in DC protection between resistant D^{k} and resistant B6, we sought to further investigate the underlying source of DC loss during MCMV infection. Ly49H

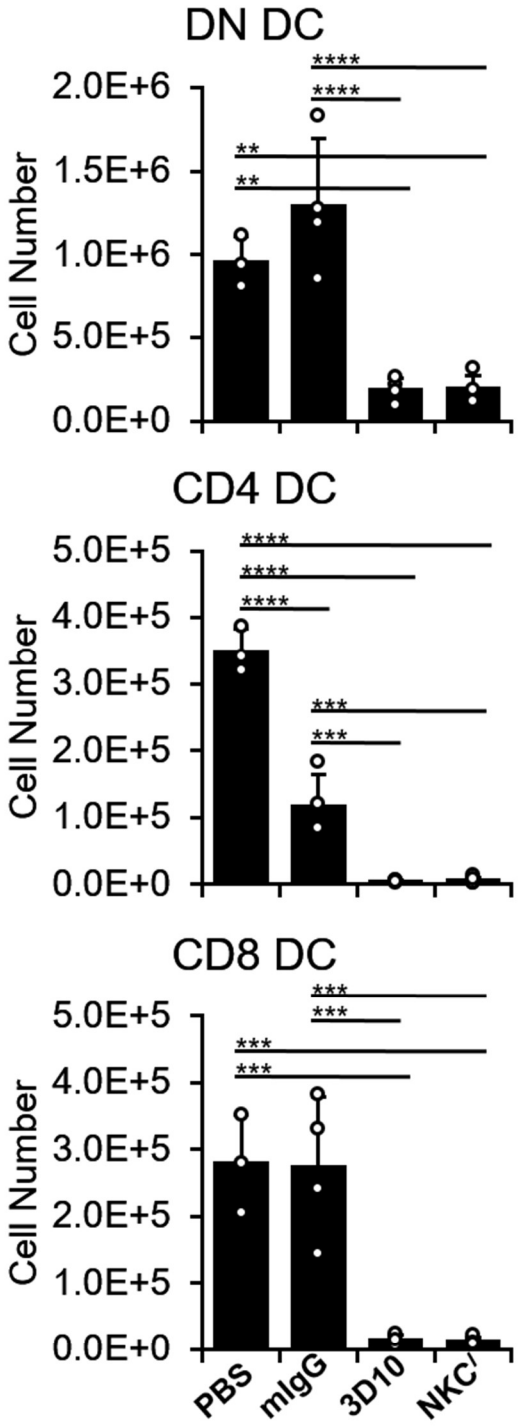


Figure 4. *Resistant B6 mice maintain DC populations*. B6 mice were infected with $5x10^4$ PFU MCMV for 3 d. Groups received either isotype control (mIgG), Ly49H blockade (3D10), or no treatment ($NKC^{-/-}$) prior to infection. PBS denotes mock infected controls. Splenocytes were analyzed by flow cytometry for DC subsets. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

expression and virus control have been associated with greatly reduced IFN-I levels during the first 36 hpi (46), but we had not yet investigated this in mice with or without G2+ NK-mediated MCMV control. Hence, IFN-I was a strong candidate since several studies invoke a role for these cytokines during splenic DC loss (46, 103, 107, 110). To determine if IFN-I toxicity could explain DC loss in resistant, D^k-expressing mice, we first interrogated IFNα levels in serum 36 hpi. This time point has been identified as a peak of inflammatory cytokine production during MCMV infection, particularly for IFN-I expression (66). Upon isolating serum and testing IFNα concentration via ELISA, we observed high levels in samples from both D^k and D^b mice at 36 and 48 hpi (Figure 5). These data suggested IFN-I toxicity could indeed explain MCMV-induced DC loss observed across resistant and susceptible C57L-derived strains.

Reducing IFN-I levels does not rescue splenic cDC subsets

To further interrogate the role of IFN-I toxicity in DC loss using our mouse model. To address this, early IFN-I levels were reduced by immunodepleting pDC before infection. We hypothesized that, since pDC are the major source of IFN-I at 36 hpi (69), depleting this population would greatly reduce the overall concentration of these cytokines and prevent them from reaching levels toxic to cDC. We treated mice with a pDC-depleting mAb, clone 927, before infection and verified that this was an effective strategy to deplete these cells for the time frame of interest. We observed a near complete absence of pDC for at least 24 h following the depletion treatment (i.e. the time of infection) and pDC frequency was greatly reduced at 4 dpi (Figure 6A). Furthermore, pDC depletion

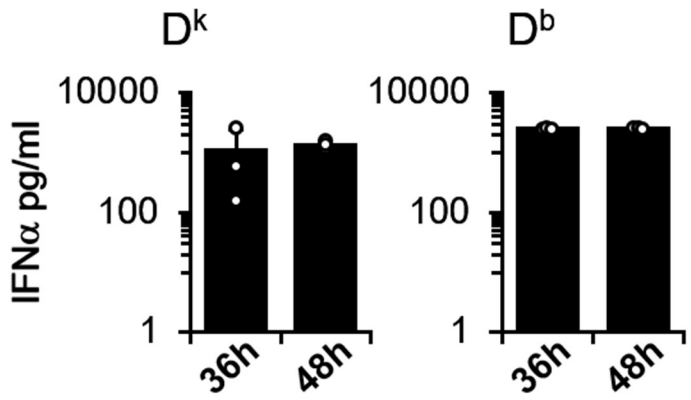


Figure 5. *IFNα is highly induced by MCMV in D^k-res and D^b-sus mice.* D^k and D^b mice (R7 and R2 respectively) were infected with $1x10^4$ PFU MCMV for 36 and 48 h. Serum was isolated and analyzed for IFNα concentration via ELISA. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

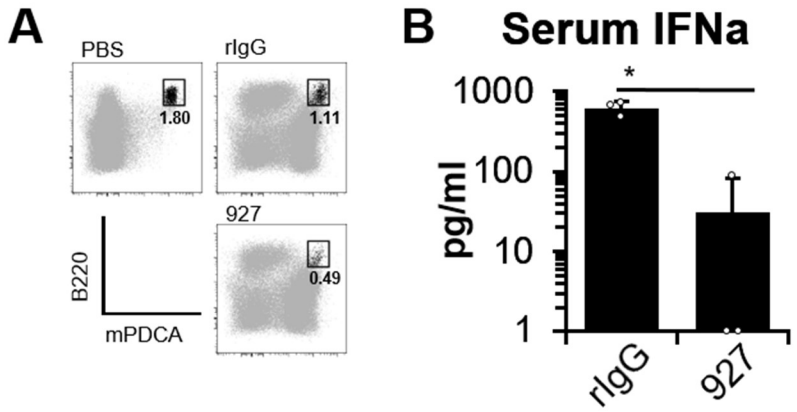


Figure 6. *pDC depletion greatly reduces serum IFNα.* D^b mice (R2) were treated with 927 pDC depleting mAb and infected with $2x10^4$ PFU MCMV. **(A)** Splenocytes were analyzed by flow cytometry for pDC frequency 4 dpi. **(B)** Serum was isolated 36-48 hpi and analyzed for IFNα concentration via ELISA. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

significantly reduced serum levels of IFNα at 36 hpi (Figure 6B). When spleens were analyzed for cDC populations at 4 dpi, the results were slightly variable between subsets. We observed similarly low numbers of CD8 DC from depleted and control rIgG treated mice, indicating a distinct lack of protection (Figure 7). Interestingly, loss of the CD11b compartment appeared less profound following pDC depletion, although this effect was slightly variable between experiments. This indicated IFN-I may have differential influence on DC subset numbers during infection (Figure 7). The data suggested that IFN-I was not a necessary factor for MCMV-induced loss of all splenic DC subsets, especially with regard to CD8 DC.

Blockade of IFN-I signaling fails to protect CD8 and CD4 DC subsets

The potential requirement for IFN-I signaling was further explored by obtaining a mAb known to block IFN-I signaling. While the IFN-I family contains 14 IFNα subtypes and a single IFNβ, all members of this cytokine family signal through the same receptor complex – here referred to as IFNAR. The mAb MAR1-5A3 has been shown to efficiently block this receptor and prevent IFN-I signaling both *in vitro* and *in vivo* (44, 45, 111). We treated susceptible D^b mice with MAR1-5A3 before infection and maintained the blockade with booster injections every 24 h for the duration of infection. Blockade was verified in the experiment by assessing ability to stain cells with MAR1-5A3 after harvest (Figure 8A) as well as evaluating expression of the early activation markers CD69 and CD86 on immune cells (Figure 8B&C). MAR1-5A3 staining was reduced to levels

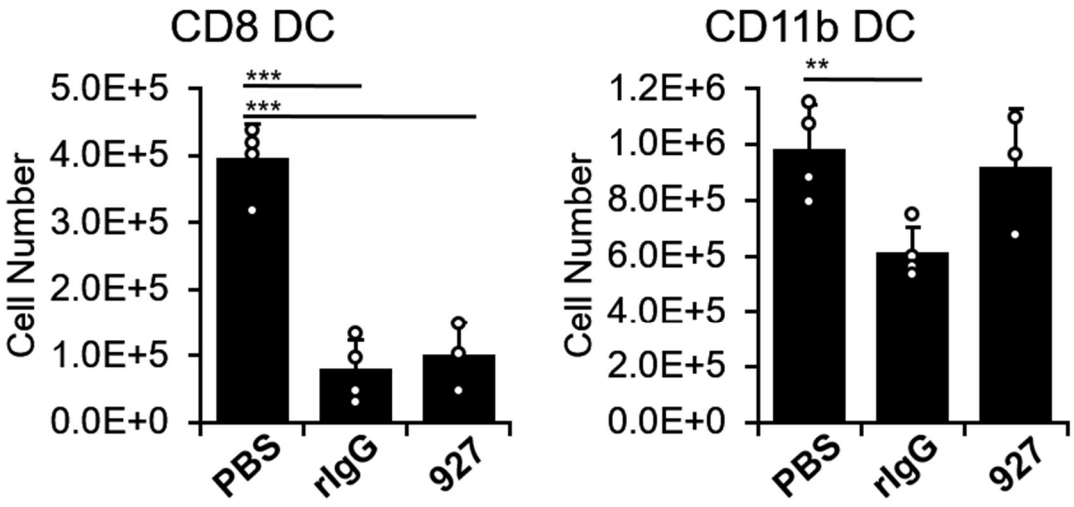


Figure 7. *pDC depletion does not rescue CD8 DC, but may protect CD11b DC.* D^b mice (R2) were treated with 927 pDC depleting mAb and infected with $2x10^4$ PFU MCMV. Splenocytes were analyzed by flow cytometry for DC populations 4 dpi. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

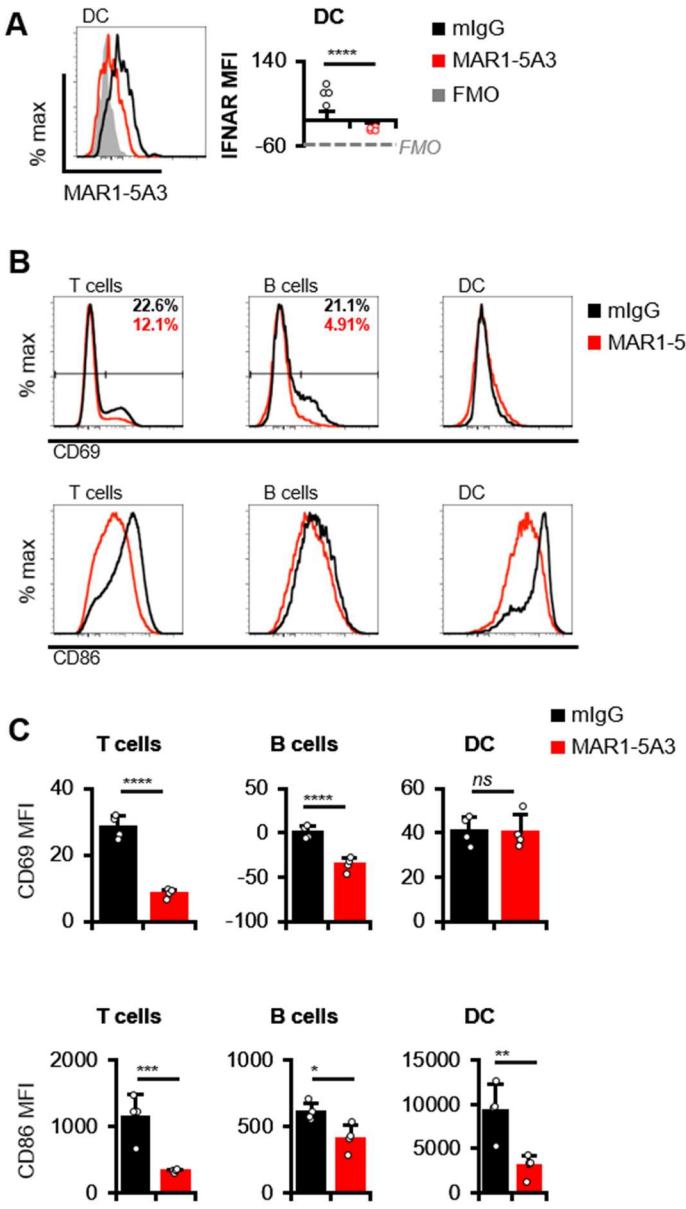


Figure 8. *Confirmation of IFNAR blockade.* D^b mice (R2) were treated with MAR1-5A3 IFNAR-blocking mAb prior to and during infection ($5x10^4$ PFU MCMV). Groups received either isotype control (mIgG; black) or MAR1-5A3 (red). Splenocytes were analyzed by flow cytometry 3 dpi for **(A)** MAR1-5A3 to staining *ex vivo* and **(B&C)** upregulation of CD69 and CD86 activation markers. C shows quantified data from B. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

comparable to the FMO and upregulation of both CD69 and CD86 was depressed for mice treated with MAR1-5A3.

IFN-I signaling has also been shown to specifically increase expression of mPDCA (112, 113). While this surface molecule is a specific marker for pDC during steady state, other cells greatly increase mPDCA expression during infection (Figure 9A). We assessed upregulation of this marker on non-pDC as an additional confirmation of IFNAR blockade. Treatment with MAR1-5A3 essentially abrogated the increases in mPDCA when compared to isotype control mice (Figure 9A).

Having verified the efficacy of IFNAR-blockade, we interrogated splenocyte populations for DC preservation. This time, we again fully broke down the cDC population into CD8, CD4, and DN subsets for analysis. Similar to the pDC depletion data, there was minimal rescue of the CD8 DC subset. A small but significant increase in CD8 DC numbers from the spleens of mice receiving MAR1-5A3 was observed, but the overall numbers were still drastically decreased compared to what would be expected in steady state conditions (Figure 9B). The CD4 DC subset displayed a similar pattern, showing a small but significant increase in numbers but also a high degree of overall loss (Figure 9B; compare cell numbers to PBS group of Figure 3). The DN DC subset, however, exhibited an increase in numbers that neared complete preservation (Figure 9B). These results confirmed what was observed during pDC depletion. While IFN-I signaling may

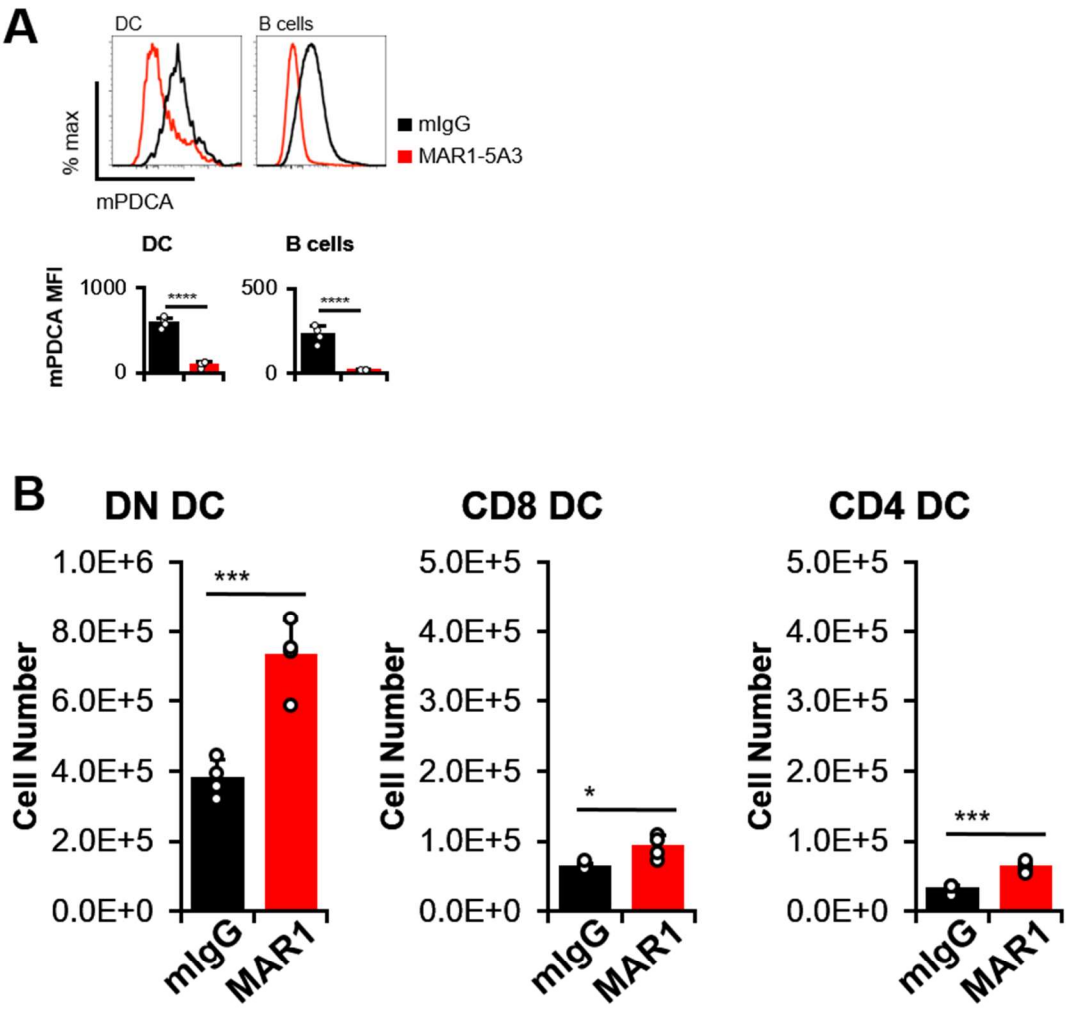


Figure 9. *IFNAR blockade prevents mPDCA upregulation and differentially protects DC subsets.* D^b mice were treated with MAR1-5A3 IFNAR-blocking mAb prior to and during infection ($5x10^4$ PFU MCMV). Groups received either isotype control (mIgG; black) or MAR1-5A3 (red). Splenocytes were analyzed by flow cytometry 3 dpi for **(A)** mPDCA upregulation and **(B)** DC subset numbers; maximum values on y-axes in B are representative of numbers normally recovered from uninfected mice in other experiments. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

have a slight impact on CD8 DC and CD4 DC numbers, it appears that the DN DC compartment is more sensitive to IFN-I signaling.

Genetic deficiency of IFNAR does not prevent DC loss

To corroborate the results obtained with IFNAR blockade, we generated mice that were MCMV susceptible and genetically IFNAR-deficient by breeding the susceptible B6 strain NKC^{l} with B6 IFNAR-KO mice (see methods). Animals from F1 litters were interbred to produce F2 litters containing NKC^{l} IFNAR$^{+/+}$ (WT), NKC^{l} IFNAR$^{+/-}$ (heterozygous), and NKC^{l} IFNAR$^{-/-}$ (KO) genotypes. These mice required no treatments or manipulations (i.e. antibody blockade or depletion) in addition to MCMV infection, allowing us to directly assess the impact of IFNAR-deficiency with minimal complicating factors. Mice received MCMV for 3 d, at which point spleens were harvested and assessed for DC subset populations. For analysis, mice were segregated into groups based on their expression of surface IFNAR protein and ability to upregulate mPDCA in response to infection. This enabled us to readily distinguish IFNAR WT, heterozygous (het), and KO mice (Figure 10).

Results from these experiments were consistent with those seen with IFNAR-blockade. The CD8 and CD4 DC subsets displayed dramatic loss of cellularity regardless of IFNAR genotype (Figure 11). The DN DC subset again exhibited much less severe loss relative to the CD8 and CD4 DC (Figure 11). In addition, despite some obvious outliers, the

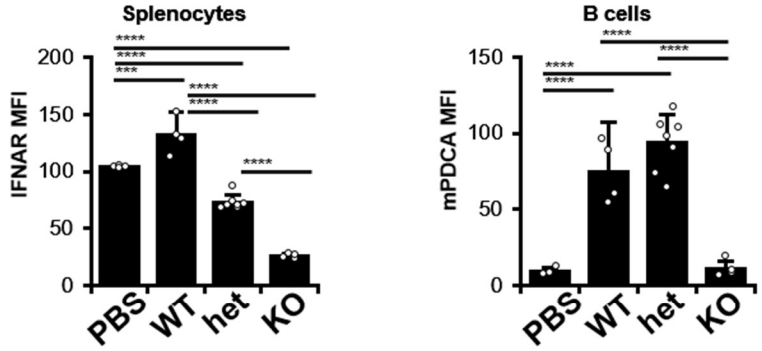


Figure 10. *Stratification of mice into experimental groups by intensity of IFNAR expression mPDCA upregulation after infection.* Litters from IFNAR-KO breeders were infected with $5x10^4$ PFU MCMV. Splenocytes were analyzed by flow cytometry 3 dpi for IFNAR expression levels and upregulation of mPDCA on B cells. Mice were sorted into WT, heterzygous, and KO phenotypes for downstream analysis of DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

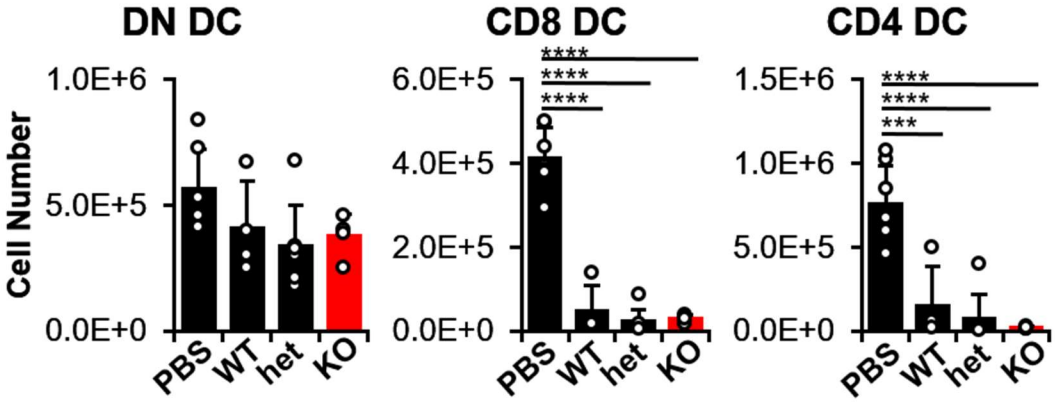


Figure 11. *Genetic deficiency of IFNAR does not protect DC subsets.* Litters from IFNAR-KO breeders were infected with $5x10^4$ PFU MCMV or mock infected (PBS). Splenocytes were analyzed by flow cytometry 3 dpi and segregated into wild type (WT), heterozygous (het), or knockout (KO) IFNAR expression groups (as shown in Figure 10) for quantification of DC subsets. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

minor effects of IFNAR could also be seen. IFNAR deficiency had very little impact on CD4 DC, but both CD8 and DN DC trended toward slight increases in numbers in KO animals (Figure 11). Despite these trends, the data show that splenic CD8 and CD4 DC subsets undergo severe contraction during MCMV infection and this is not mitigated by preventing IFN-I signaling. The IFN-I impact on DN DC is less clear. We observed a robust increase in numbers following IFNAR-blockade, but a much milder effect with genetic IFNAR-deficiency. While, this is counterintuitive, it may suggest that the interaction between DN DC and IFN-I is more complicated than anticipated.

IFN-I effects on DC are not cell-intrinsic

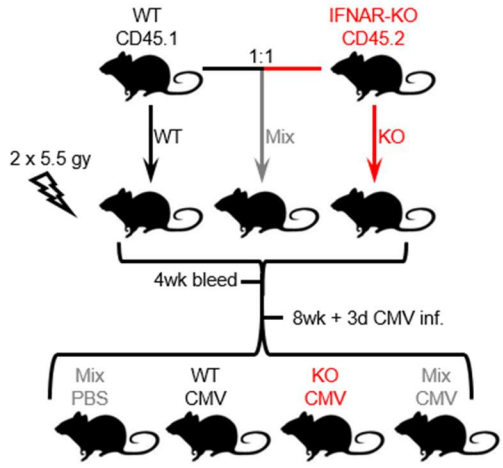
A weakness in the previous two approaches (blockade and knockout) is that these strategies result in a global defect in IFN-I signaling. This could alter cytokine expression profiles, immune cell activation and responsiveness, and host control of viral infection. Such broad effects could influence DC numbers and cause us to underestimate the role of IFN-I (49, 50). In addition, it is possible that developing in the presence of low level IFN-I signaling could alter the way DC or their precursors respond during infection. This could potentially explain differences seen in the DN DC subset between the transient blockade and complete lack of IFNAR signaling.

Hence, to address these concerns we competed IFNAR-WT and -KO DC head-to-head in an environment with a mostly intact IFN-I response. To accomplish this, we generated chimeric mice. IFNAR WT host mice were irradiated to prepare them for bone marrow

(BM) transfer. These recipients were then reconstituted with either IFNAR-WT, -KO, or mixed (1:1 WT:KO) BM for 6-8 wk. In this set up, each chimeric group has a slightly different distribution of IFNAR expression (Figure 12). Each group maintains IFN-I responsiveness in their stromal cells (main targets for early MCMV infection and replication) but only WT and mixed chimeras have intact IFNAR signaling in all or part of the hematopoietic compartment. Hence, since all cellular responses to IFN-I are maintained in mixed chimeras, the IFNAR WT and KO DC populations could be directly compared in an environment reminiscent of a WT animal. Following BM transfer (BMT), blood was drawn from recipient mice after 4 wk of reconstitution to verify chimerism (Figure 13A&B). Mice were infected between 6 and 8 wk post reconstitution and their spleens harvested 3 dpi. Splenocyte populations were analyzed for chimerism (Figure 13A), IFNAR expression (Figure 13B), mPDCA upregulation (Figure 13C), and DC subset persistence (Figure 13D). DC data were evaluated both in terms of overall total numbers and numbers of WT vs KO DC remaining in mixed chimeras. For all chimeras, cells derived from IFNAR-KO mice maintained low IFNAR expression and failed to upregulate mPDCA during infection.

The results highlighted and clarified observations made in blockade and knockout studies. First we evaluated DC populations from the unmixed chimeras (WT BMT vs KO BMT). CD4 DC from both groups exhibited dramatic cell loss with no detectable difference in numbers between WT and KO chimeras (Figure 13D). CD8 DC numbers also decreased precipitously in both groups, but there was a slight elevation in numbers

A



B

	Hematopoietic IFNAR	Stromal IFNAR
WT chimera	+	+
KO chimera	-	+
Mix chimera	50%	+

Figure 12. *Generation of IFNAR BM chimeras.* **(A)** B6 mice were irradiated with 2 doses of 5.5 Gy (11 Gy total) of whole body irradiation 2 h apart. Mice then received a total of ~4.5x10^6 donor BM cells i.v. Donor cells were harvested from the tibias and femurs of CD45.1 IFNAR-WT and CD45.2 IFNAR-KO donor mice. Donor cells were administered either singly or as a 1:1 mix into irradiated recipients. Blood was drawn at 4 wk of reconstitution to verify chimerism and infections were performed after 8 wk. Chimeras were given 3D10 prior to infection to block Ly49H-mediated MCMV control. **(B)** Table detailing IFNAR expression distribution for the 3 types of chimerias.

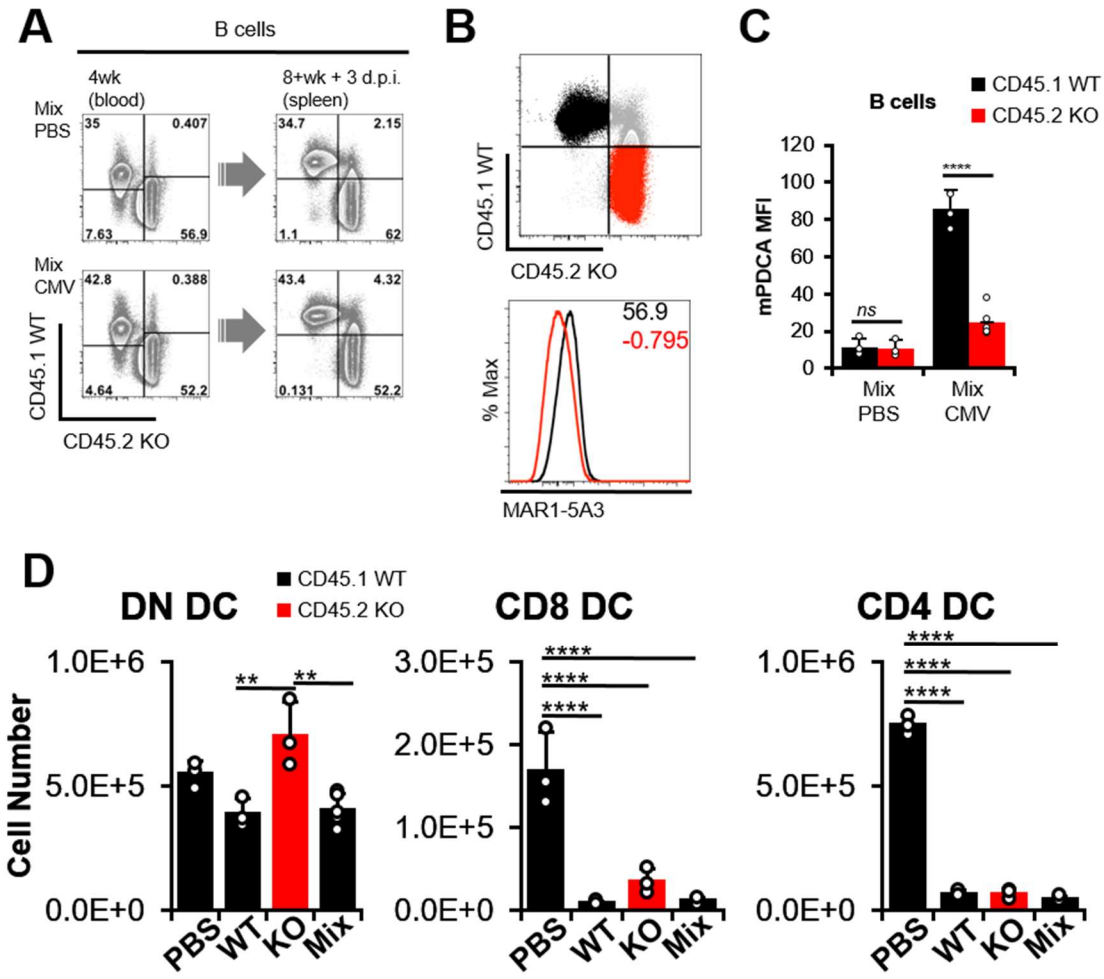


Figure 13. *Hematopoietic IFNAR deficiency has variable cell intrinsic effects on DC subsets.* **(A)** B cells from mixed BM recipients were analyzed by flow cytometry for CD45 chimerism; (*left*) peripheral blood B cells at 4 wk reconstitution (*right*) splenic B cells after 8 wk reconstitution + 3 d MCMV infection. **(B)** Donor genotypes were verified by analyzing IFNAR expression on CD45.1 (black) and CD45.2 (red) splenic B cells from mixed chimeras. Values on histogram represent IFNAR MFI. **(C)** Functional absence of IFNAR was verified by analyzing splenic B cell mPDCA upregulation 3dpi. **(D)** Total DC subset numbers from chimeric spleens 3 dpi (i.e. not separated by CD45 allele, total numbers ofCD45.1 + CD45.2). Significance is displayed as * $p <$ 0.05; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

recovered from KO chimeras over WT (Figure 13D). DN DC, on the other hand, displayed less severe loss – as could be expected given previous results. In addition, KO chimeras had significantly increased numbers of DN DC compared to WT chimeras, with the KO population reaching levels seen in uninfected mice (Figure 13). This closely reflects results seen during IFNAR-blockade. Interestingly, this may suggest that IFNAR signaling in a radioresistant cell during development is important for shaping the behavior of the DN DC compartment during inflammation, as that element would be lacking in the global IFNAR-KO background (Figure 11).

We next compared WT and KO DC populations head-to-head by analyzing their relative persistence in mixed BM chimeras. In this setting, there was no preferential retention of either WT or KO DC observed for any of the three subsets, although CD8 and DN DC showed slight, non-significant trends toward an increased presence of KO cells (Figure 14). This lack of preference showed that increased retention of the DN DC observed in the IFNAR-KO chimera (Figure 13D) was not due to an intrinsic effect of IFN-I on DN DC themselves. Hence, DN DC were sensitive to extrinsic regulation by another hematopoietic cell in conditions of high IFN-I production. When IFN-I signaling is absent in the whole hematopoietic compartment (i.e. IFNAR-KO chimeras), DN DC exhibit a significant increase in numbers; but the KO DC do not exhibit an inherent advantage over WT DC in the mixed chimera setting. Therefore, when all BM-derived cells lack IFNAR-signaling (KO chimera), DN DC display improved retention (Figure 13). But when IFNAR-signaling is restored in half of BM-derived cells (mixed chimera),

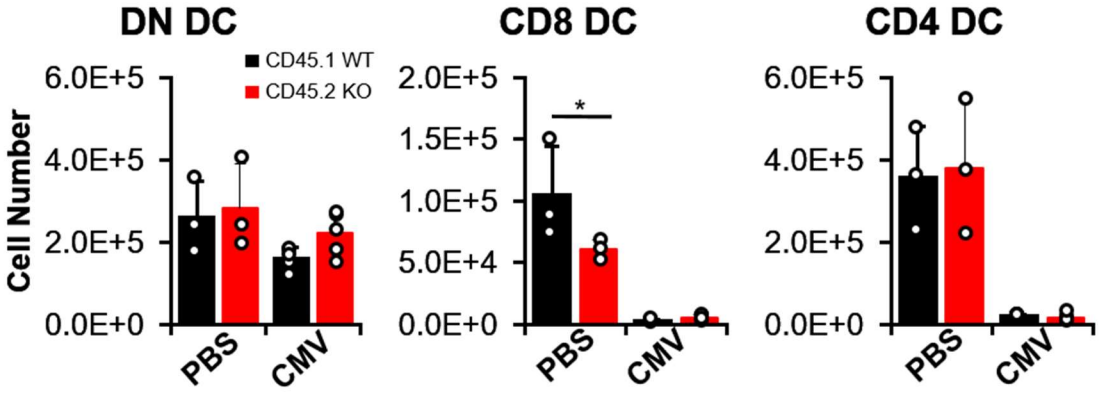


Figure 14. *IFNAR deficient DC are not preferentially retained in mixed chimeras.* DC subsets from mixed BM recipients were divided into CD45.2 and CD45.1 fractions for quantification and comparison. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

IFNAR-KO DN DC are no longer significantly retained over their IFNAR-WT counterparts (Figure 14).

Any observed increase in CD8 DC number resulting from IFNAR-KO was a minimal effect that failed to overcome the bulk of loss (Figure 13 & 14). Interestingly, uninfected mixed BM chimeras exhibited significantly higher numbers of WT CD8 DC compared to their KO counterparts (Figure 14; PBS mice). This implied that IFN-I signaling, at some low level, could aid homeostatic expansion of WT-derived CD8 DC. In support of this, a similar observation has been reported previously by Mattei et al. (107). We did not observe a similar effect in DN or CD4 DC subsets from uninfected mice, so this does not appear to be a broad effect on all leukocytes.

Discussion

Due to the nature of DC as potent modulators of the immune response, detailed understanding of their development and regulation is a primary goal in the field. While a great deal of progress has been made in the past decade for defining DC populations under various conditions (16, 20, 21, 114–116), the behavior and fate of these cells during inflammation is still not well understood. To date, IFN-I has been largely accepted as an underlying cause of DC loss during infection. Initially, we expected to confirm this concept and move on to additional areas of study. Surprisingly, though, our results did

not support the idea that IFN-I is the major driving force behind DC loss during MCMV infection.

Our data reveal several interesting and unexpected concepts. The strongest conclusion is that IFN-I is not a necessary factor for DC loss during infection, especially in regard to CD8 and CD4 DC subsets. While it is possible that these cytokines could play some role in the effect, the results detailed here definitively show that DC loss progresses in the absence of IFN-I signaling. Related to this is the observation that CD4 and DN DC subsets are completely divergent in their sensitivity to IFN-I, at least in terms of splenic retention. This is an important finding since the field currently lacks detailed information distinguishing these two populations. An interesting question that arises from this discovery is how skewed splenic retention (favoring DN DC over CD8 and CD4 DC) may influence the immune response. Functional differences that characterize the splenic DN subset are sparse, but one study has indicated that these cells are superior producers of TNFα and IL-12, compared to their CD4 counterparts, but are inefficient at CD4 T cell priming *in vivo* (23). Hence, viral inflammation that preferentially retains DN DC may generate an environment that favors inflammatory cytokine production over T cell priming in an attempt to unbalance development of immunity.

Our data also suggests that any contribution of IFN-I to DC subset loss is mediated in a DC-extrinsic fashion. There were clear differences in DN DC subset numbers between

the pure IFNAR-WT and -KO bone marrow chimeras, but no significant difference in numbers between WT and KO cell numbers in the mixed chimera setting (Figure 14). Additionally, the slight increase in CD8 DC seen between WT and KO chimeras also appeared absent from mixed chimera mice. Since the stromal cells in the chimeras were all equivalent, the implication here is that another BM-derived cell limits DC numbers (especially in the case of DN DC) in response to IFN-I signaling. Another interesting point here is that DN DC may be derived from an earlier myeloid precursor rather than pre-DC (23). With this in mind, one possibility is that IFN-I alters cytokine production in a way that favor recruitment or differentiation of inflammatory monocytes/ macrophages over DN DC, resulting in reduced persistence of this DC subset in response to DC-extrinsic IFN-I signaling.

Curiously, our conclusions seem at odds with those from other work. Fuertes Marraco et al showed that IFNAR-deficient mice were partially protected from poly I:C-induced splenic DC loss. (98). They assessed DC loss in poly I:C treated mice by comparing DC numbers between treated and control animals to calculate a fold change value. When they compared this value for poly I:C-treated IFNAR-KO and IFNAR-WT mice, the fold change was less dramatic for IFNAR- KO groups. Part of the difference for the CD8 DC subset could be due to a dual effect. Since IFNAR-KO CD8 DC do not develop as robustly as IFNAR-WT (Figure 14 & (107)), it is possible that baseline numbers were lower in their untreated IFNAR-KO mice. When coupled with the slight protective effect we see in the IFNAR-blockade and chimeric mice (Figures 9 & 13D), it is possible that

the fold change would not be as dramatic due to a larger numerator and lesser denominator. It is also important to note that the IFNAR-KO mice from the Fuertes Marraco study still exhibited a reduction in DC (fold change of ~0.7) confirming that loss is not solely dependent on IFN-I signals. It is slightly more difficult to explain the protection they observed in the CD11b compartment since steady state development of these cells is unaffected by the absence of IFNAR (Figure 14). However, since they combined CD4 and DN DC subsets by analyzing the CD11b compartment as a whole, it is possible that the less severe fold change was due to retention of the DN subset while CD4 DC receive no benefit. If this is the case, it would fit nicely with our observations.

One of the original studies that implicated IFN-I in DC loss during MCMV infection was performed by Robbins et al in 2007 (46). It is interesting to note that, while thy showed Ly49H-mediated MCMV control lowered IFN-I production from pDC and preserved cDC subset numbers, a causal link between pDC-derived IFN and cDC numbers was not established. In an attempt to further implicate IFN-I, they dosed resistant mice with IFN-I during MCMV infection to recapitulate DC loss observed in susceptible mice. To determine how much IFN-I should be used for the treatments, they quantified the IFN-I concentration in susceptible serum and calculated absolute levels of circulating cytokine using the total blood volume of mice. While this treatment did impair splenic DC retention, the degree of loss was still not comparable to that seen in susceptible animals. This further supports our conclusion that there are IFN-I-independent factors causing DC loss.

One study has followed up on the initial Robbins et al work. The aim of this second investigation was defining the transcriptional profile of DC and NK responding to IFN-I stimulation. To this end, they compared IFNAR-KO and IFNAR-WT cells using mixed BM chimeras and MCMV infection, similar to our experiments in figures 13 & 14. Although the main goal of their study was the investigation of IFNAR-dependent changes in transcriptional profiles, they also performed a cursory analysis of DC persistence during infection. Their results actually largely agree with our observations, showing a dramatic decrease in the frequency of both IFNAR-WT and IFNAR-KO DC at 3 dpi. In their hands, the IFNAR-KO DC did display a significantly higher residual frequency compared to WT counterparts, but the overall loss phenotype was still maintained. It should also be noted that they measured percentages for the DC compartment as a whole in this analysis without individual subset breakdown, which could account for any minor differences between our observations and theirs. Furthermore, the transcriptional profiles obtained in this work showed that IFNAR signaling induced both pro- and anti-apoptotic pathways. This finding led the authors to hypothesize that IFN-I is not likely to drive DC death (48). Rather, they suggested IFN-I was acting on DC precursors, ascribing the loss of DC numbers to a turnover-without-replacement effect. However, this idea was not pursued beyond the initial correlations observed. As a result, the fate of DC and the underlying cause of their disappearance in the spleen during infection is still unclear.

Due to the unexpected nature of our results, we performed an in depth survey of the literature for IFN-I/DC interactions. Our findings indicated the minimal role for IFN-I in driving DC loss may not be necessarily surprising. The majority of work performed indicates that IFN-I enhances DC functions rather than inducing outright apoptosis. Some of the work is difficult to assess due to the use of GM-CSF to differentiate DC in culture, which generates more monocytic DC rather than true conventional DC. However, we found evidence for enhanced development/differentiation (48, 107, 117–119), maturation and antigen presentation (120–122), metabolic reprogramming (123), survival (124), cross-presentation (125, 126), Tfh induction (127), and DC-dependent B cell class switching (128).

In contrast, very few papers cite apoptosis as a direct outcome of the DC response to IFN-I. Aside from the work of Fuertes Marraco et al., we identified two *in vitro* studies that cited DC apoptosis as a result of IFN-I stimulation. However, both were performed with with monocyte derived DC (moDC). In one, moDC apoptosis in response to IFN-I was only seen after maturing the cells with a cocktail of TNFα, IL-6, IL-1β, and PGE2 (129). In the other study, differentiation in the presence of IFN-I was found to sensitize the moDC to activation induced death but, again, the apoptotic effect required the addition of a maturation signal, e.g. LPS (130).

The strongest data for a contribution of IFN-I to DC apoptosis comes from a 2009 study by Mattei et al. They showed that injection of IFN-I into mice resulted in a transient

increase in DC apoptosis (Annexin V and TUNEL staining) specifically in IFNAR-WT but not IFNAR-KO mice (107). Interestingly, they saw this effect at 4 h after IFN-I injection but not at 24 h. They also never saw a reduction in DC numbers during IFN-I stimulation. They attributed this to the possibility that IFN-I preferentially causes apoptosis in older DC, allowing for fresh cells to fill the compartment. In our eyes, their data show that IFN-I can contribute to transient DC cell death, but does not result in DC loss from the spleen. It is also interesting to consider their work with the finding of Robbins et al. that IFN-I treatment during MCMV infection only induced a partial reduction of splenic DC (46). When considered together with our work, the data favor the conclusion that IFN-I can participate in DC apoptosis but also enhances DC differentiation to maintain cellularity. Therefore, IFN-I is not itself a driving force in sustained loss of splenic DC and, logically, an alternative mechanism must be involved.

The work presented in this chapter has questioned an assumption held in the field and the findings highlight a significant gap in our knowledge of DC behavior during infection. The fact that DC loss is not reliant on IFN-I signaling and is sustained in its absence necessitates a shift in focus and the exploration of alternative mechanisms. It is critical that we understand how DC are responding to infection and inflammatory stimuli *in vivo* to facilitate their manipulation in the clinic, both in terms of enhancing the generation of immunity from a vaccine standpoint or reducing potential contributions of DC to pathogenic conditions like autoimmunity. The work in the next chapter is focused

on extending this work, attempting to identify alternative mechanisms essential for DC loss.

CHAPTER II – Analysis of IFN-I Independent Splenic DC Loss

Introduction

While it is apparent from our results and the literature that DC numbers decrease in the spleen following MCMV infections, our data shows that IFN-I signaling is not the essential factor responsible for the loss of CD8 and CD4 DC subsets. Hence, something else is regulating their numbers during infection. A variety of hypotheses arise that could explain IFN-independent DC loss. First is the possibility that DC could be directly infected by MCMV and dying either due to stress or cytopathic effects of the virus. Also, if MCMV infection is not directly inducing DC death, perhaps NK cells recognize and kill infected DC. Alternatively, NK cells could play a role regardless since activated NK cells can recognize and kill immature DC (131–135), which could lead to broad reductions in DC during infection. Another possibility is that some soluble factor produced during infection is toxic to DC and responsible for the effects attributed to IFN-I. Another question that arises from this line of thinking is whether or not DC are actually dying. So far, it is more or less assumed that DC attrition is due to cell death, but this may not be the case. It is potentially possible DC are leaving the spleen en masse in either a directed (migration) or undirected (destruction of niche) manner. These possibilities form the basis of the hypotheses tested in this chapter. The following studies aimed to further assess the regulation of DC fate during infection and explore potential candidates involved in IFN-I-independent DC loss.

Results

DC loss via direct infection is unlikely

While direct infection of DC by MCMV leading to DC death has been put forth as one possibility to explain loss, we have seen little evidence in the literature or our own data to support this scenario. Incubation of DC with MCMV *in vitro* causes profound changes in DC phenotype, which we do not see recapitulated prior to loss *in vivo*. DC isolated from mouse spleens and infected in culture decrease their expression of MHC II and CD86 (Figure 15 and (136)) and become refractory to maturation signals like LPS (136). Hence, if DC were infected in the spleen during MCMV infection, we would not logically expect them to increase expression levels of MHC II and CD86, essentially taking on the appearance of mature DC. Yet this is precisely what we see (Figure 16). DC are strongly activated at time points just prior to loss of the cells at 3 dpi, with significantly increased MHC II and CD86.

NK cells are not responsible for MCMV-induced DC loss

We next wanted to explore NK cell killing as a potential source of DC loss. Previous work by Andrews et al. has assessed this in B6 by depleting total NK cells with the anti-NK1.1 mAb PK136 (106). NK-depletion in MCMV-infected mice dramatically impaired retention of CD8 DC, showing that NK cells were not necessary for DC loss. In addition,

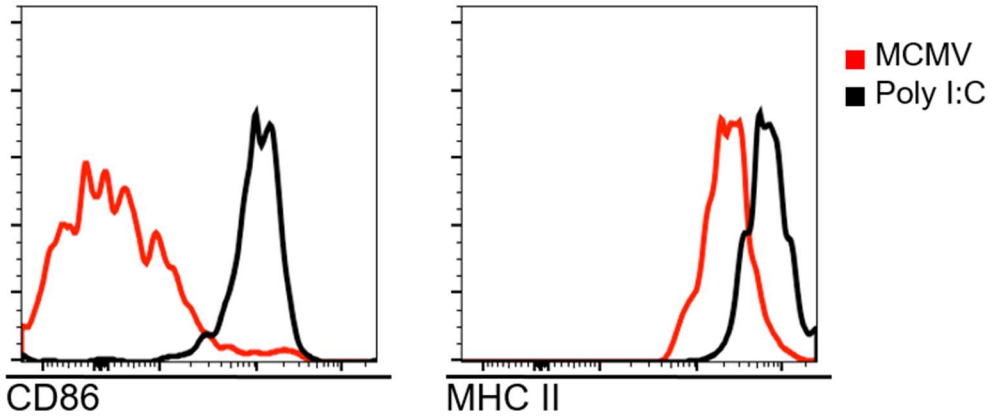


Figure 15. *Expression of activation markers by* in vitro *infected DC.* Splenocytes were isolated, CD11c sorted DC (MACS) and placed in culture with either poly I:C (50 μg/ml) or MCMV (10 MOI) for 5 h. Following incubation, DC were surface stained with mAb against CD86 and MHC II and analyzed by flow cytometry.

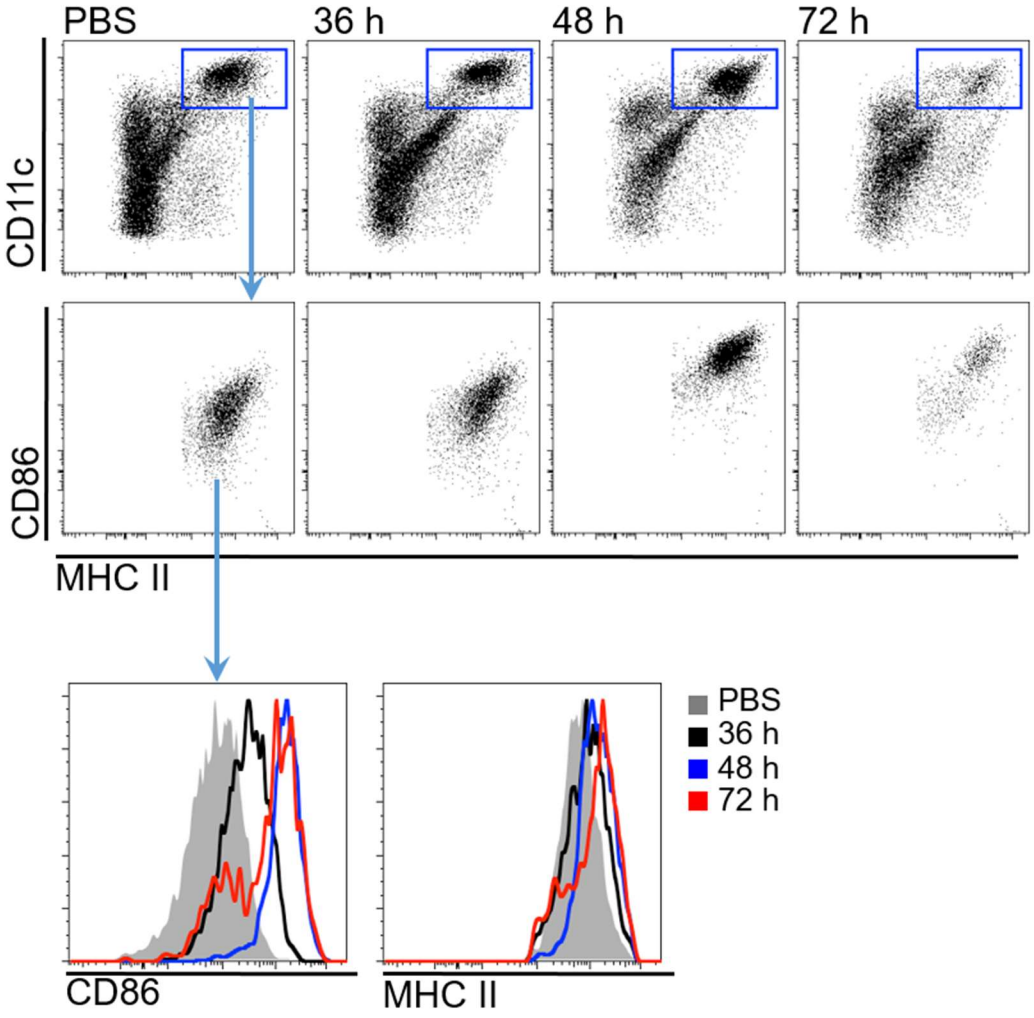


Figure 16. *DC are activated before and during MCMV-induced loss.* D^b mice (R2 and L) were infected with $2x10^4$ PFU MCMV. Splenocytes were analyzed by flow cytometry at 36, 48, and 72 hpi for DC expression levels of CD86 and MHC II. Histograms and lower dot plots show the DC population gated on in upper dot plots (gated on live cells / CD3&19-neg).

we have previously seen a similar outcome in C57L-derived mice following total NK-depletion. To verify these results, we treated mice with the NK-dpeleting PK136 mAb prior to MCMV infection. We found that the absence of NK cells did not ameliorate DC loss and, if anything, exacerbated the phenotype (Figure 17). This clearly illustrates that NK killing is not contributing to DC loss.

DC loss can be driven in the absence of MCMV infection

Aside from active infection, DC loss could be the result of inflammatory stimuli. Treatments with LPS (TLR4 agonist), poly I:C (TLR3 agonist), or CpG (TLR9 agonist) have all been shown to cause reductions in splenic DC by others (98, 103, 104, 107, 137, 138). Therefore, significant DC loss can occur in response to inflammation and in the absence of infection, supporting the conclusion that direct infection is not responsible for DC loss. We next interrogated the impact of different TLR agonists on DC subsets to determine if their effects were comparable.

TLR3, 7, and 9 are important sensors of viral infection and are variably expressed by DC subsets (27, 28, 139, 140). TLR3 is almost exclusively expressed in CD8 DC (DN DC express low levels), TLR7 is expressed in all DC except CD8 DC, and TLR9 is expressed similarly across the subsets. TLR3 and 9 especially have been identified as important players in the response to MCMV. Mutant mice deficient in either TLR3 or TLR9 exhibit drastically increased MCMV levels in spleen 4 dpi compared to control B6 mice (141).

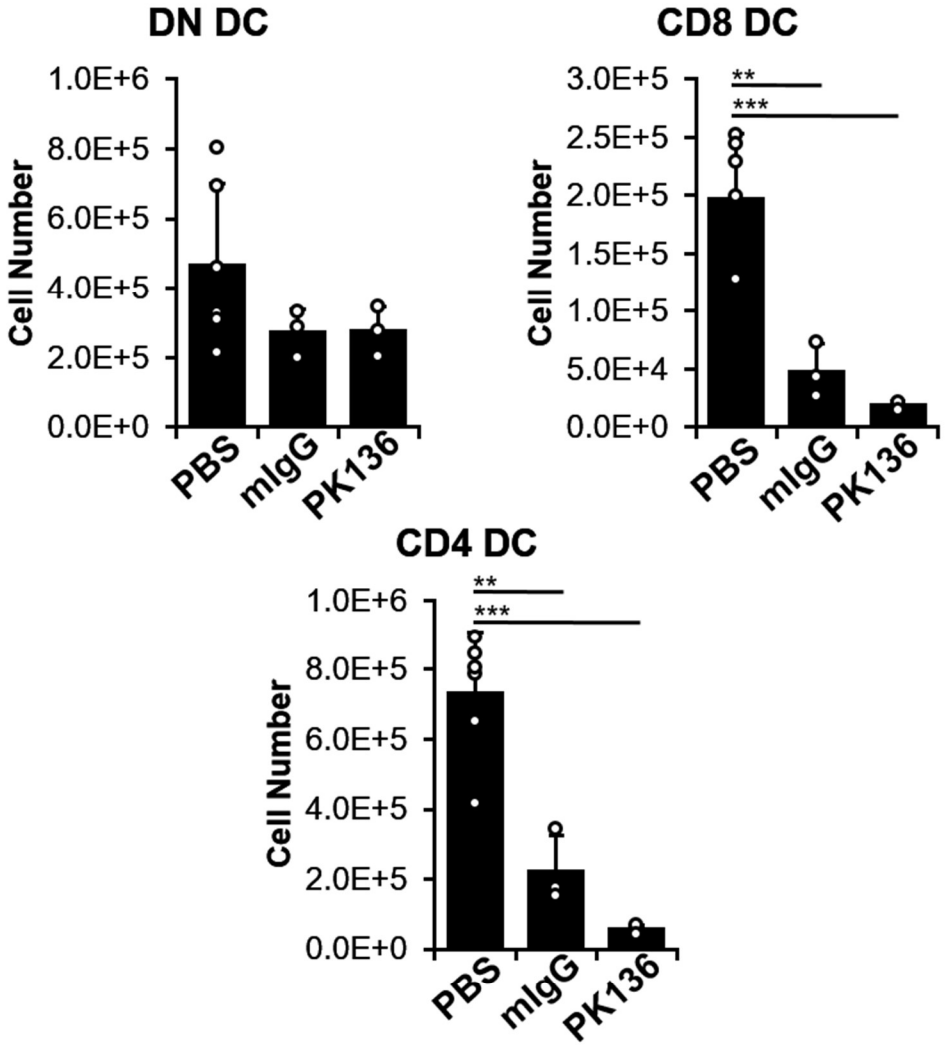


Figure 17. *DC loss occurs in the absence of NK cells.* D^k mice (R7) were treated with either isotype (mIgG) or NK depleting mAb (PK136) prior to infection with $5x10^4$ PFU MCMV. Splenocytes were analyzed at 3 dpi for DC subset numbers. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

Virus levels were greater in TLR9-KO mice than TLR3-KO, but these data exhibit the importance of both in the response to MCMV.

To compare the impacts of stimulating these TLR on DC subset loss, we tested poly I:C and two different formulations of CpG reagents (class A and class B) which signal differently through TLR9 based on their endosomal trafficking patterns (142, 143). CpG-A compounds are retained in early endosomes, preferentially inducing pDC production of IFN-I, while CpG-B compounds traffic quickly into lysosomes, triggering B cell maturation and high levels of non-IFN cytokine production (e.g. IL-6 and TNFα) (144, 145). Additionally, we were intrigued that there are no reports of DC loss following TLR7 stimulation. We hypothesized that DC loss could be a general outcome of TLR-mediated inflammation and included the TLR7 agonist imiquimod in these experiments for comparison.

Imiquimod (TLR7) and poly I:C (TLR3) were administered to mice i.p. while the two CpG reagents (TLR9) were given i.v. After 2 d, splenic cDC subsets were quantified (Figure 18). TLR7 stimulation with imiquimod produced minimal effects on DC numbers, with only a trending decrease in CD4 DC, but this was inconsistent between experiments (Figure 18). Poly I:C, on the other hand, resulted in a strong and reproducible loss of DC across all three subsets (Figure 18), as could expected based on previous studies (98). Surprisingly, we saw observed clear differences between CpG-A and CpG-B treatments. Dramatic decreases in both CD4 and CD8 DC occurred after

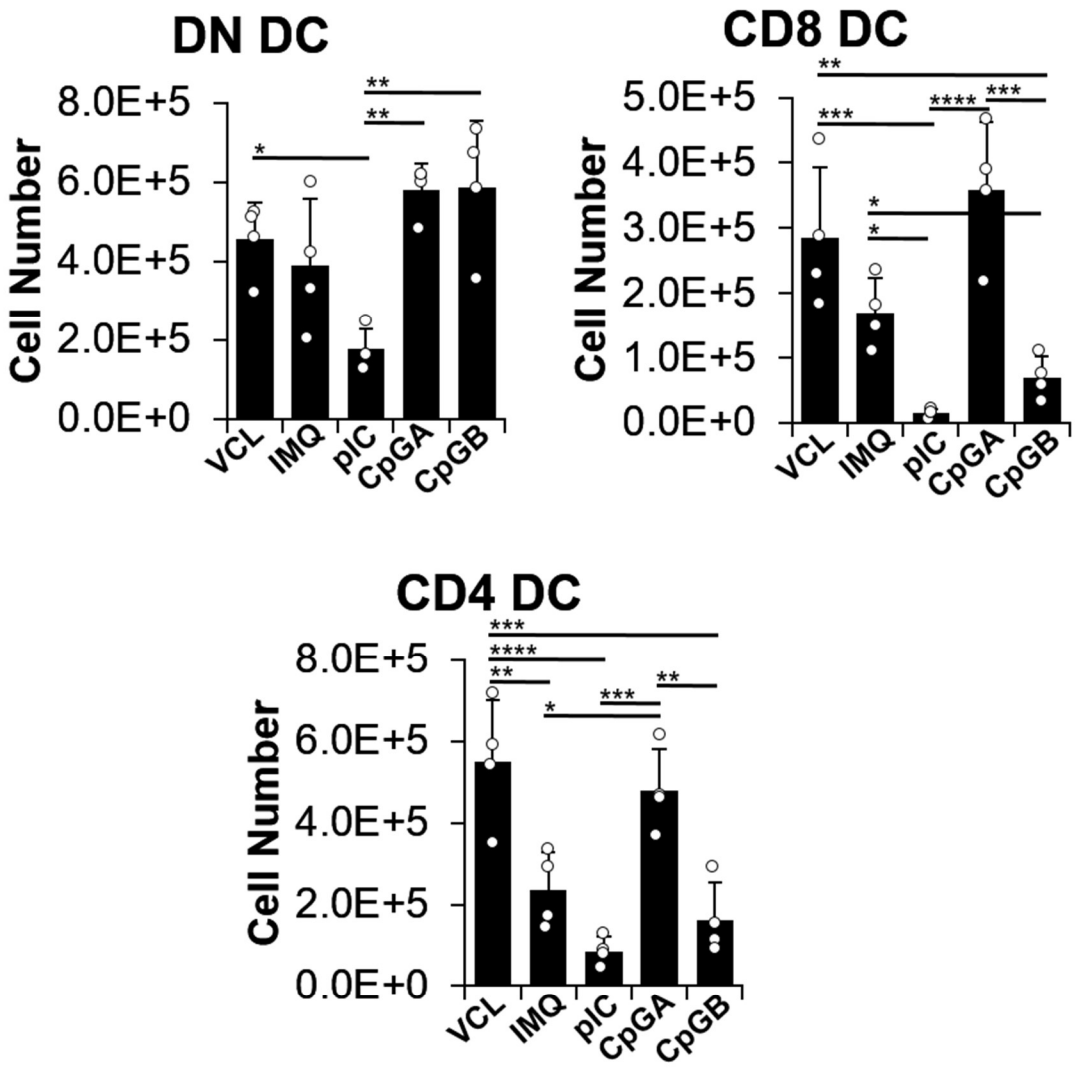


Figure 18. *DC loss occurs in the absence of virus.* B6 mice were treated with vehicle (VCL; PBS+endotoxin free water), 200μg imiquimod (IMQ), 200μg poly I:C (pIC), 75μg CpG-A, or 75μg CpG-B. Splenocytes were analyzed by flow cytometry 48 h post-treatment for DC subsets. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

CpG-B administration (DN DC were essentially spared) while CpG-A failed to induce observable loss of any subset (Figure 18). This is particularly interesting since CpG-A reagents are superior IFN-I inducers. In short, the data indicate that driving DC loss is specific to distinct types of inflammatory sensing, with DN DC being most sensitive to a TLR3 signature and CD4 and CD8 DC responding to both TLR3 and lysosomal TLR9 signals. However, DC loss is not a general function TLR-mediated inflammation since TLR7 and CpG-A stimulations had minimal effects.

Soluble factors do not potently induce DC loss

We were interested by the differences we observed between CpG-A and B induction of DC loss. Upon investigating the two compounds, we found that they diverge in their potency for IL-6 induction (information published on Invivogen's website (146)). Higher IL-6 levels are induced with CpG-B reagents than with CpG-A. We performed a luminex study to assess cytokines produced during MCMV infection and found high levels of IL-6 in serum from both resistant (D^k) and susceptible (D^b) infected mice (Figure 19), suggesting that IL-6 could reasonably play a role in DC loss.

We tested this hypothesis during infection by neutralizing IL-6 with a specific blocking mAb (clone MP5-20F3). Ab injections were given approximately 18 h prior to infection and every 24 h after infection. The results showed a minimal increase in DC numbers from IL-6-neutralized mice; however, DC loss was still prominent and there were no

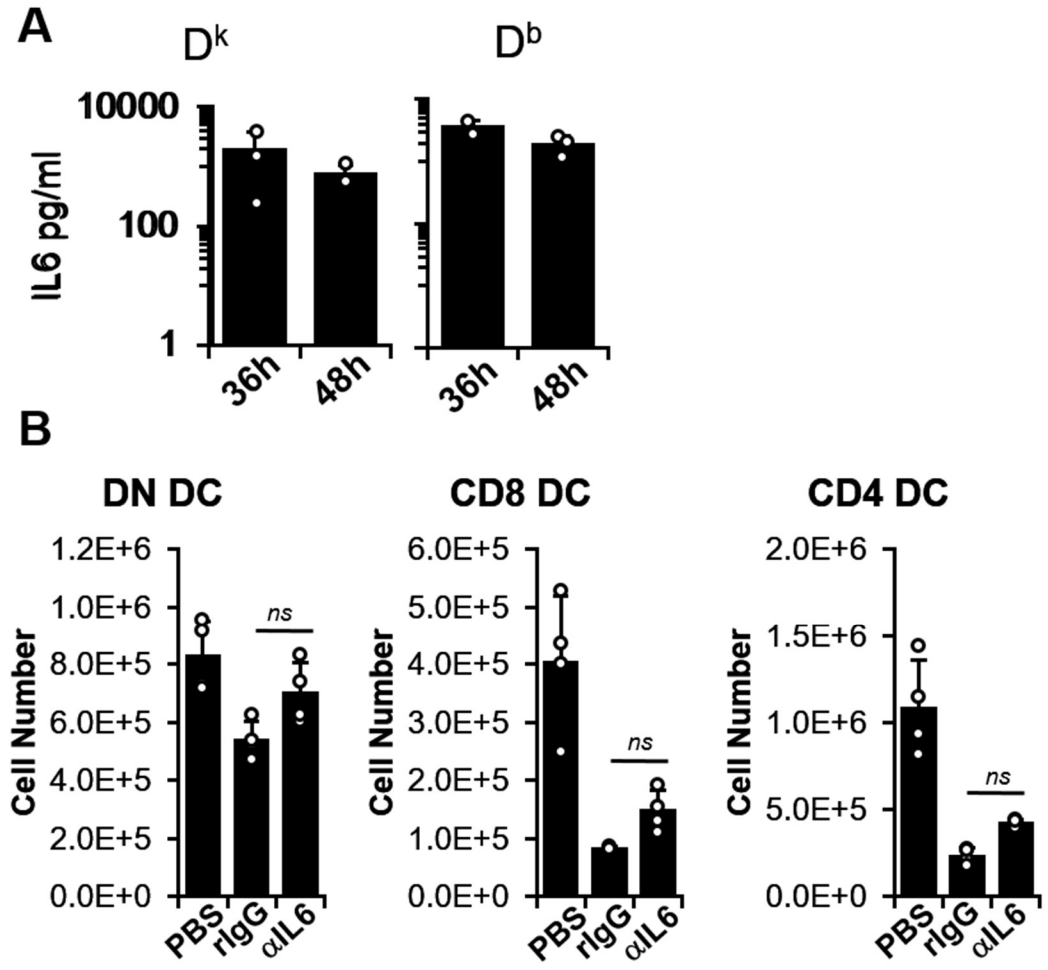


Figure 19. *IL6 blockade does not rescue DC numbers.* **(A)** D^k and D^b mice (R7 and R2 respectively) were infected with with $1x10^4$ PFU MCMV and serum was collected 36 and 48 hpi. Cytokines were quantified via Luminex by the UVA flow core. **(B)** B6 mice were treated with 300μg rIgG or IL6-blocking mAb (MP5-20F3) ~18 h prior to infection ($5x10^4$ PFU MCMV) followed by boosts 1 and 2 dpi. Mice also received 200μg mAb 3D10 with infection to block Ly49H resistance. Spleens were harvested for DC analysis 3 dpi. *ns* = not significant. Error bars represent SD.

significant differences between mAb treated and untreated groups (Figure 19). Hence, IL-6 levels are not controlling DC loss.

Since IL6-neutralization did not prevent DC loss, we next assessed if any MCMV-induced, soluble factor to negatively regulate DC numbers. If secreted factors are responsible, a transfer of infected serum into naïve mice should result in decreased splenic DC retention. Serum was isolated from MCMV-infected or PBS-treated mice at the peak of cytokine production (~40 hpi; (70)). Blood for serum isolation was collected from donor mice via post-mortem cardiac puncture and isolated serum was pooled within groups to ensure homogeneity of the transfer material. The total volume of collected serum was then split evenly among the same number of recipient mice as donors, essentially a 1 donor:1 recipient transfer ratio. Splenic DC subsets were quantified ~40 h post transfer.

Interestingly, no negative impact of MCMV serum on DC retention was observed (Figure 20A). There was no reduction in any of the DC subsets in MCMV-serum recipients and, if anything, slight trends toward increased numbers when compared to the PBS-serum recipients. We also did not observe increased CD86 expression on DC after MCMV-serum transfer, indicating that the cytokines present in serum at 2 dpi are not sufficient for strong activation of naïve DC (Figure 21). Interestingly, though, the mass of MCMV-serum recipient spleens increased relative to PBS-serum spleens, indicating a bulk increase in cellularity; an outcome likely due in part to increased

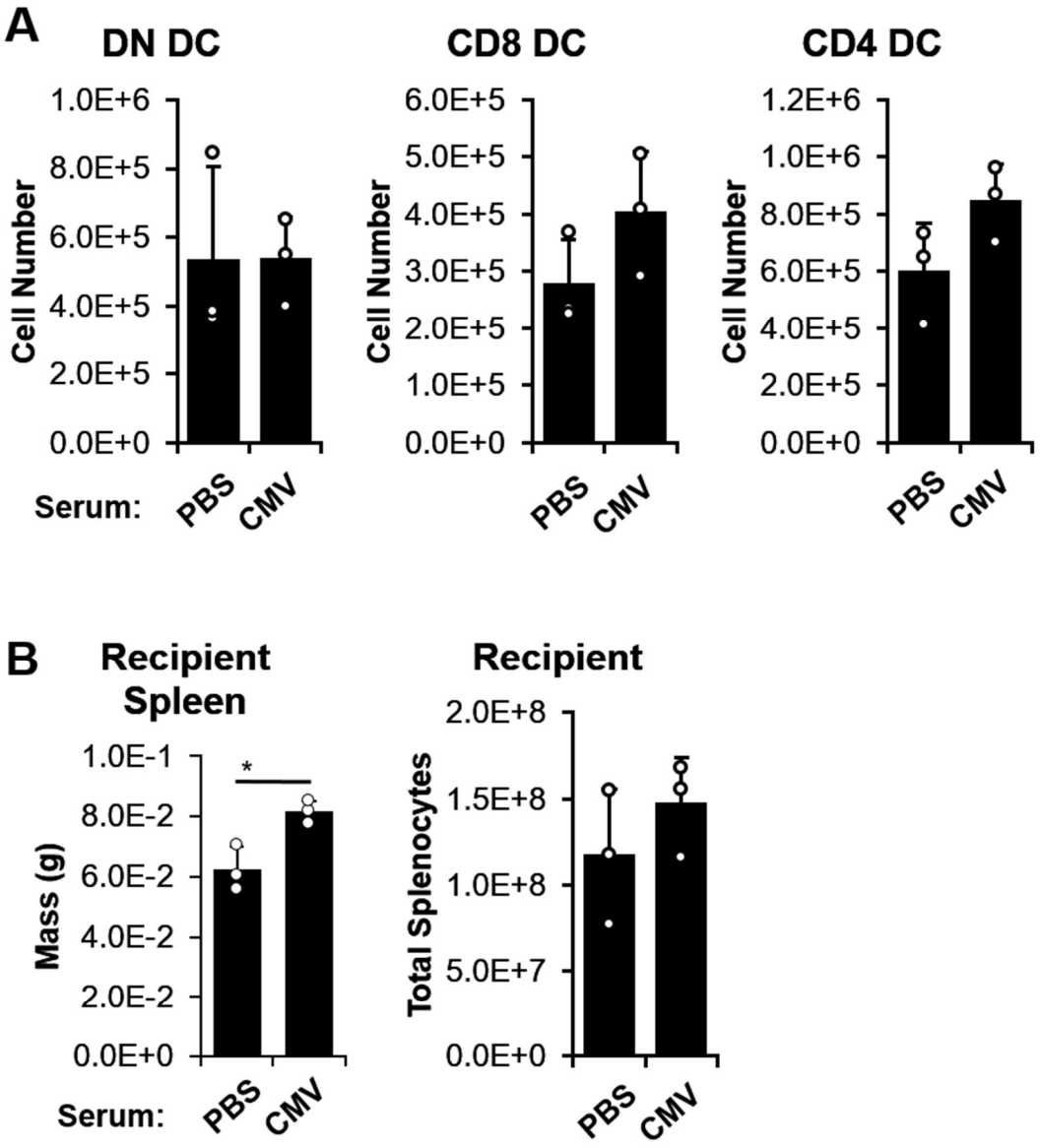


Figure 20. *Serum from infected mice does not transfer DC loss.* Donor B6 mice were 3D10 treated 1 d prior to infection (3 mice; $5x10^4$ PFU MCMV) or PBS mock infection (3 mice). Serum was collected between 36 and 48 hpi via post mortem cardiac puncture and the total volume of collected serum was divided between recipient mice for each group. Serum from 3 donors was transferred to 3 recipients for each group. Recipient splenocytes were analyzed by flow cytometry 24 h post transfer. **(A)** DC subset quantification. **(B)** Spleen mass and total splenocyte count from serum recipients. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

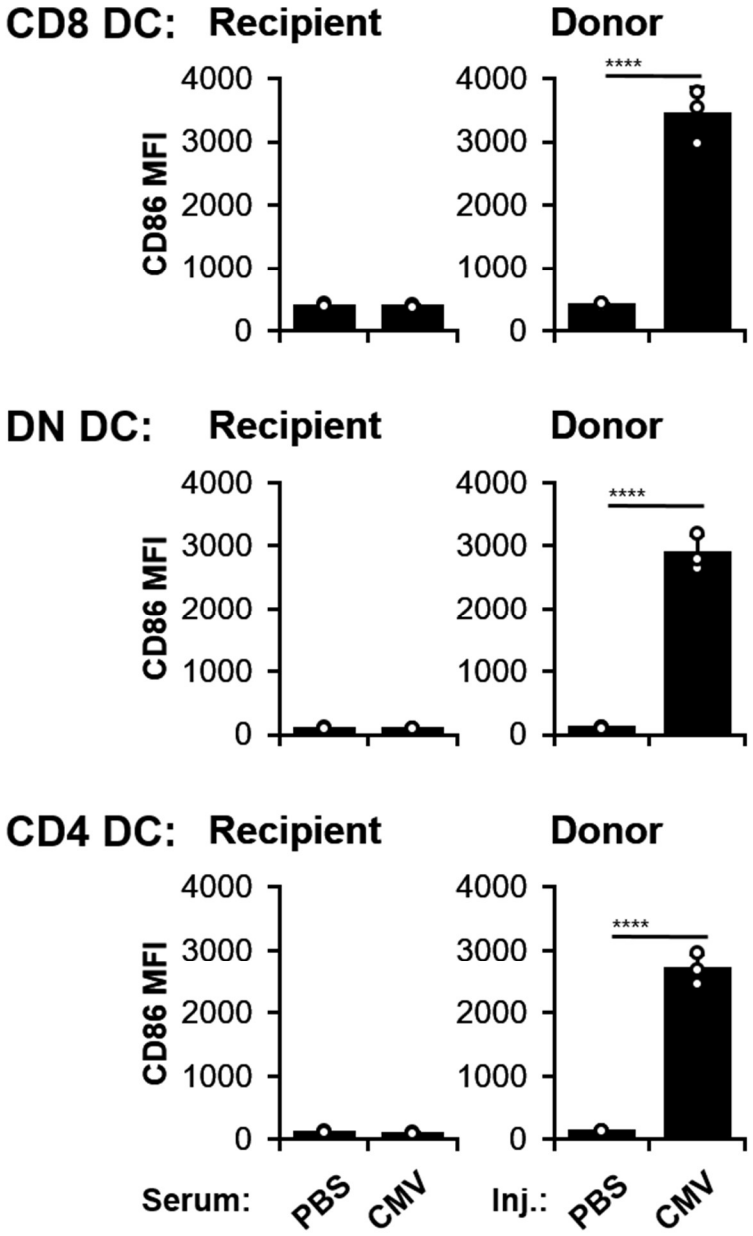


Figure 21. *Serum from infected mice does not activate DC.* Donor B6 mice were 3D10 treated 1 d prior to infection (3 mice; $5x10^4$ PFU MCMV) or PBS mock infection (3 mice). Serum was collected between 36 and 48 hpi via post mortem cardiac puncture and the total volume of collected serum was divided between recipient mice for each group. Serum from 3 donors was transferred to 3 recipients for each group. Splenocytes from donors and recipients were analyzed by flow cytometry for CD86 upregulation on DC subsets. Inj. = injection material. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

erythropoiesis since bulk splenocytes were not significantly increased (Figure 20B). Jordan et al. have previously shown that increased spleen weight following MCMV infection correlates with large increases in TER-119^{+} erythroid lineage cells (147). Regardless, despite the lack of DC changes, this increase in mass shows that MCMV-serum was able to stimulate other changes in recipient spleens.

MCMV infection does not overtly dysregulate putative pre-cDC compartment

As mentioned above, Baranek et al. suggested DC loss could be due to a decrease in splenic DC precursors (pre-cDC), leading to turnover-without-replacement and a gradual DC attrition (48). Using flow cytometry to detect pre-cDC, they observed reduced numbers of splenic pre-cDC during MCMV infection We have also attempted to distinguish intrasplenic pre-cDC through the use of multicolor flow cytometry. Guided by the characterization of Naik et al. (114), we stained for cells that were Lin^{-} CD11c^{int} SIRPα^{int} CD43^{int} (with Lineage markers represented by CD3, CD19, CD4, CD8, NKp46, and MHC II). The frequency of the population identified with this strategy was between .03-.05% of total splenocytes at steady state (Figure 22A), which matches what would be expected for pre-cDC (114). Unlike the Baranek et al. study, we did not detect any loss of this population during infection; in fact, numbers of these cells trended toward an increase (not significant) (Figure 22B).

DC do not exhibit increases in apoptosis or necroptosis during MCMV infection

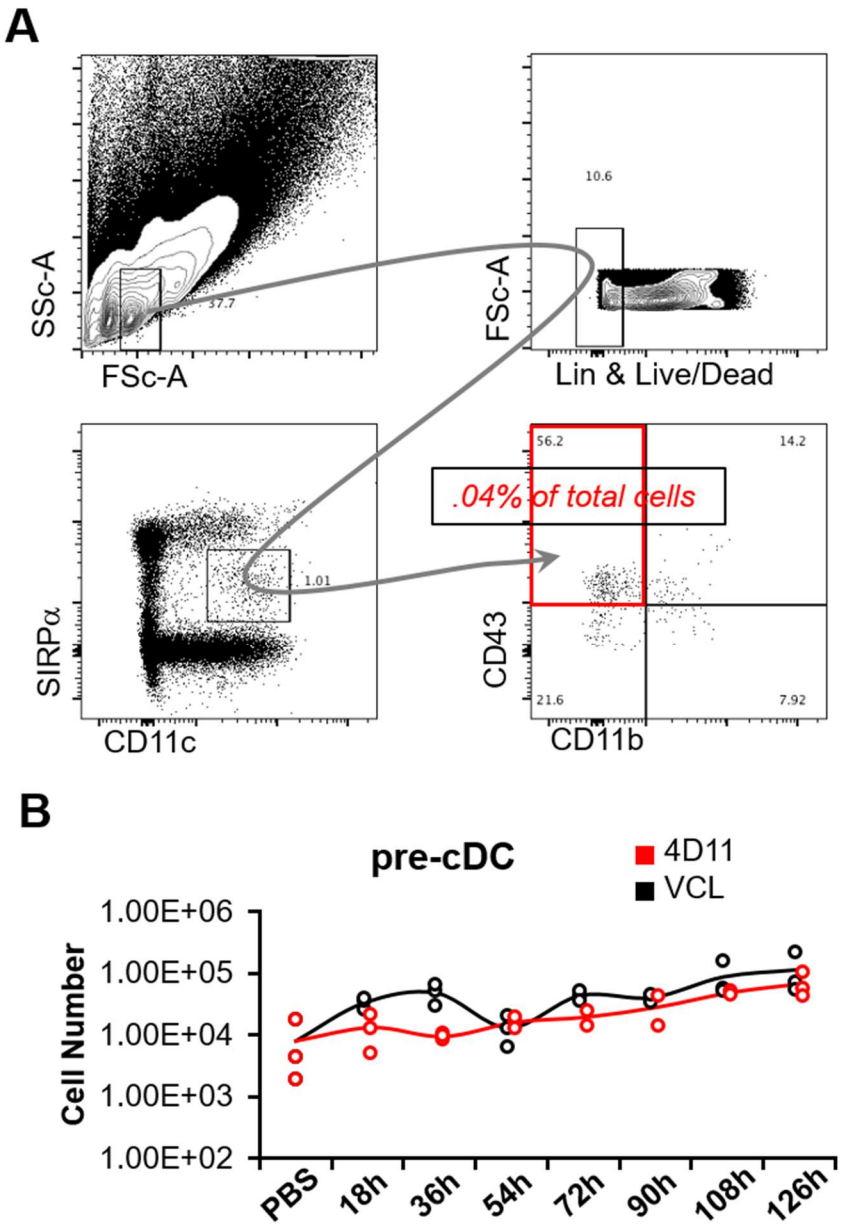


Figure 22. *Putative pre-cDC population does not decrease during infection.* D^k mice (R7 and R12) were treated with either vehicle control (VCL) or a Ly49G2 depleting mAb (4D11) 36 h before infection with $2x10^4$ PFU MCMV for 18, 36, 54, 72, 90, 108, and 126 h. Boosters of 4D11 were given to mice at 3 dpi to maintain G2 depletion. Splenocytes were analyzed by flow for cells expressing a pre-cDC phenotype (Lin^- Ssc^{lo} $SIRP\alpha^{int}$ $CD11c^{int}$ $CD43^{int}$). **(A)** Gating strategy for pre-cDC shown for a mock-infected animal. Lin cocktail included CD3, CD19, NKp46, and MHC II. **(B)** Qantification of pre-cDC-like population over time.

Since we did not detect a reduction in prec-cDC-like cells over the course of infection, we viewed inefficient replenishment of DC as a less likely explanation for DC loss. We next interrogated the fate of DC themselves during infection, turning to the investigation of cell death pathways. To test the hypothesis that increased DC death was indeed leading to their loss, we first analyzed DC death rates via multiparametric flow cytometry. We used Annexin V staining of surface phosphatidylserine and propidium iodide (PI) to detect loss of membrane maintenance and/or increases in cell permeability (respectively).

Surprisingly, when spleens were harvested at 60 and 72 hpi (the window when DC numbers begin to decline), we did not observe increased indicators of cell death in the $CD11c^{hi}$ DC compartment (Figure 23). Rather, the frequencies of Annexin V^+ and PI^+ $CD11c^{hi}$ cells decreased over time from uninfected to 60 hpi to 72 hpi. This was in stark contrast to $CD11b^+$ cells which exhibited increases in both Annexin V and PI positive frequencies at 60 and 72 hpi.

While we were unable to detect increases cell death by these methods, the possibility remained that artifacts in the experiment were interfering with our ability to find dying DC. For example, if rapid *in vivo* clearance of infected DC is occurring or DC are dying off too quickly during *ex vivo* organ processing, we may not be able to accurately determine the degree of splenic DC death occurring during MCMV infection. Hence, we next aimed to interfere with traditional cell death pathways for apoptosis and

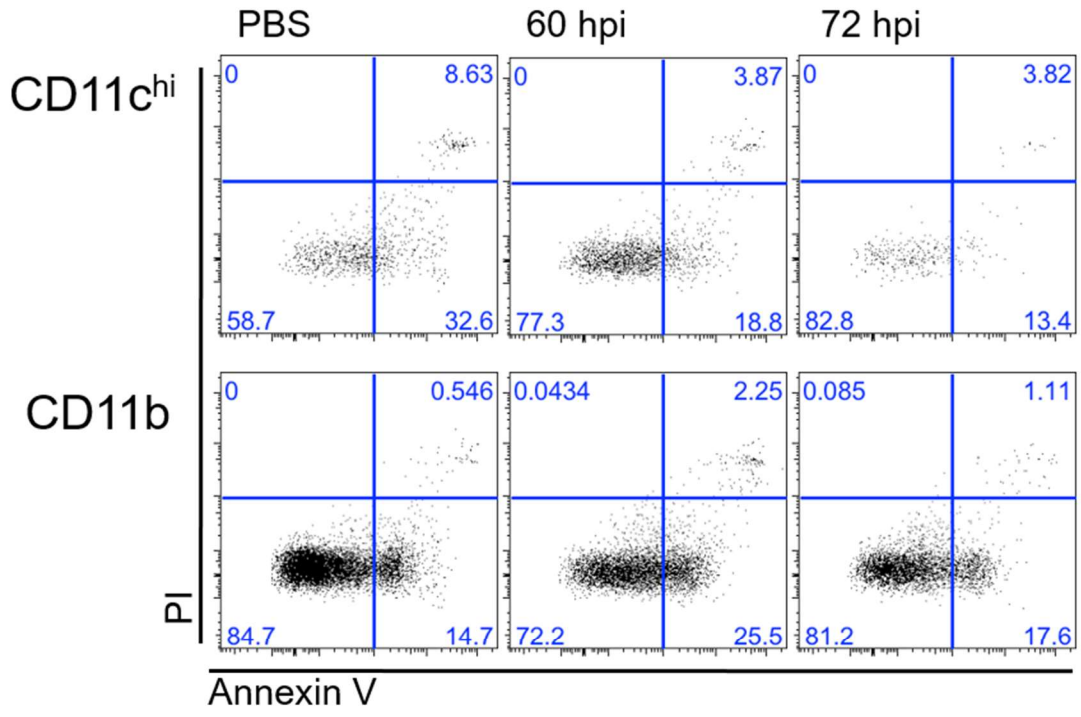


Figure 23. *DC do not exhibit increases in cell death during loss.* D^k mice (R7) received 200μg 4D11 to abrogate Ly49G2 resistance 48h prior to infection with $5x10^4$ PFU MCMV. Splenocytes were analyzed by flow cytometry at 60 and 72 hpi for DC and markers of cells death.

necroptosis (a.k.a. regulated necrosis) and assess any protective effects on splenic DC subset numbers. Generally speaking, caspase activation is an important step for the propagation of apoptotic cell death (148, 149). To block this, mice were treated with an irreversible caspase inhibitor, z-VAD-fmk. Necroptosis, on the other hand, is now known to rely on RIP3 kinase phosphorylation of MLKL (150, 151). We obtained mice deficient in RIP3 to investigate the role of necroptosis in DC death during infection (kind gift from Dr. John Lukens). Since treating cells with z-VAD-fmk can drive them toward necroptosis as a compensatory death pathway (152), we decided to investigate DC subsets in RIP3-KO mice with or without z-VAD-fmk treatment. This way, if necroptosis is playing an important role, both groups should exhibit DC retention vs RIP3-WT mice; whereas if apoptosis is the important death mechanism only z-VAD-fmk treated mice should manifest DC protection.

Amazingly, neither experimental group exhibited improved retention of DC numbers at 3 dpi (Figure 24A). Z-VAD-fmk treatment appeared to be working since mice treated with this inhibitor exhibited an increase in CD3 cell numbers (Figure 24B) and a proportion of T cells can undergo apoptosis during acute infection (153–155). We therefore infer that neither classical apoptosis nor necroptosis are involved in loss of splenic DC during MCMV infection.

DC trafficking is a viable hypothesis

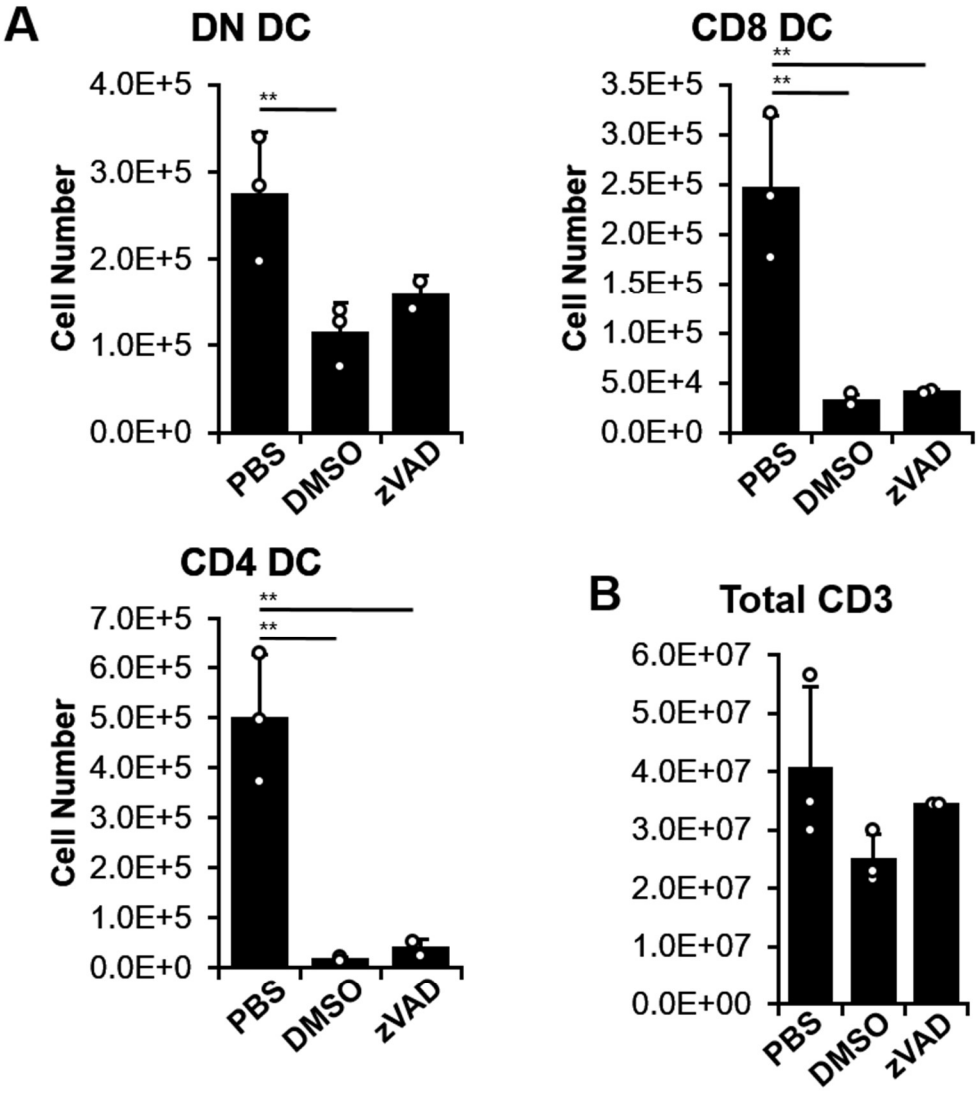


Figure 24. *Interfering with death pathways does not preserve DC.* RIP3-KO mice were treated with 3D10 1 d prior to infection ($5x10^4$ PFU MCMV). At 2 and 2.5 dpi mice were treated with vehicle (10% DMSO) or 500μg of the caspase inhibitor z-VAD-fmk i.p. Splenocytes were analyzed by flow cytometry at 3 dpi. **(A)** Quantification of DC subsets. **(B)** Quantification of $CD3^+$ cells. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

Our inability to detect increased DC death or rescue cells via disruption of death pathways spurred us toward attempts to track DC. We have performed preliminary studies to evaluate whether cellular migration is a reasonable hypothesis to pursue. We surveyed $CD11c^+$ $MHCII^+$ cells in peripheral sites 3 dpi – including blood, spleen, liver, kidney, lung, and mesenteric nodes. Since CD8 is specifically expressed on lymphoid tissue-resident DC, we hypothesized that, if splenic emigration is occurring, we should detect an increase in the representation of $CD8^+$ $CD11c^+$ $MHCII^+$ cells at peripheral locations concomitant with their reduction in the spleen. Interestingly, we observed a potential increase in this population in the liver of infected mice (Figure 25A). Moreover, the increase appeared to track with the severity of splenic loss. Resistant D^k mice (rIgG) exhibited moderate levels of splenic CD8 DC loss accompanied by a similarly moderate increase of CD8 DC in the liver. On the other hand, D^k mice made susceptible by Ly49G2 depletion (4D11) displayed a greater degree of both splenic loss and liver increase relative to the isotype-treated D^k mice (Figure 25A). We also observed an increase of $CD11c^+$ $MHCII^+$ cells in the blood (Figure 25B). However, we could not detect CD8 on these cells and they are likely monocyte/macrophage populations. Additionally, though, this could represent an increased presence of DC precursors in the circulation undergoing active differentiation. These cells may be shuttled to the liver during infection. Nevertheless, the data indicates that DC trafficking is a potential contributor to the decreases observed in splenic DC subsets.

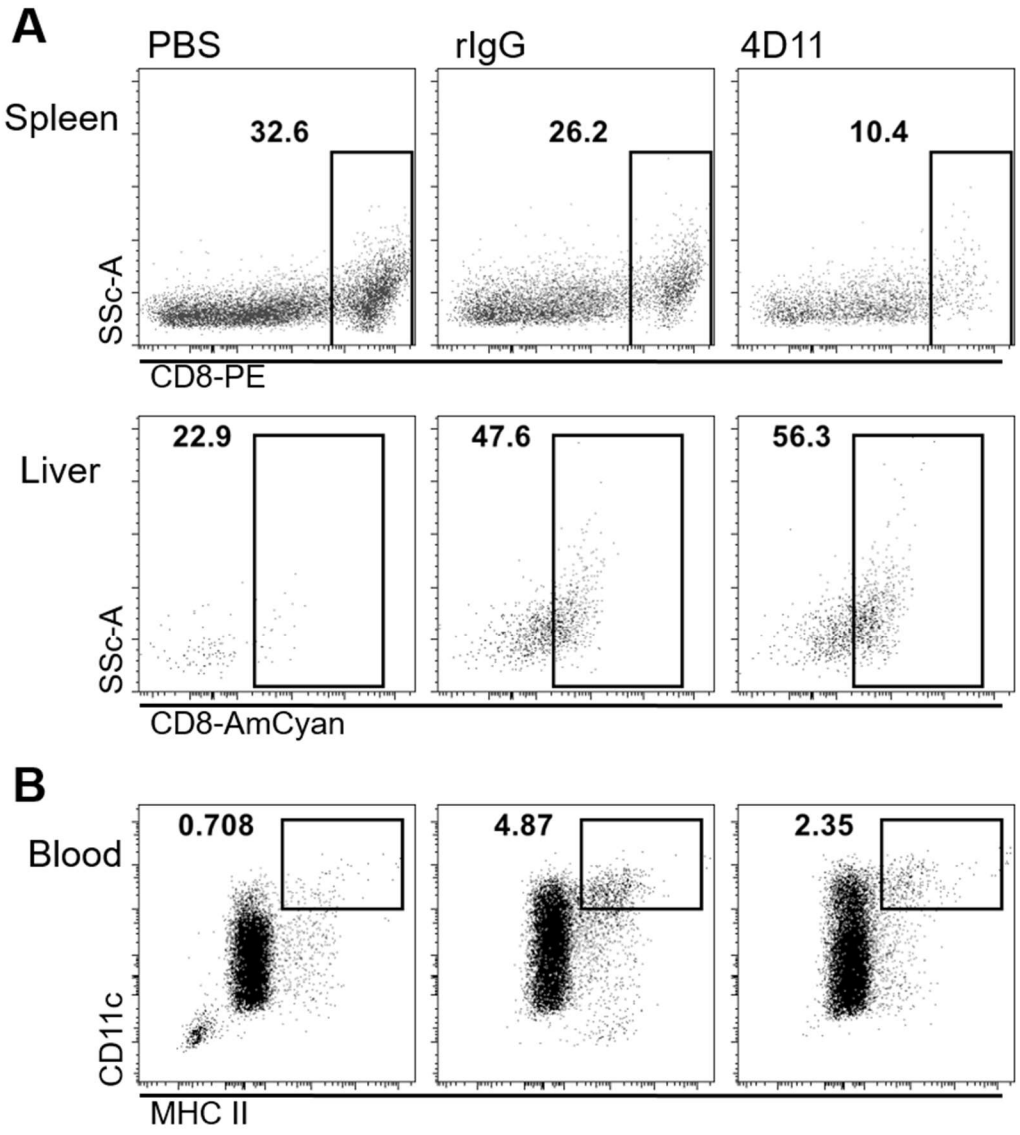


Figure 25. *Liver may acquire DC with CD8 expression during infection.* D^k mice (R7) were treated with isotype (rIgG) or Ly49G2 depleting mAb (4D11) 48 h prior to infection ($5x10^4$ PFU MCMV). Organs were harvested 3 dpi and assessed for DC. **(A)** CD8 DC from spleen and liver, gated on single cells / CD3&19-neg / CD11c&MHCII-hi. **(B)** CD11c & MHC II expression in the blood; gated on single / CD3&19-neg cells

In another attempt to assess DC migration, we injected a small bolus of fluorescent anti-CD11c (PE conjugated; clone N418) into the bloodstream of mice 2 dpi. This will quickly circulate to the spleen and liver and label any DC present at the time of injection. The 2 dpi time point was chosen since minimal DC loss has occurred at this time, allowing us to label the bulk of the population 24 h prior to their dramatic decline. We hypothesized this would allow us to track CD11c-PE accumulation at extrasplenic sites upon organ collection at 3 dpi. We would also be able to determine if any labeled cells were retained in the spleen with altered surface marker expression (i.e. DC loss due to cellular conversion). PE was chosen for the fluorescent tag due to its high level of stability across a range of pHs, temperatures, and light exposure times (156).

We observed that there was no CD11c-PE staining on remaining DC from the spleens of infected mice (Figure 26A). This was puzzling since we were able to detect CD11c on the cells *ex vivo* with a separate Ab clone (HL3) conjugated to APC-Cy7. To determine if the APC-Cy7^{+} PE^{-} cells had freshly upregulated or recycled their CD11c expression, we repeated the experiment but harvested spleens 30 minutes after CD11c-PE injection instead of 24 h. Again, we observed very little PE staining on DC from infected spleens, indicating that the Ab was unable to bind CD11c on DC *in vivo* at 2 dpi (Figure 26B). Interestingly, NK cell *in vivo* staining with CD11c-PE was unaffected by infection, showing that the inability of N418 to bind CD11c *in vivo* may be specific to DC.

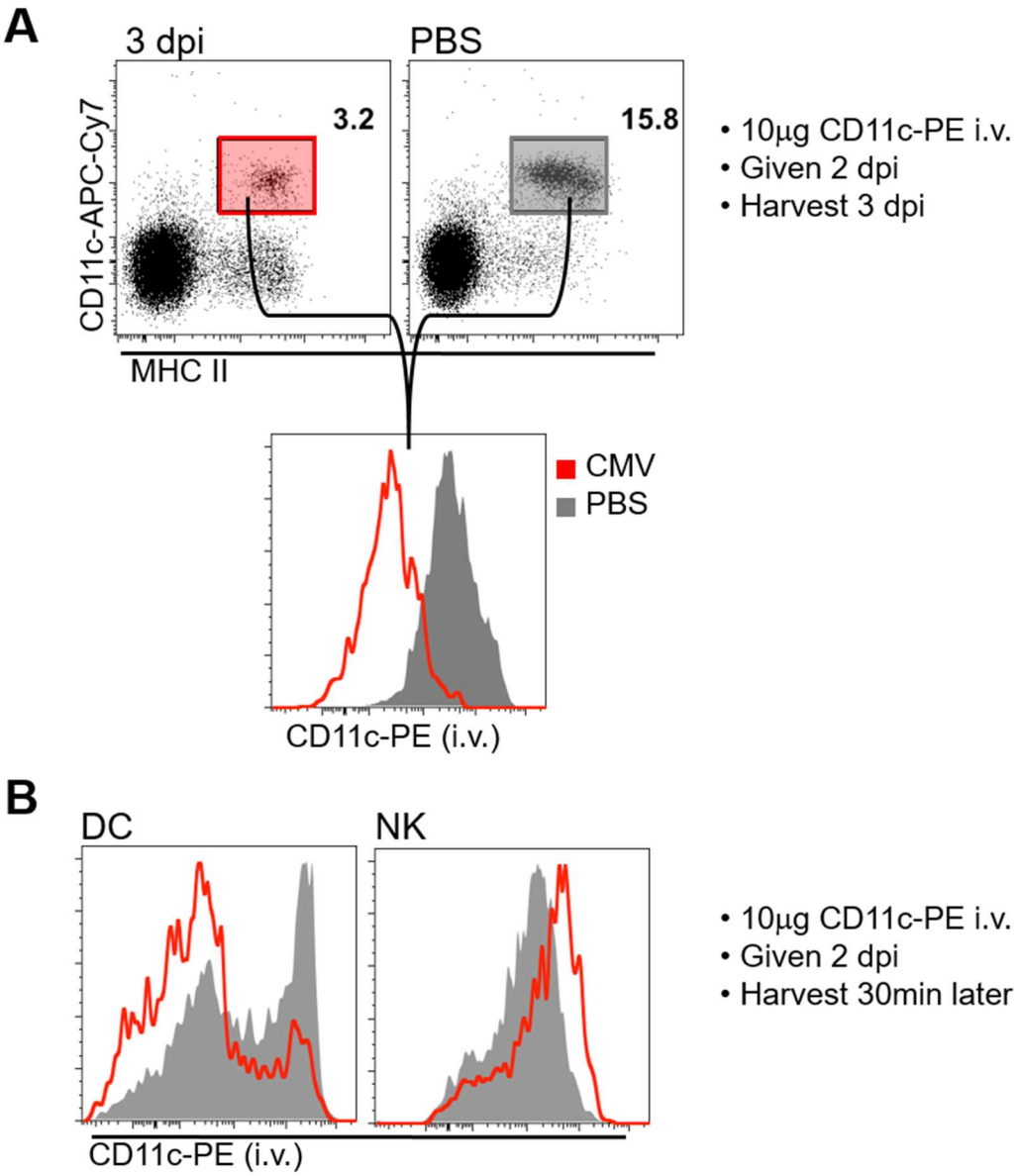


Figure 26. *CD11c is not available to circulating Ab during infection.* Mice were infected with $5x10^4$ PFU MCMV or mock infected with PBS. **(A)** Susceptible mice (4D11 treated R7) were i.v. injected with CD11c-PE at 2 dpi. Splenocytes were analyzed by flow cytometry 3 dpi (24 h after CD11c injection). **(B)** Susceptible mice (3D10 treated B6) were i.v. injected with CD11c-PE at 2 dpi. Splenocytes were collected 30min after injection an analyzed by flow cytometry.

Discussion

Like IFN-I toxicity toward DC, splenic DC fate is another topic that has been subjected to some unexplored assumptions. It used to be thought that once DC entered a lymph node (LN), they remained there until they died. However, it is now understood that a small fraction of DC are capable of emigrating from the node to enter the blood stream through the thoracic duct (30, 34). Hence, it is no longer believed that LNs are terminal organs for DC. What is still not well understood, however, is the trafficking potential of resident DC that develop within secondary lymphoid tissue (SLO). Are these cells migrating and entering circulation like peripheral tissue DC or are they a static population that never leaves its specific tissue? This question is even more prominent for the spleen, which can retain DC populations but does not actively recruit them (30). DC enter the spleen passively due to its connection to the circulation, but splenic flux beyond that is rather mysterious. Yet, based on the results obtained in this section, trafficking out of the spleen may indeed be a mechanism to explain DC loss from the spleen. Currently, this conclusion has been derived from a process of elimination thought process, as studies investigating other potential mechanisms have all yielded negative data.

Work investigating the role of direct infection of DC by MCMV yielded evidence that this didn't make logical sense as an explanation. Direct infection of DC is difficult to test due to the phagocytic nature of these cells. Many DC may take up infected cells or

material from infected cells, leading to false positives and an over-estimation of direct infection frequency. This is especially true for CD8 DC which are specialized for the uptake of dead or dying cells. Hence, the use of viruses encoding fluorescent proteins is not ideal for such studies. In addition, infiltrating monocytic cells may be included in DC populations from infected spleen if care is not taken to exclude these cells during flow cytometric analysis. Such cells are more prone to infection than DC *in vivo* (157) and therefore could artificially increase the estimation of infected DC.

One study used a cre-expressing MCMV and a ROSA26-*flox*-STOP-*flox*-YFP reporter mouse strain to try to quantify DC infection (158). In these mice, MCMV infection excises the STOP codon preceding the YFP protein to allow for transcription from the ROSA26 promoter. However, ubiquitous expression from the ROSA26 promoter recapitulates many of the same issues as a virus encoding a fluorescent protein. It would be more precise to test this system with a mouse encoding a fluorescent protein downstream of the CD11c promoter or, better yet, the promoter for the DC specific gene zbtb46. This would significantly reduce background and potential for false positive results. These tools do not exist yet but are likely not far off. Despite the issues with these systems, though, there is a rough consensus in the literature that, at most, < 4% of DC are likely to be infected during peaks of infection (110, 120, 158, 159). This level of infection does not seem nearly high enough to explain the dramatic and rapid loss we observe.

A study performed by Andrews et al (136) examining DC infection suggests that infected DC are transitioning into "virally-altered DC" displaying reduced CD11c and greatly restricted MHC II expression, which is a potential possibility. However, there is little evidence presented to indicate that this $CD11c^{+}$ MHC II^{lo} population which arises in their mice is not a separate, non-DC population altogether. These cells match the phenotype of both monocytes and NK cells which could both be increasing in proportion during MCMV infection. This could make it appear that DC progressively convert into "virally-altered" cells, when in fact DC numbers are dropping and an entirely different cell type is recruited or expanded. Furthermore, they show a near complete absence of cDC by 4 dpi, which would suggest near 100% DC-infection frequency (well above anything reported elsewhere). Considering these studies alongside our TLR stimulations and evidence for DC maturation during infection, we have concluded that there is no strong evidence for direct infection as a major source of splenic DC loss.

While examples of NK cell cytolytic killing of DC populations exist in the literature, there is really no evidence that this occurs during MCMV infection. The data from our lab showing sustained DC loss in the absence of NK cells corroborates the results seen by others. Andrews et al. showed that NK depletion in B6 mice, which normally retain their DC, resulted in significant loss of CD8 DC during MCMV infection (106). Mitrovic et al. has also investigated the role of NK cytotoxicity using mice deficient for the cytolytic protein perforin. They do not show the data in their report, but relate that they

witness dramatic reductions in cDC during MCMV infection of the perforin-knockout mice (110). Presumably, therefore, DC loss is not reliant on perforin-dependent cytotoxicity from NK cells (or T cells). This collection of evidence rules out a role for NK in enforcing DC loss.

Since DC activation precedes loss, we have considered the possibility for an activation-induced cell death mechanism. Logically, TLR stimulation would be a strong candidate as the inducer of this type of pathway since they are directly upstream of IFN-I expression. This could explain why IFN-I appeared responsible for DC loss. If DC were dying in response to TLR activation, IFN-I expression would be inherently correlated to the phenotype.

However, one puzzling aspect from the TLR data is that CD4 DC are reduced in response to stimulation of TLR they do not express. CD4 DC lack expression of TLR3 (27, 28) but still exhibit strong loss following poly I:C injection, suggesting CD4 DC loss is not dependent on direct activation by TLR3. Hence, the implication is that CD4 DC numbers are being regulated by stimulation of a separate, $TLR3^+$ cell. Alternatively, CD DC could be responding to TLR3 through cytosolic DNA sensors (e.g. MDA-5), but stimulation of these sensors generally require an additional reagent to target poly I:C to the cytosol after injection. This line of evidence indicates direct TLR stimulation is not necessarily involved in the DC loss, but to conclude this firmly we will need to run experiments with MyD88 and TRIF knockout DC. These studies will likely require

mixed BM chimeras since we will need to directly compare WT and KO DC to determine the cell intrinsic effects, much like what was done in figure 14 for IFN-I effects.

Interestingly, the TLR data do present the possibility that DC loss is being regulated in a cell-exogenous manner. DN DC express some TLR3 (but not to the same levels as CD8 DC), which could indicate that TLR3 stimulation is responsible for the loss of these cells. However, they also express significant levels of TLR7 and 9 but exhibited no loss in response to imiquimod, CpG-A, or CpG-B. This further indicates loss is not a general effect of TLR stimulation and may require activation of an accessory cell. CD8 DC express both TLR3 and 9, but not TLR7. In light of this, direct stimulation of these cells could be leading to their loss since only poly I:C and CpG-B induced their attrition. However, it is still unclear why CpG-A would have no effect while CpG-B potently induces loss. This will require more in depth analysis of the similarities and differences between CpG-A, CpG-B, and poly I:C to discern aspects (e.g. cytokines, cells activated, morphology and localization changes in the spleen) of each response that correspond to DC loss.

While our initial experiments have indicated soluble factors are not responsible for DC loss, this finding still requires independent verification. The serum transfer study, while interesting, has distinct caveats that prevent firm conclusions from being made. First is the fact that a 1:1 transfer of serum (serum collected from 3 mice and transferred into 3

mice) means there is still at least a 50% dilution of any factors present. Once the material is injected into the blood stream of the recipient, the infected serum fraction is, at most, half of the total serum volume of in the mouse. Additionally, we only administered a single bolus of serum, which likely differs from the cumulative, sustained production of cytokine that is likely occurring during active infection. With this in mind, it may be necessary to administer serum as a concentrated bolus or via repeated transfers to see a significant effect. Another potential issue is that the serum was administered systemically. Hence, if local concentration or intrasplenic gradients are important in any way, these effects will not necessarily be recapitulated. This requires more in depth experimentation to make firm conclusions about the role of secreted factors and future work will address these issues. However, what can be taken from this data currently is that there does not appear to be a potent, soluble regulator of DC numbers present in ~2 d-infected serum, the peak of cytokine production.

Similarly, our work on cDC precursors (pre-cDC) is far from conclusive. To truly call these DC precursors, we will need to confirm the capability of the population to exclusively generate cDC upon transfer into hosts and in culture. Once we verify the homeostatic population as a true pre-cDC, we will need to isolate the cells from infected mice and test their differentiation potential. This is important since they may be functionally impaired. If pre-cDC are unable to differentiate in an MCMV environment, the fact that we do not see the population decrease in numbers during infection becomes a moot point.

While we cannot yet make firm conclusions about the impact of MCMV on DC precursors, we do have good data indicating that the cells are not being remove through apoptosis or necroptosis. Our inability to detect increased DC death or restore DC numbers by death inhibition strongly indicates that these mechanisms are not responsible. Interestingly, while DC generally have fairly rapid turnover times at steady state (107, 160) they aquire resistance to some forms of death signaling after activation/maturation. For example, immature DC are susceptible to the external pathway of Fas-mediated cell death, but upon activation become resistant to Fas engagement (161–163). Interestingly, they also increase anti-apoptotic proteins in response to IFN-I and/or TNF signaling (48, 164).

Since we have found no role for cell death, DC trafficking has become a main focus moving forward. This is, however, a daunting task which will likely require innovative models and strategies to investigate. Ideally, we need a way to track DC that originated specifically in the spleen and detect them leaving, in transit, and increasing in numbers at another anatomical location. Without knowing where the DC might go, though, we are currently unsure where to look to detect increased accumulation of spleen derived DC. However, our initial results from figure 25 suggest the liver is a good candidate. Moving forward, future studies on trafficking will require use of zbtb46^{gfp} mice, since this will allow us to define DC populations with minimal effort and high specificity.

However, we will still require a way to specifically mark spleen DC at some point. Without a way of determining the source location of the liver CD8 DC, we will be unable to know if they immigrated from the spleen or are locally expanded/differentiated, recruited from the blood, or entering from a lymph node. Also, the apparent correlation between increased cells in the liver and splenic loss could be due to differing levels of prolonged inflammation and sustained viral loads in the G2+ NK depleted mice. This is something we are looking into (perhaps through exploiting tissue specific promoters), but time will be required to develop a suitable strategy.

This work has begun the exploration of IFN-I-independent mechanisms of DC loss, but there is still a great much to be done on this topic. It is difficult to draw firm conclusions from the experiments presented here due to the lack of clear positive results, but some elements are clear. We now know that virus and NK are not required for DC loss and strong DC activation precedes the phenomenon. We also know that the process can occur in the absence of virus, but may be dependent specific types of stimulation/inflammation. The role of soluble factors merits more investigation, but for now it appears there is no potent toxicity present in infected serum. This means that, if a soluble component is involved, it could be dependent on local production in the spleen or *in vivo* gradients that are not recapitulated by serum transfer. Additionally, we have not observed significant fluctuations in a putative pre-cDC population and the fact that IFNAR blockade or knockout does not increase DC numbers suggests that IFN-I is not impairing splenic precursor populations. To date, we have not observed a role for

apoptosis or necroptosis, but this does not rule out the possibility of alternative mechanisms of cell death. Apparently, there is a mechanism of B cell and DC death that is driven by MHC II and is independent of caspase activation but dependent on mitochondrial calcium flux (165–168). This may merit investigation as well. Alternatively, a role for cell trafficking has not been formally ruled out, but this will require sophisticated systems to investigate thoroughly. Overall, what we have seen so far is a process that is dependent on inflammatory stimuli leading to DC activation followed by rapid loss, either through migration or alternative death pathways.

CHAPTER III – Licensed Ly49G2$^+$ NK Cells Enhance Splenic DC Recovery

Introduction

In addition to their prominent role in innate immune protection during viral infection, recent reports have shown NK cell responses can also lead to dampened T cell immunity (169). This can occur through either direct T cell killing or reducing the amount of antigen available for DC priming of virus-specific T cells (63, 110, 170–172). Despite these findings, we have previously shown that licensed NK cells which can specifically respond to MCMV infection promote an accelerated accumulation of virus-specific T cells and efficient viral clearance (109). Since MCMV is well known for modulating dendritic cell (DC) numbers during infection, it is possible that the retention of these cells influences the kinetics of T cell priming. Indeed, Stadnisky et al showed a rapid recovery of splenic DC in mice with an efficient licensed-NK cell response. Here, we investigated the ability of the Ly49G2$^+$ NK cells that have been licensed by self expression of H-D^k to support DC recovery during MCMV infection.

It is well known that there is intimate cross-talk between NK and DC. Previous work has shown that Ly49H$^+$ NK cells play a significant role in preserving DC populations during MCMV infection (106). In the Ly49H model, DC are essentially preserved with no significant initial loss, although one could imagine that pushing the dose high enough would manifest DC loss similar to susceptible mice. One particularly interesting

observation from our MCMV studies in the C57L background is that despite an initial DC loss, mice with a licensed Ly49G2 population mounted a protective NK response and rapidly reconstituted their DC compartment within ~24-36 h of loss (Figure 3 & (109). This contrasts with what has been observed in other resistant strains and strains lacking efficient NK protection. Rather than resulting in DC maintenance (other resistant mice) or prolonged DC suppression (susceptible mice), we observed a third profile marked by initial DC loss followed by rapid reconstitution. We therefore hypothesized that the licensed NK response could be promoting DC expansion and recovery.

Results

Ly49G2$^+$ NK cells are necessary for rapid DC recovery

Both D^k and D^b mice contain Ly49G2$^+$ NK cells (G2+ NK), but only mice expressing the MHC I-D^k allele license this population at steady state. To address a potential role for licensed NK cells in promoting DC recovery following MCMV-induced loss, we first looked at DC in infected D^k and D^b strains at time points after loss. Our earlier experiments with these MHC I-disparate mice clearly revealed the importance of the D^k molecule in rapid DC recovery at 4 dpi (see Figure 3). In fact, by 4 dpi, DC subsets from D^k mice trended toward increased numbers relative to uninfected mice (red lines = average number of DC seen in uninfected mice).

We also examined a specific role for G2+ NK cells in splenic DC protection and recovery. Depletion of G2+ NK cells prior to MCMV infection increased the severity of DC loss in D^k mice to similar levels as those seen in D^b animals at 3 dpi (Figure 27A). Furthermore, G2-depletion abrogated the rapid accumulation of splenic DC observed in D^k mice; rather, splenic DC numbers remained low through 4 dpi (Figure 27B). Hence, even in the presence of residual NK cells, the licensed $G2^+$ NK cells are necessary for D^k mice to efficiently reconstitute splenic DC during MCMV infection.

Ly49G2⁺ NK cells interact with DC

NK cells have been shown to acquire surface proteins via a process termed "trogocytosis" (cell eating) (42, 173–176). Upon interacting with other cells, NK sample their partner's membrane, allowing the transfer of surface molecules to the NK. An example of this is the transfer of MHC II which is highly expressed on DC. The NK cells themselves do not express mRNA transcript for MHC class II production, but, upon generating cell-cell contacts with DC, acquire enough surface MHC class II protein for clear detection during flow cytometric analysis (42). This is, therefore, a surrogate marker for interactions between NK and DC in mice. We evaluated this phenomenon in by staining for MHC II on the surface of NK cells during infection. $NKp46^+$ cells from D^k mice displayed greatly increased staining intensity of surface MHC II by 54.hpi when compared to uninfected MHC II levels (Figure 28A). NK cells from D^b mice also had increased surface levels of MHC II compared to uninfected mice, but this paled in comparison to the increase seen from D^k mice (Figure 28A). When NK cells from D^k mice

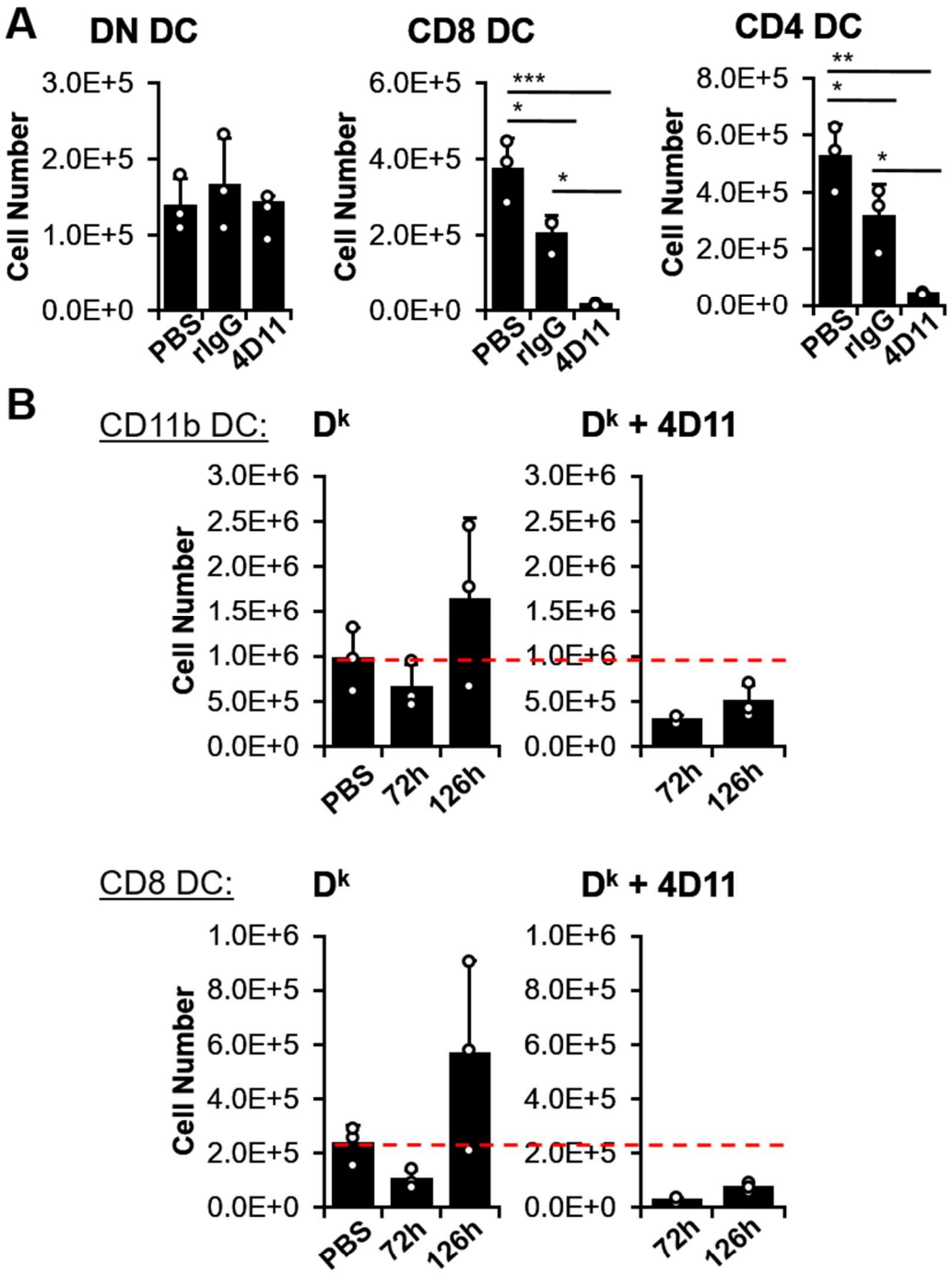


Figure 27. *Ly49G2 depeletion impairs retention and expansion of DC subsets.* **(A)** D^k Mice (R7) were treated with isotype (rIgG) or Ly49G2-depleting mAb (4D11) 48 h prior to infection with $5x10^4$ PFU MCMV. Splenic DC were analyzed 3 dpi **(B)** D^k mice (R7 and R12) were given either vehicle (PBS) or Ly49G2-depleting mAb 36 h prior to infection with $2x10^4$ PFU MCMV. Splenic DC were analyzed 72 and 126 hpi. Red line denotes value from mock infected mice. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

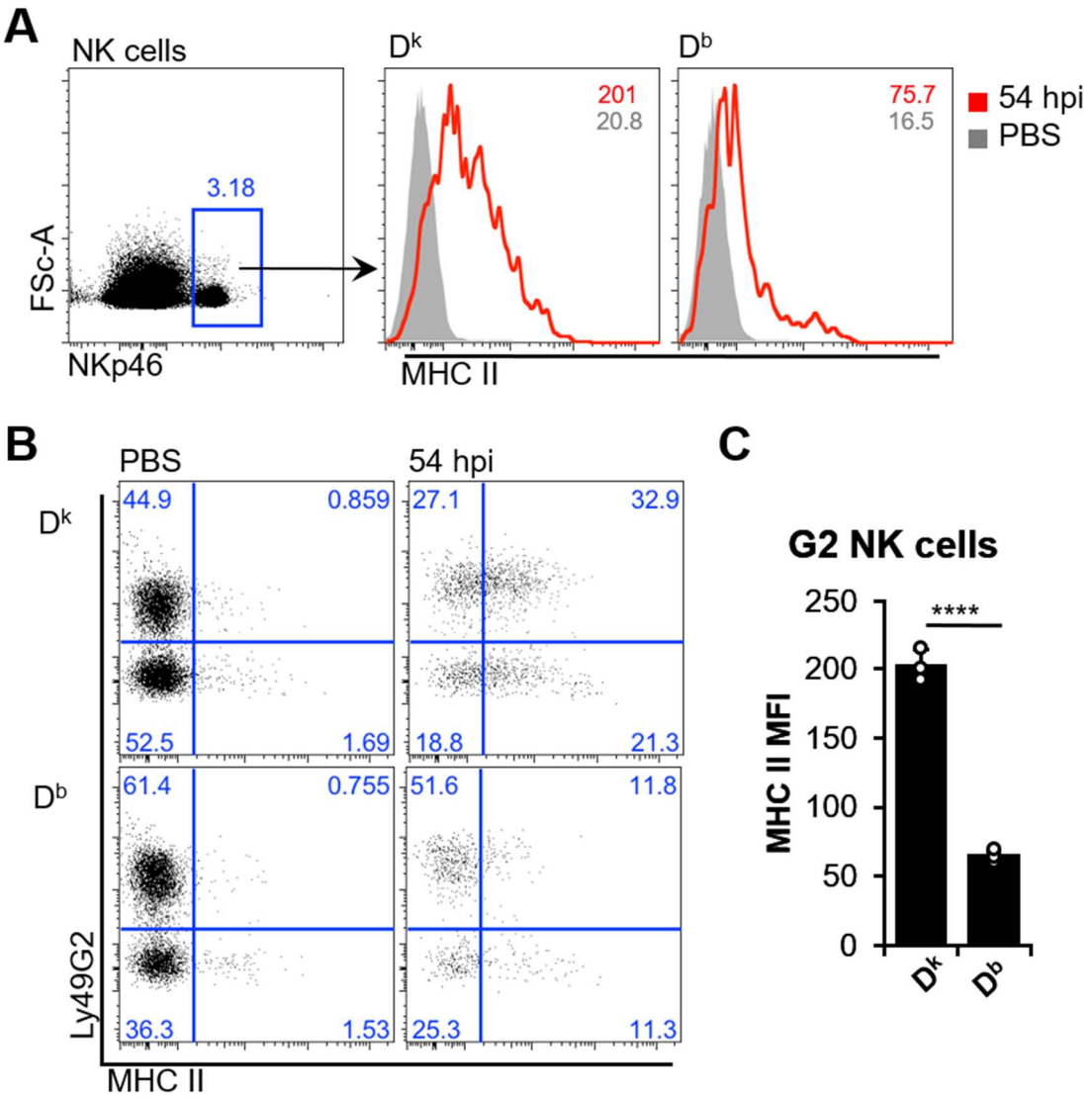


Figure 28. *Licensed NK exhibit greater MHC II acquisition*. D^k and D^b mice (R7 and R2 respectively) were infected with $2x10^4$ PFU MCMV. Splenocytes were isolated at 54 hpi for analysis. **(A)** $NKp46^+$ cells were evaluated for MHC II intensity at 54 hpi. **(B)** NK cells were separated into $Ly49G2^+$ and $G2^-$ subsets and their acquisition of surface MHC II was compared. **(C)** Comparison of Overall MFI of MHC II on $Ly49G2^+$ cells from infected D^k and D^b. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

were separated into Ly49G2^{+} an Ly49G2^{-} subsets, there did not appear to be a preference for MHC II acquisition by either subset. Slightly over 50% of G2^{+} and G2^{-} cells had increased MHC II staining. Hence, NK-DC interactions are generally increased during MCMV infection in the presence of a specific licensed NK response.

Licensed Ly49G2^{+} NK cells protect splenic DC accumulation in D^{k} mice

DC recovery and accumulation above homeostatic levels in D^{k} mice prompted us to investigate if they were expanding in response to NK-mediated virus control. For this, we used BrdU incorporation assays as a measure of proliferation (pulse of BrdU administered 3 h prior to analysis). At 90 hpi, Dk mice had more BrdU+ CD11b DC, both in terms of absolute numbers and frequency, than uninfected mice (Figure 29). In contrast, the CD11b DC from Db mice had a slightly increased frequency of BrdU+ cells, but no increase in numbers over uninfected mice. This suggests that both strains have more cells undergoing division during MCMV infection (i.e. increased rate of proliferation), but only the Dk mice are able to maintain their CD11b DC population in substantial numbers. We observed similar results with the splenic CD8 DC subset. Both Dk and Db mice exhibited increased BrdU frequencies at 90 hpi, but only Dk mice displayed increased numbers (Figure 30). This, again, indicated that DC from both D^{b} and D^{k} mice had proliferated during the 3 h BrdU pulse and were cycling at higher rates than uninfected mice, but only D^{k} mice were capable of retaining numbers within both subsets. In other words, D^{b} mice were unable to accumulate DC numbers in the spleen despite increases in proliferation rates over uninfected mice.

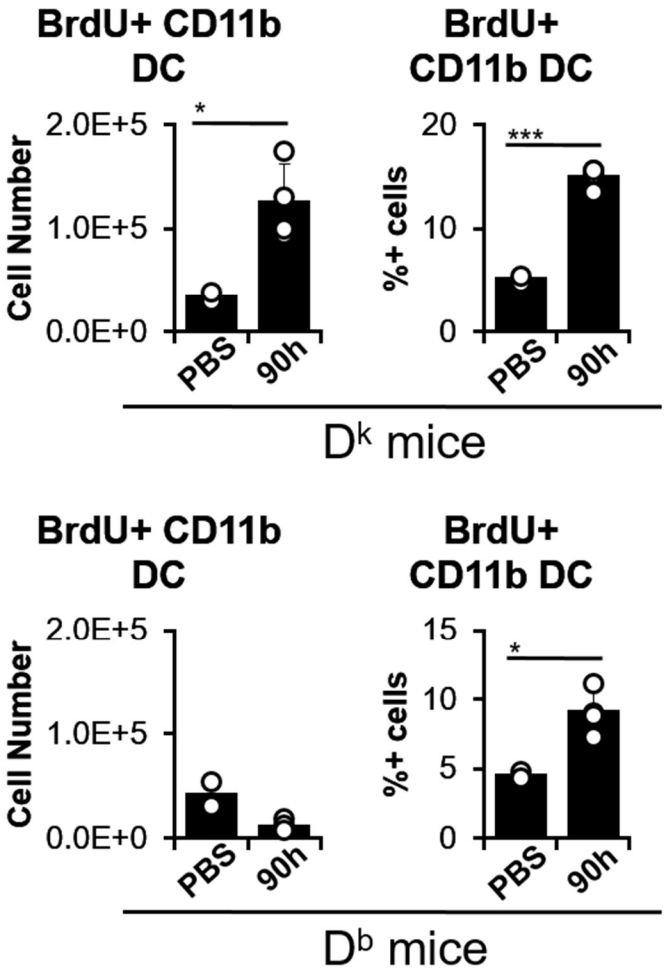


Figure 29. *D^k mice accumulate BrdU$^+$ CD11b DC.* Dk and Db mice (R7 and R2 respectively) were infected with 1x104 PFU MCMV. 3 h before organ harvest the mice were dosed with 1mg BrdU each i.p. Spleens were collected 90 hpi and assessed for DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

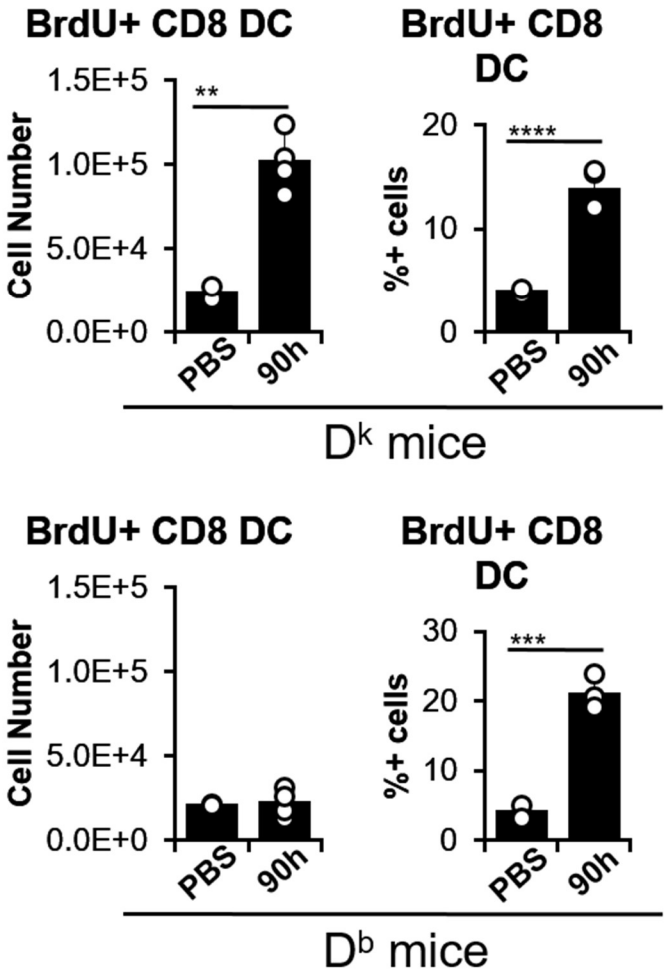


Figure 30. *D^k mice accumulate BrdU$^+$ CD8 DC.* Dk and Db mice (R7 and R2 respectively) were infected with 1x104 PFU MCMV. 3 h before organ harvest the mice were dosed with 1mg BrdU each i.p. Spleens were collected 90 hpi and assessed for DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

Productive Ly49G2^{+} NK-licensing is required for enhanced DC recovery during MCMV infection

We have shown previously that, in bone marrow chimeric mice, productive licensing of Ly49G2 NK cells requires D^{k} expression on both hematopoietic and non-hematopoietic cells (78, 100). Using C57L mouse strains with differential expression of a D^{k} transgene (C57L.Tg-D^{k} (a.k.a. Tg or T) and C57L littermates (a.k.a. non-Tg or N)), we generated bone marrow (BM) chimeras. Chimeras were created by reconstituting T host mice with either T or N donor BM (T:T and N:T) as well as reconstituting N host mice with T donor BM (T:N). Of these combinations, only the T:T mice will have homogeneous expression of the Ly49G2-licensing ligand D^{k}. In the other chimeras, G2+ NK are only exposed to D^{k} expression on the hematopoietic (T:N) or stromal (N:T) compartments. Despite availability of D^{k} in all mice, only the T:T chimeras exhibited productive G2+ NK licensing and MCMV control (100); demonstrating the importance of MHC expression by both hematopoietic and non-hematopoietic cells for proper NK licensing and responsiveness.

Thus, we assessed DC recovery during MCMV infection in the context of fully competent or impaired licensed-NK cells. The results from these chimeric mice confirmed our previous results that DC recovery tracks with licensed NK cells. At 90 hpi, only the T:T chimeras exhibit increases in DC numbers (Figure 31). Hence, DC recovery is not due to expression of D^{k} on the DC, since low DC numbers were

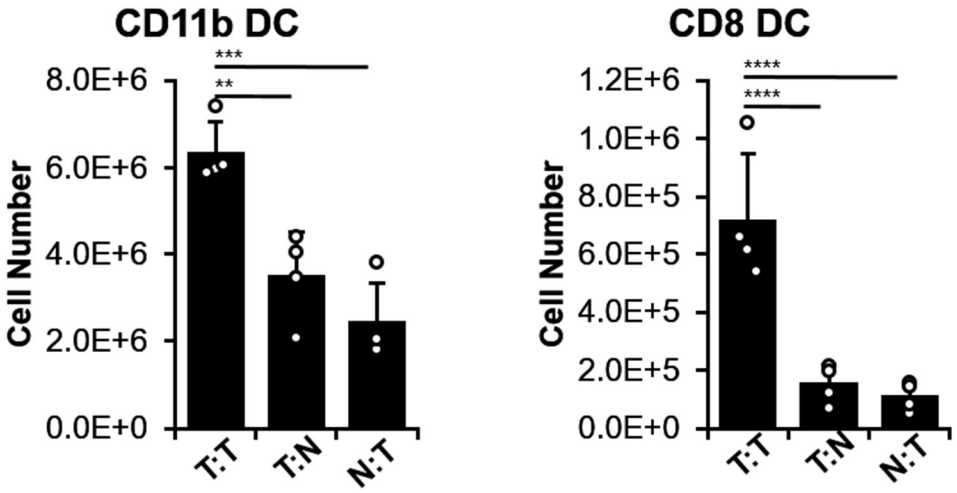


Figure 31. *Only T:T chimeras harboring licensed NK cells show signs of expanding DC at 90 hpi.* BM chimeras were generated by irradiating D^k-transgenic (C57L.Tg3-D^k; T) or non-transgenic (C57L littermates; N) recipients and transferring BM from T or N donors to establish T:T, T:N, and N:T chimeric mice. Mice were infected with $2x10^4$ PFU MCMV and splenocytes analyzed by flow cytometry at 90 hpi for DC populations. Significance is displayed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Error bars represent SD.

maintained in T:N mice. Rather, the results suggest that, in this context, NK cell licensing is important for DC recovery.

DC suppression is dependent on virus dose

We next assessed if DC loss was responsive to virus dose and if this could overcome any protection granted by NK. Since mice without licensed G2+ NK always exhibit higher residual virus levels, we wanted to examine DC recovery in a situation where virus levels were equal between D^k and D^b mice.

We have run experiments investigating this by infecting mice with a range of virus inoculums ranging from $2x10^4$ PFU to $1x10^5$ PFU MCMV. The results indicated that retention of cDC in D^k mice was dose dependent up to a point (see Figure 2). Doses of $5x10^4$ and $1x10^5$ PFU exhibited essentially the same degree of DC loss, but both were more severe than the $2x10^4$ PFU dose. Hence, the degree of DC loss at 3 dpi can be modulated in resistant mice.

We next investigated the impact of higher doses on DC recovery. When infecting with inoculums between $2\text{-}5x10^4$ PFU, we see robust DC recovery in D^k mice by 4 dpi (see Figure 3). We wanted to test the hypothesis that licensed NK cells provide a direct benefit to DC. If this is the case, Dk mice should display strong DC recovery regardless of virus dose. Therefore, we compared DC recovery in D^k mice infected with $1x10^5$ PFU to D^b mice infected with $1x10^3$ PFU. (According to previous experiments, we knew these

doses should result in similar virus levels between the two groups 4 dpi). We also included a group of D^k mice infected with $1x10^4$ PFU MCMV. We knew this group would exhibit strong DC recovery 4 dpi and planned to use it as a benchmark for "expected" DC recovery.

The results revealed that, while we saw high numbers of DC in the $1x10^4$-infected D^k, DC did not recover in the D^k mice infected with $1x10^5$ PFU (Figure 32A). DC numbers were comparable between the high-dose D^k and low-doseD^b mice (Figure 32A). As planned, virus levels were also similar between these two groups (Figure 32B). This indicates that sustained virus levels is likely a key factor regulating DC numbers.

MCMV-induces prolonged DC suppression in mice without Ly49G2$^+$ NK control

Since NK cell-mediated restriction of MCMV appeared to be a key element governing DC recovery, we next investigated the extent of DC suppression in the absence of an efficient G2+ NK response. In the context of licensed G2+ NK cell MCMV control (D^k mice), we observed that splenic DC numbers (both CD8 and CD11b subsets) were recovering by 90 hpi and exhibited a robust expansion by 6 dpi which began returning to baseline by 12 dpi (Figure 33). However, in mice lacking the protective G2+ NK response (D^b), DC numbers displayed a different pattern. The CD8 DC subset was affected most severely, exhibiting extremely low numbers through 6 dpi but finally recovering by 12 dpi. CD11b DC appeared to mostly recover by 6 dpi, but the effect was rather variable between mice, with some animals still well below baseline cell numbers

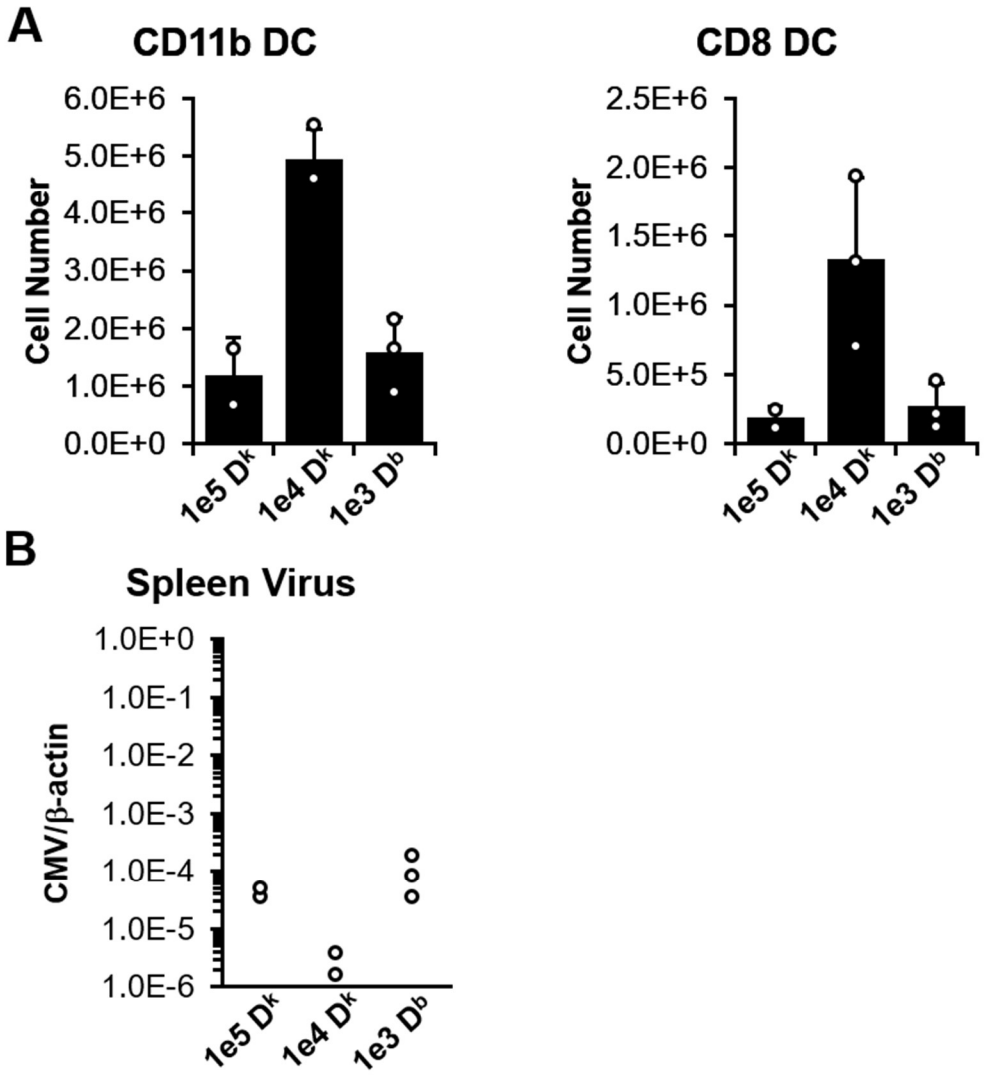


Figure 32. *High virus doses restrict rapid DC recovery.* D^k mice (R7) were infected with $1x10^5$ (1e5 D^k)or $1x10^4$ (1e4 Dk) PFU MCMV; D^b mice (R2) were infected with $1x10^3$ (1e3 D^b) PFU MCMV. Spleens were analyzed at 4 dpi **(A)** Quantification of DC numbers by flow cytometry. **(B)** Splenic viral titers at 4 dpi.

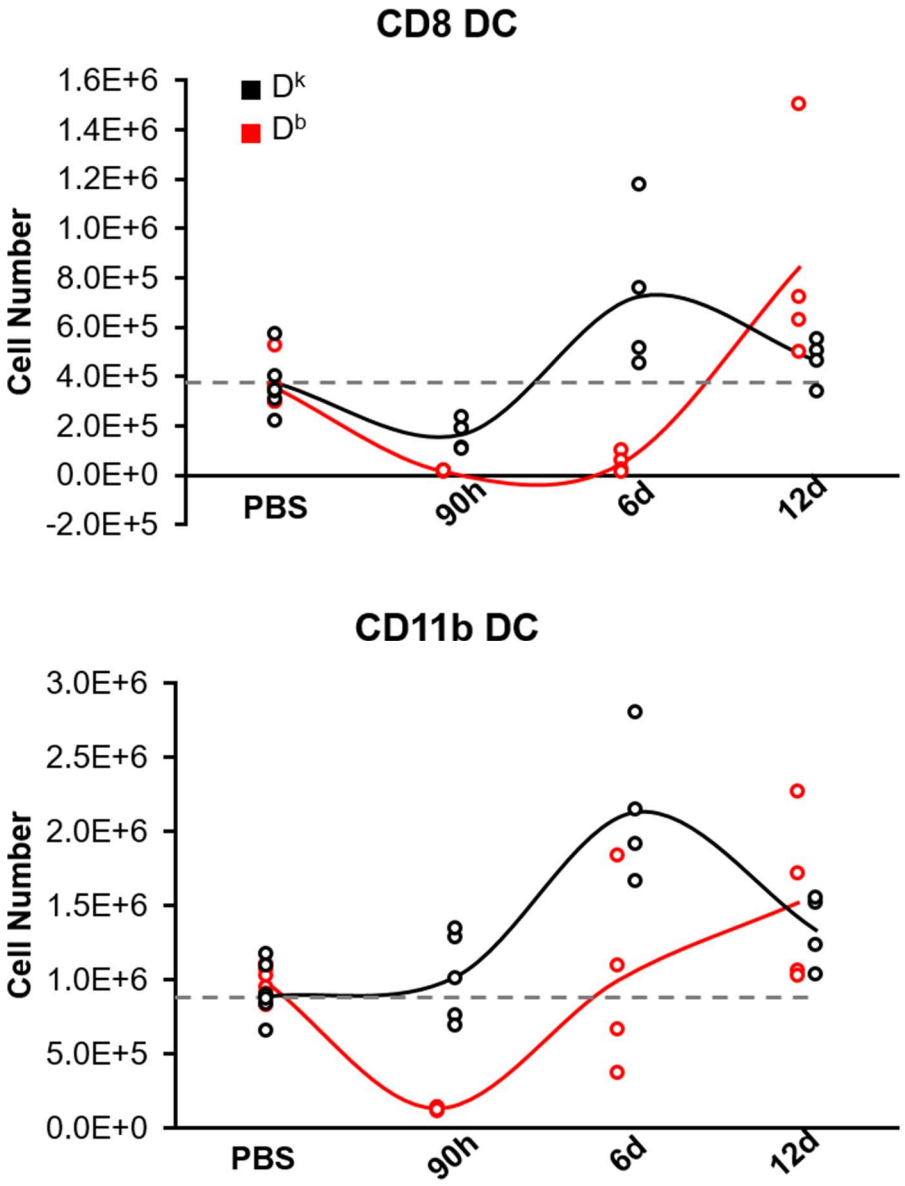


Figure 33. *DC recovery is dealayed in D^b mice.* D^k and D^b mice (R7 and L respectively) were infected with $2x10^4$ PFU MCMV. Splenocytes were analyzed by flow cytometry at 90 hpi, 6 dpi, and 12 dpi for DC populations. Time points were collected on separate days which does not allow for formal statistical comparison.

(Figure 33). Interestingly, the results show that Db mice exhibit a DC expansion around 12 dpi, while DC from mice with a licensed NK response are contracting (Figure 33). This appears to coincide with a time frame when these mice have finally significantly controlled their MCMV levels(109).

A specific NK response accelerates DC accumulation

Although sustained virus levels appear to be a dominant element controlling DC suppression, we wanted to know if a specific NK response might enhance DC expansion following the restriction of viral load. We compared DC numbers at defined time points following injection of a general NK stimulus (poly I:C; TLR3 agonist and strong inducer of IFN-I) or a specific NK stimulus (MCMV; specific stimulus for licensed G2+ NK in D^k mice). Poly I:C alone causes a transient inflammation without any antigen for specific recognition or a replicating agent to sustain DC suppression. MCMV, on the other hand, will generate a specific response in G2+ NK and cause sustained DC suppression in mice without D^k.

Interestingly, we did not observe DC loss in D^k mice during this experiment, likely due to the dose and stock used. However, since Db mice gradually lost DC populations over the course of the experiment the stock used was clearly capable of causing infection and DC suppression. This must mean that the D^k mice rapidly controlled the MCMV infection. Despite such a rapid control of virus, we still observed robust expansion of splenic DC subsets from 60-96 hpi (Figure 34 A&B). Hence, DC accumulation follows the

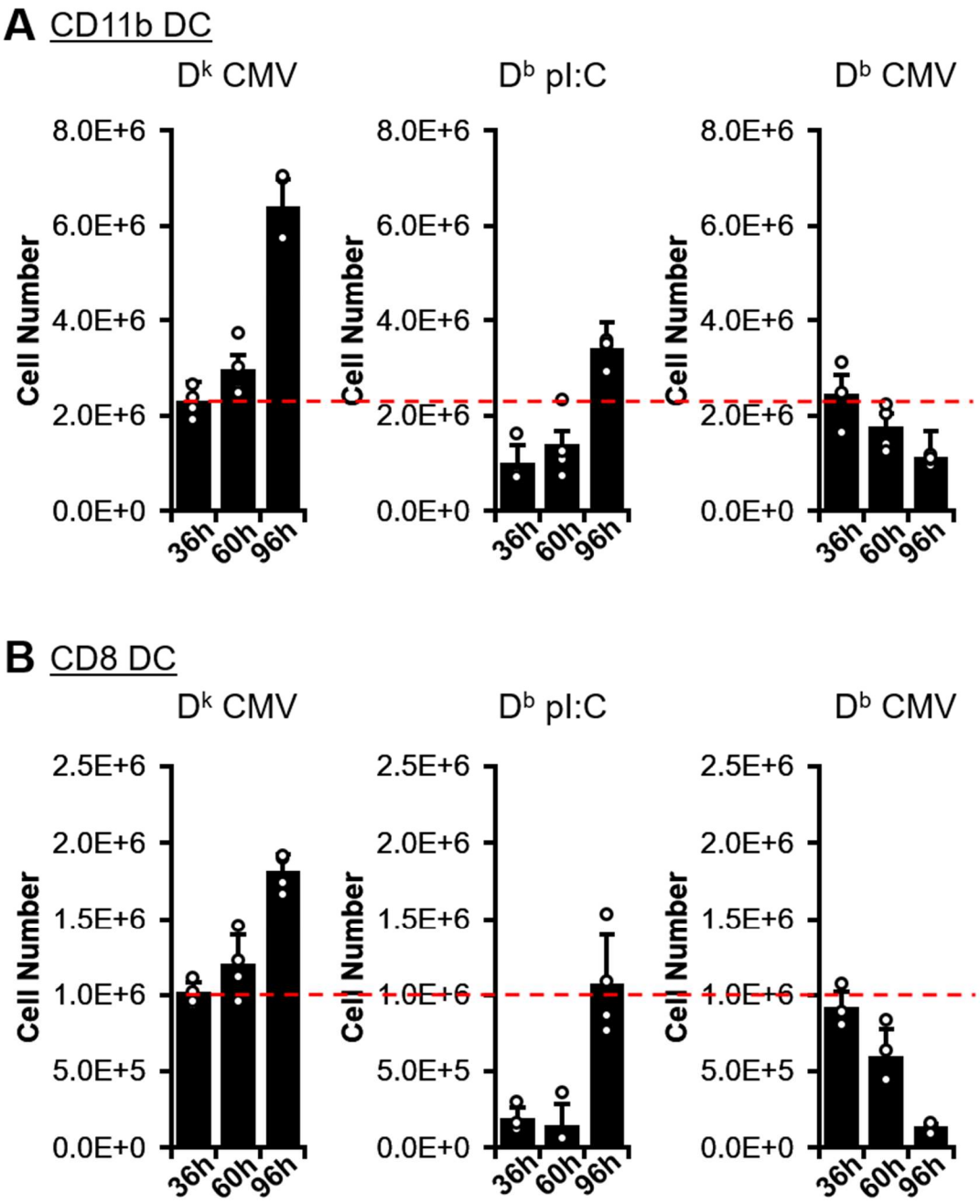


Figure 34. *Licensed NK-dependant viral control reverses DC suppression.* D^k mice (R7) were infected with $2x10^4$ PFU MCMV, D^b mice (R2) were injected with 200µg poly I:C or $2x10^4$ PFU MCMV. Splenocytes were analyzed for DC subsets by flow cytometry at 36, 60, and 96 hpi. **(A)** Quantification of CD11b DC numbers. **(B)** Quantification of CD8 DC numbers. Time point data was collected on separate days, which does not formally allow for direct statistical comparison. Red lines indicate value for D^k DC at 36 hpi for visual comparison (no gross changes are seen in DC numbers at 36 hpi, so value should be similar to numbers from an uninfected animal).

licensed response even when virus levels do not reach suppressive levels. Moreover, this expansion happens rapidly in the context of a licensed G2+ NK response, potentially faster than DC recovery after poly I:C.

During MCMV infection, pronounced DC loss generally occurs at 3 dpi (see Figure 27). Following poly I:C, significant loss begins at 36 h post injection (Figure 34). Yet DC recovery happens by 4 d in both scenarios. Essentially, this means DC recovery takes about 60 h after poly I:C and 36 h during MCMV. Unfortunately, the CMV and poly I:C groups in figure 34 are not easily compared, but the individual pattern of each supports the hypothesis that a specific NK response can induce rapid accumulation of splenic DC subsets, possibly with accelerated kinetics compared to homeostatic reconstitution.

DC expansion is not exclusive to Ly49G2 responses

To further evaluate the potential benefits of a licensed antiviral response on splenic DC populations, we compared our Ly49G2+ NK model to Ly49H-mediated control. The hypothesis here being if licensed NK are specifically supporting DC numbers, we should see DC expansion in mice with a G2-mediated response and not in those reliant on an H-response. We wanted to compare these systems on the same genetic background and generated two new mouse strains. To produce licensed Ly49G2+ NK cells in B6 mice, we introgressed our D^k transgene and the C57L NKC into the B6 background using traditional speed congenic approaches (90). This resulted in two new strains: B6.Tg-D^k.NKC^{b6} and B6.Tg-D^k.Cg-*NKC*^{l}. While both strains express MHC I-D^k, only the mice

with NKC^{l} express a Ly49G2 that binds and licenses on it. The NKC^{b6} mice, on the other hand, express the activating receptor Ly49H, which mediates MCMV recognition and control by recognizing the viral protein m157. Using these mice, we compared DC loss and expansion in the context of a licensed G2 response or unlicensed H response. Splenic DC were analyzed 2, 4, and 6 dpi. As expected, the NKC^{b6} mice did not lose DC during the course of the experiment. However, DC loss was observed in the NKC^{l} at 4 dpi (Figure 35). At 6 dpi, both strains expanded their splenic DC populations to similar levels. This suggested that H-dependent MCMV control can also promote DC expansion. According to these results, the important element for DC accumulation appears to be the ability to mount a specific response to infection

Discussion

While we have published extensively on the ability of licensed G2+ NK to control MCMV (77, 78, 100, 109), this is the first time we have formally shown a requirement for these cells in DC recovery and maintenance via several approaches. Infecting mice without the licensing ligand (D^{k}), an intact G2+ NK population, or productive licensing due to chimeric MHC I expression all lead to an inefficient NK response and inability to recover DC in a timely fashion. In fact, these mice exhibit suppressed DC numbers for extended periods (through 6 dpi) while mice with licensed G2+ NK cells promote

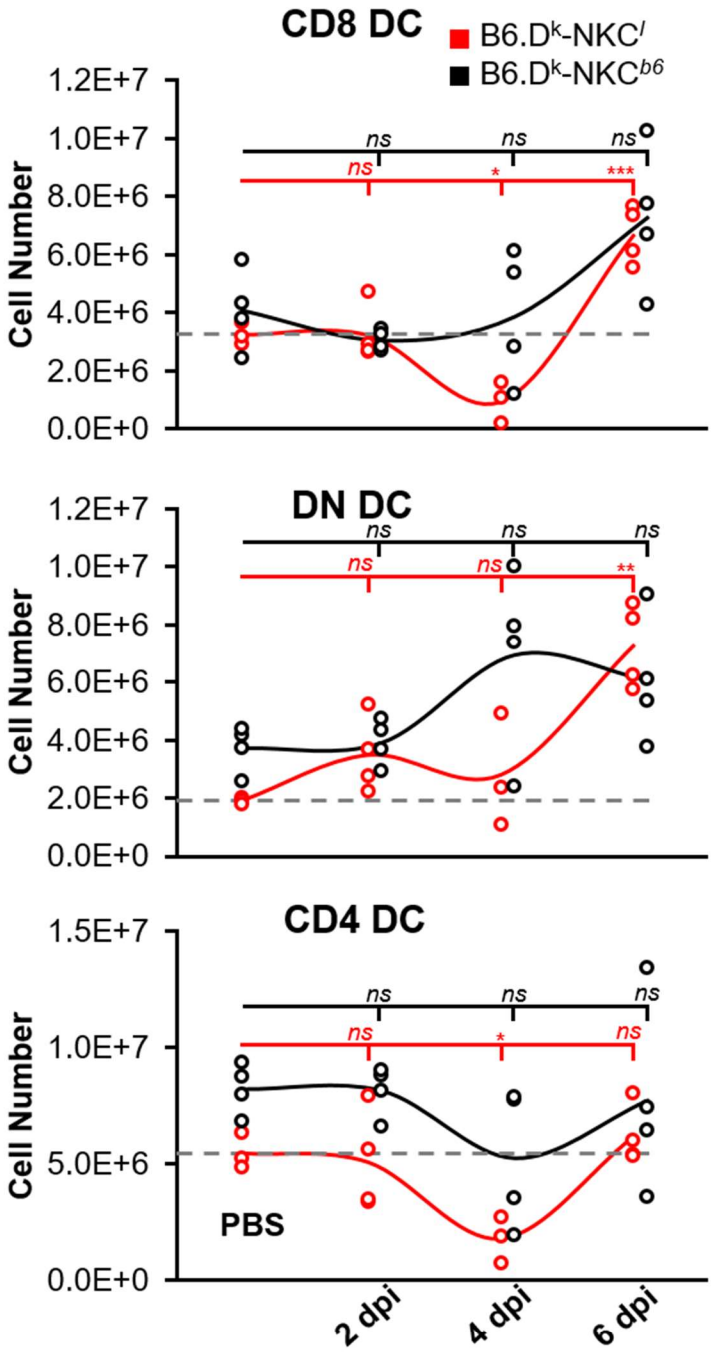


Figure 35. *DC populations expand following specific NK responses.* B6.D^k-NKC^l or B6.D^k-NKC^{b6} mice were infected with 5x10^4 PFU MCMV for 2d, 4d, or 6d or PBS as a mock infection. Splenocytes were analyzed by flow cytometry for DC subsets. Significance was determined with Dunnett's multiple comparison test, comparing each time point back to the appropriate PBS control.

splenic DC accumulation. Hence, we show that licensed G2+ NK are critical to enhancing DC reconstitution kinetics.

It has long been appreciated that NK and DC engage in extensive crosstalk. Since initial work by Fernandez et al. showed DC are capable of directly triggering NK to recognize target cells (177), there has been an underlying interest in the field to better define their interactions. Another early study showed that the crosstalk is truly bidirectional. Mocikat et al. found that, in response to injection of MHC class I-low targets, NK cells primed DC to initiate an adaptive CD8 T cell response (178). The work performed in this section extends our knowledge of the interactions between licensed NK cells and DC during MCMV infection.

While Ly49H-dependent control of MCMV has been shown to essentially preserve splenic DC during MCMV infection (46, 110), we observe a unique pattern of dendritic cell regulation with G2+ NK-mediated control. In our model, resistant mice first lose, then quickly recover, and finally expand their splenic DC populations over the course of infection. To our knowledge, there has been very little investigation into the dynamics and fate of the DC compartment beyond 4 d post-MCMV and it is unclear if this late expansion occurs in other models of MCMV control.

Furthermore, it is still unclear what this expansion means for the immune response. Presumably, having a transiently expanded DC compartment between 4 and 12 dpi

would be beneficial for rapidly priming a robust T cell response. This matches well with our previously published work and unpublished data from ongoing studies (109), but we have not yet proven a role for increased DC numbers in enhancing the T cell response. For the time being, though, it is a valid hypothesis.

If this is the case, our work becomes incredibly interesting from a vaccine development standpoint. A means of exploiting NK-DC crosstalk to enhance adaptive responses priming is a clear interest in the field. In general, there has been a focus toward using innate immune mechanisms to enhance vaccine efficacy, both for protective and therapeutic vaccine strategies. The concept of using dendritic cells for cancer vaccines has been around for years and is still a popular approach being developed and refined as a clinical tool (87, 179, 180). Recently, there has also been interest in taking advantage of NK cells as DC vaccine enhancers (89, 181). We have seen clear evidence in these studies that licensed NK are viable candidates for such tasks.

One of the strongest observations made from the above data is that DC are unable to recover efficiently in the presence of elevated virus levels. This is made clear by figure 32 in which increasing the viral inoculum resulted in sustained virus at levels similar to those seen in mice without licensed NK protection. Both of these groups failed to recover and expand their splenic DC, indicating that virus control is the primary benefit of licensed NK in these studies. Hence, an efficient NK response establishes an environment conducive to DC accumulation. This is supported by the BrdU

incorporation data. D^k mice have comparable frequencies of $BrdU^+$ DC to D^b mice which means their compartments are proliferating at similar rates. However, the accumulation of cells seen in D^k mice implies that the environment is conducive to DC retention.

If trafficking is involved in DC loss as, Chapter II may suggest, perhaps this is due to protection of stromal cells that produce CCL19 and 21 or at least reducing viral interference with these pathways. Such an effect has been seen by Benedict et al. (159). MCMV infected spleens express less CCL21, a ligand for CCR7 and an important factor for bringing cells into the T cell zones of lymphoid tissue. DC upregulate CCR7 upon activation and this generally allows them to interact with T cells during inflammation. However, if the levels of CCL21 are reduced enough it may lead to impaired DC retention upon upregulation f CCR7. Hence, licensed NK cells may be supporting stromal cells and chemokine networks to keep maintain proper localization of DC and other immune cells.

Additionally, since our data suggested DC themselves weren't necessarily proliferating at a higher rate, the increased number of BrdU+ DC from D^k mice could imply greater precursor efficiency for DC differentiation in the context of licensed NK cell virus control. One hypothesis is that licensed NK cells could be secreting Flt3 ligand (Flt3L) to support DC generation. Apparently, in the spleen, Flt3Lexpression by immune cells is required for the development of local DC (182). Furthermore, NK cells secrete the most Flt3L compared to other immune cells (182). This is an interesting prospect because

Flt3L is also required for developing DC to become competent inducers of NK activation, particularly during MCMV infection (183). DC from mice with a defect in Flt3 signaling succumb more easily to MCMV infection due to suboptimal crosstalk of DC with NK.

While virus control appears to be the most critical function of the licensed NK cell does not preclude the possibility that they could still have additional roles in enhancing DC recovery. It is very interesting to note that NK are exhibiting immune-regulatory effects early on during MCMV infection. By 54 hpi, mice with a licensed G2+ NK response exhibit an NK cell compartment that has undergone extensive interactions with DC – as exhibited by increases in MHC class II on NK cells. The fact that MHC class II acquisition is much less dramatic on NK cells from susceptible D^b mice is telling, but it is also quite interesting that all NK cells in D^k mice derive a benefit from having licensed G2+ NK cells. The MHC class II increase is not limited to G2+ NK. Rather, it appears to be equally spread around the NK compartment, implying that all NK subsets are interacting with DC to a greater degree. Somehow, the licensed G2+ NK cells are enforcing an environment conducive to crosstalk during the first 54 h of the response to MCMV. This function could be critical in enhancing vaccine efficacy and merits further study.

An indication that the licensed NK could be providing an additional benefit to DC recovery is seen when comparing the time to recovery/expansion between poly I:C

treatment (Figure 34) and infected D^k mice. While both scenarios induce inflammation, DC loss, and NK cell activation, only MCMV will generate a licensed response due to specific MCMV recognition by the G2+ NK. In this comparison, an infected D^k mouse will generally lose DC on d 3 and recover by d4, followed by a period of increased DC numbers. In the case of non-specific NK activation with poly I:C, however, DC are lost by d 1.5 and do not recover until d 4. While this is not a conclusive analysis, it does provide evidence for the hypothesis that post-infection DC recovery in the presence of a licensed NK response is faster than baseline repopulation.

Interestingly, by 12 d, D^b mice appeared to have an expansion of DC similar to what was seen in D^k mice between 4 and 6 dpi. This would suggest that expansion of DC can occur in the absence of a specific NK response, but it is difficult to claim this conclusively. A variety of factors could be at play by 12 dpi. For example, maybe the T cell response to virus is contributing to DC expansion or perhaps by this time there has been a shift in the infection to allow specific recognition by another NK subset. The result from our B6 strains also indicate that DC can expand independently of a licensed response. The indication from the B6 mice was that a Ly49H dominated response is capable of allowing splenic DC to increase their numbers, hence reinforcing the primacy of virus control for DC expansion. However, there is still a possibility that licensed NK are playing a role in the B6 mice. Ly49A is an inhibitory receptor encoded by the B6 NKC and it can also bind to D^k, similar to Ly49G2 from C57L mice. Ly49A is expressed at much lower levels and on fewer NK cells than Ly49G2, but it is possible that Ly49A is

generating a licensed response in our B6.D^k-NKC^{b6} mice and influencing DC recovery. We will have to test this further by including B6 mice without D^k expression as a comparator.

In summary, this work shows a dominant role MCMV titers in enforcing DC suppression. Some aspect of the licensed NK cell allows for enhanced DC interactions, likely leading to full functionality of the NK response. Furthermore, the presence of the licensed NK allows permits the rest of the NK compartment to interact with DC as well. The mechanism and outcome of this in our model is currently unknown, but NK-DC interactions generally increase functionality of one or both of the cells. If virus cannot be cleared efficiently, elevated levels of virus are driving conditions that maintain low splenic DC count. Hence, the foremost function of the licensed NK cell from D^k mice is to accelerate virus control. This control establishes conditions that protect the accumulation of DC that are proliferating in the spleen. In conditions without efficient control, DC proliferation appears to be occurring, but sustained virus levels are inducing either rapid efflux or rapid elimination of any DC that arise in the spleen. Hence, a licensed G2+ NK cell is promoting the recovery of splenic DC.

CONCLUSIONS & FUTURE DIRECTIONS

Interestingly, recent work for both type I IFN and NK cells has highlighted the duality of each in terms of their abilities as immune modulators capable of either benefiting or hindering immunity. While the many beneficial effects of IFN-I have been long appreciated and range from directly limiting viral spread to B cell activation and class switching (49), there are circumstances in which the benefit of IFN-I is markedly diminished. For example, during chronic LCMV infection (clone 13), two groups have found that blocking the IFN-I receptor results in a relief of immunosuppression and enhanced clearance of persistent virus (44, 45). NK cells are now also known to manifest a diverse array of functions. Like IFN-I they are involved in limiting early virus spread by recognizing and eliminating infected cells. They can also participate in the switch to adaptive immunity through their feedback on DC (35, 84, 89, 181). However, recent evidence also points to NK cells restricting immunity and persistent virus control (171). Depletion of NK cells from mice latently infected with LCMV results in a renewed T cell response and improved viral clearance. Intriguingly, IFN-I has come up frequently as a modulator of the immune dampening potential of NK cells. Two studies have shown a role for IFN-I in protecting virus specific T cells during an immune response, although they cited different mechanisms of action. Crouse et al. found that CD8 T cells reduced their levels of NK-activating ligands in response to IFN-I signaling which allowed them to escape NK killing (63). Xu et al., on the other hand, observed increased expression of MHC class I and Ib by CD8 T cells in response to IFN-I. This protected the T cells by

binding NK cell inhibitory receptors, signaling them to spare the T cells (170). Yet another report, by Madera et al., showed that IFN-I was necessary for NK cell expansion in response to viral infection (64). Without IFN-I signaling, proliferating NK cells increased their expression of activating ligands for the NK receptor NKG2D, making them susceptible to fratricide.

However, it is important to keep in mind that many of these detrimental scenarios occur in an artificial system (i.e. complete lack of IFN-I signaling) or in the context of a viral infection that has evolved to destabilize the immune response for prolonged periods. Here, we have investigated the interplay between MCMV-induced inflammation and licensed NK-dependent virus control and the ensuing impact on integrity of the dendritic cell compartment. Overall, we observed minimal negative impacts of IFN-I and a direct benefit of licensed NK cells on splenic DC numbers. Hence, in the context of an effective, licensed NK cell response to infection, the pathogenic properties of IFN-I and NK cells appear largely mitigated in favor of cooperation and an optimized antiviral response.

IFN-I impact on DC

We showed that splenic DC subsets differ in their sensitivity to loss in general and, specifically, in their susceptibility to IFN-I. The DN DC subset is most resistant to MCMV-induced loss, but appears to be most sensitive to IFN-I regulation. CD4 DC, on

the other hand, are severely decreased during MCMV infection but in a largely IFN-I independent manner. This distinction is an important point due to our minimal knowledge about the differences between DN and CD4 DC. The fact that these two subsets are so often combined as the "CD11b^{+}" or "CD8^{-}" subset and analyzed together is potentially troubling since we understand so little about the potential differences that may exist between them. The most detailed information to date describing these cells comes from work by Lewis et al. (23). As stated previously, they determined that Notch2 signaling controlled development of the CD4 DC subset specifically and that mice deficient for this population had severe impairments in CD4 T cell priming. On the other hand, the DN subset exhibited greater capacity for cytokine (IL-12, TNFα) production. We are just now beginning to define the differences between DC within the CD11b subset, but our finding of divergent sensitivity to IFN-I regulation contributes to this knowledge.

CD8 DC numbers showed a small, generally insignificant increase in response to removal of IFN signaling, but loss in response to MCMV is maintained regardless of IFN-I signals. Of the splenic subsets these cells are the best characterized to date. CD8 DC are premier cross-presenters, able to take up exogenous proteins or dead/dying cells and process the material for display on MHC class I. These cells rely on the Batf3 transcription factor for development and initial work with Batf3-KO mice by Hildner et al. (22) proved the importance of this population for CD8 T cell stimulation, in response to viral infection as well as tumor challenge. Interestingly, infection of Batf3-KO mice

with MCMV gives mixed results. Initial priming of virus specific T cells is greatly impaired in Batf3-KO, but as the infection progresses, direct presentation is capable of simulating an inflationary T cell response (184). A similar finding was reported by Ong et al. when they used a CpG reagent to transiently deplete DC before infection (103).

There are issues with these experiments, however since IL-12 signaling can induce development of CD8 DC in Batf3-KO mice and pretreatment with CpG is going to prime the immune system prior to infection. More definitive studies are needed to truly rule out an important role for CD8 DC during MCMV infection. However, the fact that cross-presenting DC appear dispensable during MCMV infection does not reduce their general importance. Hence, our conclusions extend beyond MCMV since we have essentially investigated the necessity of IFN-I in establishing a condition of reduced DC numbers. From this work, we can strongly conclude that IFN-I signaling is not required for loss of CD8 and CD4 splenic DC.

Alternative mechanisms of DC loss

While IFN-I may participate in DC loss on some level, especially for DN DC, it is quite clear that the bulk of DC loss within the CD8 and CD4 subsets is sustained in the absence of IFN-I signaling. Therefore, there must logically be other factors with a strong ability to drive DC loss during infection. Due to the assumption that IFN-I was responsible for DC toxicity, the area of alternative regulation has remained largely

unexplored in the literature. We have been unable to definitively implicate another source of DC loss as of yet, but the experiments here allow us to refine the future of the investigation. Thus far, we can firmly conclude that DC loss occurs via a mechanism that is pDC-independent, NK-independent, virus-independent, IFNAR-independent, but inflammation dependent. It is likely that DC activation and maturation plays a role in sensitizing them to this mechanism of loss, but this will require further study. The fact that DC loss can proceed independently of IFN-I is an important advance in the field itself, though. This has been fully unappreciated until now, which will allow us to further pursue alternative mechanisms of DC loss during inflammation.

The CpG reagent results present an interesting opportunity for future study. Since both signal through TLR9 but have completely divergent effects on DC. While the CpG-A reagent generated no loss in any DC subset, CpG-B treatment resulted in substantial decreases in CD4 and CD8 DC compartments. Hence, a more complete profiling of the effects of each treatment should provide us with a better understanding of the conditions leading to DC loss. It is quite interesting that CpG-B is a more potent activator of B cells since they are involved quite early in the response to MCMV. As noted in the introduction, LTα/β signaling from B cells to stromal cells is responsible for the very first wave of IFN-I in response to MCMV (153). Additionally, TLR9 is known to be strongly activated during the response to MCMV and is actually heavily involved in controlling virus levels (141). The involvement of TLR9 in the anti-MCMV response means we know that its ligands are present. This creates the potential to stimulate both

B cells and pDC. Hence, the knowledge of a mechanism where activated B cells are generating an environment that suppresses DC it would be a significant advance in the field. To test this, CpG-B stimulations in Rag-KO or other B cell deficient mouse strain can be performed and DC loss compared to control mice reconstituted with B cells. If DC are protected during CpG-B treatment of B cell deficient mice, then this would be a strong indication they are involved. However, total B cell deficiencies may influence the structure and development of lymphoid tissue and an alternate approach would likely be required for verification. In this case, TLR9-KO mice could be of use. For these studies, bone marrow chimeras could be made by reconstituting mice with a mix of TLR9-KO and Rag-KO bone marrow. In these chimeras, all T and B cells that develop will be TLR9-KO while all other cell compartments will have half of their cells expressing TLR9. Using these models and CpG-B treatments will greatly assist in parsing out the requirement for B cell TLR9 signaling in splenic DC loss.

Another strong candidate to be tested is cell trafficking. Traditionally, spleen is thought of as an end-point organ for DC. As such, their emigration from this organ has really not been characterized or studied. However, there are some indications that DC may be able to re-enter circulation. Some have detected a small percentage of CD11c+ cells in the lymph moving into the thoracic duct for redistribution to the blood stream (31). These cells lacked other lineage markers for T, B, and NK cell. Another line of evidence for potential DC circulation is that, during influenza infection, virus specific T cells become primed in the spleen and develop into functional memory cells, indicating there is an

antigen bearing DC present that has likely come into contact with the infection (32). Yet another indicator that DC may enter circulation and migrate to new sites comes from work by Reinhardt et al. They found that during *Listeria monocytogenes* or LPS treatment, DC rapidly accumulated in lymph nodes around high endothelial venules and the accumulation could be inhibited with Pertussis toxin treatment which inhibits most G protein-coupled receptor (GPCR)-mediated migration signals (33).

Hence, considering this evidence and the fact that we have been unable to detect increased death or rescue DC by blocking cell death, DC emigration from the spleen is a good hypothesis to test. These studies should be done with zbtb46^{gfp} in order to easily identify and track DC. As a broad approach, treating mice with pertussis toxin during infection to broadly inhibit cell migration could be performed. In this scenario, I would expect most populations to be largely static, without a lot of movement in or out of most peripheral sites. If DC still decreased in number in the spleen under these circumstances, the possible interpretations would be *(1)* the cells are dying in the spleen *(2)* the cells are leaving the spleen in a GPCR-independent manner. These two possibilities also carry their own complications. For example, if the cells die in response to pertussis toxin treatment, is it because they normally die during infection or is it because their GPCR signaling is being interfered with or because a separate cell has been dysregulated. In short, this is a blunt approach that must not be over-interpreted.

Alternatively, for a more restricted approach, the drug Ciglitazone blocks migration in response to the chemokine receptor CCR7(185). As mentioned previously, CCR7 is an attractive candidate since it is only upregulated in DC during activation and, in our experience, DC activation always precedes loss. Also, when Idoyaga et al. (138) used an anti-langerin antibody to label CD8 DC in spleen sections after poly I:C treatment, they observed clustering of the langerin^{+} DC in the T cell zone just prior to poly I:C-induced DC loss. Hence, since CCR7 controls localization of mature DC to T cell zones, and DC appear to cluster in T cell zones prior to loss, CCR7 could play a role in the progression of splenic DC loss. However, if Ciglitazone treatment preserves splenic DC numbers, we still will not know why CCR7 is important and would have to perform further tests to determine more detailed information. For example, we still wouldn't know if CCR7 was mediating trafficking out of the spleen after clustering the T cell zone or if the something happens to the DC after entering the T cell area that leads to the demise of the cell. Perhaps the most rigorous way to test for DC trafficking or migration would be through a spleen transplant. If we surgically implanted the spleen of a zbtb46^{gfp} mouse into a non-gfp mouse, it would be possible to easily identify cells at peripheral locations that originated from the spleen. The biggest concern with this approach would be if the inflammation of a major surgery alters DC distribution prior to running the infection and completing the experiment.

Related to the DC clustering observation made by Idoyaga et al. (138), an alternative mechanism of cell death that requires DC clustering could explain the DC loss as well.

According to a study by Leverkus et al. (165), MHC class II ligation on activated DC drives a rapid apoptosis. Through several experiments, they defined some of the characteristics of this mechanism. First, DC must be activated and mature before MCH II-ligation induces death. Second, DC-DC homotypic interactions promote the progression of this mechanism (i.e. clustering). Third, uncoupling actin filament bundling prevented death. Fourth, the process was independent of caspases. And fifth, Bcl-2 overexpression could not prevent it (165). Several of these elements fit well with what we see during MCMV or TLR-induced DC loss (DC activation, caspase independent, clustering, rapid death). The most feasible and cost-effective way to test this possibility would likely be with an MHC II blocking Ab. Interestingly, this does exist (clone M5/114) and could be tested easily. Treatment with the antibody would be transient, lasting from 2 dpi to 3 dpi to cover the window of dramatic DC loss and therefore shouldn't manifest many off target effects. If the DC are dying in a MCH II-dependent manner, this should interfere with the process since it is capable of blocking T cell responses (186).

Before performing any interventional experiments, though, it may be useful to generate spleen sections from infected zbtb^{gfp} mice and follow DC during infection. We propose this because the context and environment of the DC prior to loss is an important factor that we do not yet fully understand. We were also very intrigued that we lost the ability to label DC *in vivo* at 2 dpi, presumably indicating that either the DC themselves or the receptor were unavailable for binding and recognition. Interestingly, the antibody clone

used (N418) has blocking functionality for CD11c, which means if CD11c is bound by a ligand N418 may not be able to recognize its epitope (187). Determining exactly which cells DC are interacting with during splenic loss would be conducive to identifying additional candidate pathways and interaction to test for a role DC loss.

Support functions of the licensed Ly49G2+ NK cell response

In terms of DC recovery, we have shown a requirement for virus control and a role for licensed G2+ NK cells in driving DC expansion rapidly after controlling infection. The exact role of licensing NK beyond virus control is still in question, though and requires more detailed analysis. A benefit to CD8 DC maintenance has previously been shown in the context of a Ly49H unlicensed response, so if there are no additional benefits of the licensed NK cell, the process becomes more confirmatory of those data(106). However, the fact that a licensed NK population is mediating virus control followed by DC protection and expansion is still a novel finding in itself.

To further investigate the question of whether or not licensed NK cells provide additional benefits, the overall strategy will be two-fold. First, we will attempt to see if DC expand to a similar degree when infection is controlled independently of licensed NK cells. Second, we will attempt to expand naïve DC by manipulating licensed NK cells.

We have acquired a strain of MCMV that expresses the herpes simplex virus thymidine kinase (TK) (188). Expression of this kinase molecule enhances the incorporation of antiviral drugs (like Acyclovir or Ganciclovir) into the genome of replicating herpesviruses and destabilizes their DNA. This potentially allows for exogenous viral control in susceptible mice. Using this MCMV-TK and the antiviral Ganciclovir, we plan to investigate the ability of exogenous virus control to expand DC after infection. This will speak to the effect of virus control in general on DC recovery and will allow us to evaluate if the licensed NK cell is necessary for DC expansion.

The second approach will be more focused on whether a licensed NK cell response is sufficient for DC expansion. There is very little information in the literature investigating specifically licensed NK support of DC. However, we were able to find one report showing licensed human NK cells preferentially promote DC maturation by expression of the TNF superfamily member LIGHT. This occurs after the NK receive activation signals – either CD16 stimulation, IL-2 and IL-15, or stimulation with tumor targets (189).

Ideally we want to know if the licensed NK cell response is enhancing DC numbers *in vivo.* We will employ 3 strategies to test this. First, NK cells from 3 d and 4 d infected mice will be isolated. It will be informative to perform NK transfers of MCMV-experience NK into naïve recipients to determine if this causes an expansion in DC. A second strategy will be to deplete G2+ NK at discreet time points during the course of

infection (i.e. before infection vs d 1 depletion vs d 2 depletion etc.) and determine if the extent or kinetics of DC recovery are impacted. Finally, a third approach is to inject MHC class I-low target cells or MHC-mismatched cells into D^k mice. Licensed NK cells are uniquely tuned to respond to differences in MHC class I so either of these should activate G2+ NK. This type of recognition is termed a "missing" or "altered" self response. We currently hypothesize that this is how the G2+ NK are responding to MCMV-infected cells. So, in this approach, we will be able to specifically induce licensed NK cell activation in the absence of virus. Following injection of the target cells, DC populations will be evaluated over 4 d for any fluctuations in numbers. Hence this will speak directly to the question of whether or not an activated licensed NK cell can drive DC expansion.

Closing remarks

Interestingly, while our licensed NK cells are effective at restricting viral load, they may be slightly less efficient than a cell that recognizes infected cells purely through an activating receptor. This has very interesting theoretical implications for the immunomodulatory aspects of an NK cell response. If, for some reason, the licensed response takes longer to initiate recognition of infection, this means that there will be a brief period for viral replication and expansion. This will result in a transiently increased viral titer and proportionally increased inflammation in response to virus (i.e. a stronger IFN-I response). Both of these elements contribute to a productive adaptive response.

With more antigen available, DC can prime more T cells and higher levels of IFN-I will protect T cells from NK and NK from each other (63, 64, 110, 170). Furthermore, since the NK are still capable of specifically recognizing infected cells, destruction of those cells makes viral antigen available to DC while also controlling viral load and allowing for DC recovery. Hence, we put forth the assertion that a licensed NK cell response, by its nature and kinetics of response, can modulate the timing and severity of infection for the progression of an optimal immune response with minimal pathology. This model fits with our observations to date and is completely unique in the field.

Here, we have shown a function for licensed NK cells that benefits and stabilizes the immune system during MCMV infection, enacting pathogen clearance, resolution of inflammation, and immune cell reconstitution. An element that is, however, still unclear is the actual impact of DC loss on immunity. The assumption has been made that DC loss prior to 4 dpi will impair T cell priming, but this may not be the case. While we know that loss in the spleen occurs and that NK can prevent/counter it, we do not know if DC are leaving to prime T cells at a separate location or if they are dying because they have completed their role in the immune response. It is distinctly possible that DC are disappearing by 3 dpi because they have already performed their function and set the stage for an ensuing T cell response. This may be a failsafe of the immune system since systems that enforce prolonged DC survival can result in autoimmunity (190–192). However, it is precisely for reasons like this that it is important to understand the dynamics regulating DC responses and homeostasis; some of the studies proposed

above will speak to these issues. Furthermore, understanding the elements of DC recovery during infection could prove important for patient care following viral infection. It is not a stretch to imagine that conditions exhibiting prolonged periods of decreased DC (i.e. susceptible mice infected with MCMV or humans infected with HIV) make the host more susceptible to secondary infections. To limit these effects, it will be crucial for us to effectively regulate DC numbers, survival, and function in the clinic.

REFERENCES

1. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice I. Morphology, Quantitation, Tissue Distribution. *J. Exp. Med.* 137: 1142–1162.
2. Steinman, R. M., and Z. A. Cohn. 1974. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice Ii. Functional Properties in Vitro. *J. Exp. Med.* 139: 380–397.
3. Steinman, R. M., D. S. Lustig, and Z. A. Cohn. 1974. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice Iii. Functional Properties in Vivo. *J. Exp. Med.* 139: 1431–1445.
4. Steinman, R. M., J. C. Adams, and Z. A. Cohn. 1975. Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. *J. Exp. Med.* 141: 804–820.
5. Steinman, R. M., G. Kaplan, M. D. Witmer, and Z. A. Cohn. 1979. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. *J. Exp. Med.* 149: 1–16.
6. Steinman, R. M., and M. D. Witmer. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci.* 75: 5132–5136.
7. Nussenzweig, M. C., and R. M. Steinman. 1980. Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. *J. Exp. Med.* 151: 1196–1212.
8. Nussenzweig, M. C., and I. Mellman. 2011. Ralph Steinman (1943-2011). *Nature* 478: 460–460.
9. Rowley, D. A., and F. W. Fitch. 2012. The road to the discovery of dendritic cells, a tribute to Ralph Steinman. *Cell. Immunol.* 273: 95–98.
10. Hume, D. A. 2008. Macrophages as APC and the Dendritic Cell Myth. *J. Immunol.* 181: 5829–5835.
11. Mosier, D. E. 1967. A Requirement for Two Cell Types for Antibody Formation in vitro. *Science* 158: 1573–1575.
12. Mosier, D. E., and L. W. Coppleson. 1968. A THREE-CELL INTERACTION REQUIRED FOR THE INDUCTION OF THE PRIMARY IMMUNE RESPONSE in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* 61: 542–547.
13. Gilbertson, S. M., P. D. Shah, and D. A. Rowley. 1986. NK cells suppress the generation of Lyt-2+ cytolytic T cells by suppressing or eliminating dendritic cells. *J. Immunol.* 136: 3567–3571.
14. Shah, P. D., S. M. Gilbertson, and D. A. Rowley. 1985. Dendritic cells that have interacted with antigen are targets for natural killer cells. *J. Exp. Med.* 162: 625–636.

15. Shah, P. D., J. Keij, S. M. Gilbertson, and D. A. Rowley. 1986. Thy-1+ and Thy-1- natural killer cells. Only Thy-1- natural killer cells suppress dendritic cells. *J. Exp. Med.* 163: 1012–1017.
16. Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting. *Annu. Rev. Immunol.* 31: 563–604.
17. Naik, S. H. 2008. Demystifying the development of dendritic cell subtypes, a little. *Immunol. Cell Biol.* 86: 439–452.
18. Satpathy, A. T., X. Wu, J. C. Albring, and K. M. Murphy. 2012. Re(de)fining the dendritic cell lineage. *Nat. Immunol.* 13: 1145–1154.
19. Manz, M. G., D. Traver, T. Miyamoto, I. L. Weissman, and K. Akashi. 2001. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 97: 3333–3341.
20. Meredith, M. M., K. Liu, G. Darrasse-Jeze, A. O. Kamphorst, H. A. Schreiber, P. Guermonprez, J. Idoyaga, C. Cheong, K.-H. Yao, R. E. Niec, and M. C. Nussenzweig. 2012. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J. Exp. Med.* 209: 1153–1165.
21. Satpathy, A. T., W. Kc, J. C. Albring, B. T. Edelson, N. M. Kretzer, D. Bhattacharya, T. L. Murphy, and K. M. Murphy. 2012. Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *J. Exp. Med.* 209: 1135–1152.
22. Hildner, K., B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, and K. M. Murphy. 2008. Batf3 Deficiency Reveals a Critical Role for CD8α+ Dendritic Cells in Cytotoxic T Cell Immunity. *Science* 322: 1097–1100.
23. Lewis, K. L., M. L. Caton, M. Bogunovic, M. Greter, L. T. Grajkowska, D. Ng, A. Klinakis, I. F. Charo, S. Jung, J. L. Gommerman, I. I. Ivanov, K. Liu, M. Merad, and B. Reizis. 2011. Notch2 Receptor Signaling Controls Functional Differentiation of Dendritic Cells in the Spleen and Intestine. *Immunity* 35: 780–791.
24. Satpathy, A. T., C. G. Briseño, J. S. Lee, D. Ng, N. A. Manieri, W. Kc, X. Wu, S. R. Thomas, W.-L. Lee, M. Turkoz, K. G. McDonald, M. M. Meredith, C. Song, C. J. Guidos, R. D. Newberry, W. Ouyang, T. L. Murphy, T. S. Stappenbeck, J. L. Gommerman, M. C. Nussenzweig, M. Colonna, R. Kopan, and K. M. Murphy. 2013. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat. Immunol.* 14: 937–948.
25. Fan, X., Z. Liu, H. Jin, J. Yan, H. Liang, X. Fan, Z. Liu, H. Jin, J. Yan, and H. Liang. 2015. Alterations of Dendritic Cells in Sepsis: Featured Role in Immunoparalysis, Alterations of Dendritic Cells in Sepsis: Featured Role in Immunoparalysis. *BioMed Res. Int. BioMed Res. Int.* 2015, 2015: e903720.
26. Mildner, A., and S. Jung. 2014. Development and Function of Dendritic Cell Subsets. *Immunity* 40: 642–656.
27. Edwards, A. D., S. S. Diebold, E. M. C. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. R. e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack

of TLR7 expression by CD8α+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33: 827–833.
28. Luber, C. A., J. Cox, H. Lauterbach, B. Fancke, M. Selbach, J. Tschopp, S. Akira, M. Wiegand, H. Hochrein, M. O'Keeffe, and M. Mann. 2010. Quantitative Proteomics Reveals Subset-Specific Viral Recognition in Dendritic Cells. *Immunity* 32: 279–289.
29. Mildner, A., and S. Jung. 2014. Development and Function of Dendritic Cell Subsets. *Immunity* 40: 642–656.
30. Alvarez, D., E. H. Vollmann, and U. H. von Andrian. 2008. Mechanisms and Consequences of Dendritic Cell Migration. *Immunity* 29: 325–342.
31. Cavanagh, L. L., R. Bonasio, I. B. Mazo, C. Halin, G. Cheng, A. W. M. van der Velden, A. Cariappa, C. Chase, P. Russell, M. N. Starnbach, P. A. Koni, S. Pillai, W. Weninger, and U. H. von Andrian. 2005. Activation of bone marrow–resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat. Immunol.* 6: 1029–1037.
32. Turner, D. L., K. L. Bickham, D. L. Farber, and L. Lefrançois. 2013. Splenic Priming of Virus-Specific CD8 T Cells following Influenza Virus Infection. *J. Virol.* 87: 4496–4506.
33. Reinhardt, R. L., S. Hong, S.-J. Kang, Z. Wang, and R. M. Locksley. 2006. Visualization of IL-12/23p40 In Vivo Reveals Immunostimulatory Dendritic Cell Migrants that Promote Th1 Differentiation. *J. Immunol.* 177: 1618–1627.
34. Bonasio, R., and U. H. von Andrian. 2006. Generation, migration and function of circulating dendritic cells. *Curr. Opin. Immunol.* 18: 503–511.
35. Ferlazzo, G., and B. Morandi. 2014. Cross-talks between natural killer cells and distinct subsets of dendritic cells. *NK Cell Biol.* 5: 159.
36. Walzer, T., M. Dalod, S. H. Robbins, L. Zitvogel, and E. Vivier. 2005. Natural-killer cells and dendritic cells: "l'union fait la force." *Blood* 106: 2252–2258.
37. Moretta, A. 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat. Rev. Immunol.* 2: 957–965.
38. Lanier, L. L. 2008. Evolutionary struggles between NK cells and viruses. *Nat. Rev. Immunol.* 8: 259–268.
39. Martinet, L., and M. J. Smyth. 2015. Balancing natural killer cell activation through paired receptors. *Nat. Rev. Immunol.* 15: 243–254.
40. Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. Dendritic Cells Prime Natural Killer Cells by trans-Presenting Interleukin 15. *Immunity* 26: 503–517.
41. Castillo, E. F., S. W. Stonier, L. Frasca, and K. S. Schluns. 2009. Dendritic Cells Support the In Vivo Development and Maintenance of NK Cells via IL-15 Trans-Presentation. *J. Immunol.* 183: 4948–4956.
42. Nakayama, M., K. Takeda, M. Kawano, T. Takai, N. Ishii, and K. Ogasawara. 2011. Natural killer (NK)–dendritic cell interactions generate MHC class II-dressed NK cells that regulate CD4+ T cells. *Proc. Natl. Acad. Sci.* 108: 18360–18365.
43. Trifilo, M. J., B. Hahm, E. I. Zuniga, K. H. Edelmann, and M. B. A. Oldstone. 2006. Dendritic Cell Inhibition: Memoirs from Immunosuppressive Viruses. *J. Infect. Dis.* 194: S3–S10.

44. Teijaro, J. R., C. Ng, A. M. Lee, B. M. Sullivan, K. C. F. Sheehan, M. Welch, R. D. Schreiber, J. C. de la Torre, and M. B. A. Oldstone. 2013. Persistent LCMV Infection Is Controlled by Blockade of Type I Interferon Signaling. *Science* 340: 207–211.
45. Wilson, E. B., D. H. Yamada, H. Elsaesser, J. Herskovitz, J. Deng, G. Cheng, B. J. Aronow, C. L. Karp, and D. G. Brooks. 2013. Blockade of Chronic Type I Interferon Signaling to Control Persistent LCMV Infection. *Science* 340: 202–207.
46. Robbins, S. H., G. Bessou, A. Cornillon, N. Zucchini, B. Rupp, Z. Ruzsics, T. Sacher, E. Tomasello, E. Vivier, U. H. Koszinowski, and M. Dalod. 2007. Natural Killer Cells Promote Early CD8 T Cell Responses against Cytomegalovirus. *PLoS Pathog* 3: e123.
47. McNab, F., K. Mayer-Barber, A. Sher, A. Wack, and A. O'Garra. 2015. Type I interferons in infectious disease. *Nat. Rev. Immunol.* 15: 87–103.
48. Baranek, T., T.-P. Vu Manh, Y. Alexandre, M. A. Maqbool, J. Z. Cabeza, E. Tomasello, K. Crozat, G. Bessou, N. Zucchini, S. H. Robbins, E. Vivier, U. Kalinke, P. Ferrier, and M. Dalod. 2012. Differential Responses of Immune Cells to Type I Interferon Contribute to Host Resistance to Viral Infection. *Cell Host Microbe* 12: 571–584.
49. McNab, F., K. Mayer-Barber, A. Sher, A. Wack, and A. O'Garra. 2015. Type I interferons in infectious disease. *Nat. Rev. Immunol.* 15: 87–103.
50. Swiecki, M., and M. Colonna. 2011. Type I interferons: diversity of sources, production pathways and effects on immune responses. *Curr. Opin. Virol.* 1: 463–475.
51. Hervas-Stubbs, S., J. L. Perez-Gracia, A. Rouzaut, M. F. Sanmamed, A. L. Bon, and I. Melero. 2011. Direct Effects of Type I Interferons on Cells of the Immune System. *Clin. Cancer Res.* 17: 2619–2627.
52. Mattei, F., G. Schiavoni, and D. F. Tough. 2010. Regulation of immune cell homeostasis by type I interferons. *Cytokine Growth Factor Rev.* 21: 227–236.
53. Castellaneta, A., O. Yoshida, S. Kimura, S. Yokota, D. A. Geller, N. Murase, and A. W. Thomson. 2014. Plasmacytoid dendritic cell-derived IFN-α promotes murine liver ischemia/reperfusion injury by induction of hepatocyte IRF-1. *Hepatology* 60: 267–277.
54. Trinchieri, G. 2010. Type I interferon: friend or foe? *J. Exp. Med.* 207: 2053–2063.
55. Crow, M. K. 2014. Advances in understanding the role of type I interferons in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* 26: 467–474.
56. Davidson, S., M. K. Maini, and A. Wack. 2015. Disease-Promoting Effects of Type I Interferons in Viral, Bacterial, and Coinfections. *J. Interferon Cytokine Res.* 35: 252–264.
57. Jewell, A. P., C. P. Worman, P. M. Lydyard, K. L. Yong, F. J. Giles, and A. H. Goldstone. 1994. Interferon-alpha up-regulates bcl-2 expression and protects B-CLL cells from apoptosis in vitro and in vivo. *Br. J. Haematol.* 88: 268–274.
58. Tomic, J., B. Lichty, and D. E. Spaner. 2011. Aberrant interferon-signaling is associated with aggressive chronic lymphocytic leukemia. *Blood* 117: 2668–2680.
59. McNally, J. M., C. C. Zarozinski, M.-Y. Lin, M. A. Brehm, H. D. Chen, and R. M. Welsh. 2001. Attrition of Bystander CD8 T Cells during Virus-Induced T-Cell and Interferon Responses. *J. Virol.* 75: 5965–5976.
60. Bahl, K., S.-K. Kim, C. Calcagno, D. Ghersi, R. Puzone, F. Celada, L. K. Selin, and R. M. Welsh. 2006. IFN-Induced Attrition of CD8 T Cells in the Presence or Absence of Cognate Antigen during the Early Stages of Viral Infections. *J. Immunol.* 176: 4284–4295.

61. Crouse, J., U. Kalinke, and A. Oxenius. 2015. Regulation of antiviral T cell responses by type I interferons. *Nat. Rev. Immunol.* 15: 231–242.
62. Xu, H. C., M. Grusdat, A. A. Pandyra, R. Polz, J. Huang, P. Sharma, R. Deenen, K. Köhrer, R. Rahbar, A. Diefenbach, K. Gibbert, M. Löhning, L. Höcker, Z. Waibler, D. Häussinger, T. W. Mak, P. S. Ohashi, K. S. Lang, and P. A. Lang. 2014. Type I Interferon Protects Antiviral CD8+ T Cells from NK Cell Cytotoxicity. *Immunity* 40: 949–960.
63. Crouse, J., G. Bedenikovic, M. Wiesel, M. Ibberson, I. Xenarios, D. Von Laer, U. Kalinke, E. Vivier, S. Jonjic, and A. Oxenius. 2014. Type I Interferons Protect T Cells against NK Cell Attack Mediated by the Activating Receptor NCR1. *Immunity* 40: 961–973.
64. Madera, S., M. Rapp, M. A. Firth, J. N. Beilke, L. L. Lanier, and J. C. Sun. 2016. Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J. Exp. Med.* jem.20150712.
65. Hsu, K. M., J. R. Pratt, W. J. Akers, S. I. Achilefu, and W. M. Yokoyama. 2009. Murine cytomegalovirus displays selective infection of cells within hours after systemic administration. *J. Gen. Virol.* 90: 33–43.
66. Loewendorf, A., and C. A. Benedict. 2010. Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. *J. Intern. Med.* 267: 483–501.
67. Schneider, K., A. Loewendorf, C. De Trez, J. Fulton, A. Rhode, H. Shumway, S. Ha, G. Patterson, K. Pfeffer, S. A. Nedospasov, C. F. Ware, and C. A. Benedict. 2008. Lymphotoxin-Mediated Crosstalk between B Cells and Splenic Stroma Promotes the Initial Type I Interferon Response to Cytomegalovirus. *Cell Host Microbe* 3: 67–76.
68. Verma, S., Q. Wang, G. Chodaczek, and C. A. Benedict. 2013. Lymphoid-Tissue Stromal Cells Coordinate Innate Defense to Cytomegalovirus. *J. Virol.* 87: 6201–6210.
69. Swiecki, M., S. Gilfillan, W. Vermi, Y. Wang, and M. Colonna. 2010. Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* 33: 955–966.
70. Ruzek, M. C., A. H. Miller, S. M. Opal, B. D. Pearce, and C. A. Biron. 1997. Characterization of Early Cytokine Responses and an Interleukin (IL)-6–dependent Pathway of Endogenous Glucocorticoid Induction during Murine Cytomegalovirus Infection. *J. Exp. Med.* 185: 1185–1192.
71. Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. C. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama. 2001. Vital Involvement of a Natural Killer Cell Activation Receptor in Resistance to Viral Infection. *Science* 292: 934–937.
72. Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. Direct Recognition of Cytomegalovirus by Activating and Inhibitory NK Cell Receptors. *Science* 296: 1323–1326.
73. Smith, H. R. C., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc. Natl. Acad. Sci.* 99: 8826–8831.

74. Chalmer, J. E., J. S. Mackenzie, and N. F. Stanley. 1977. Resistance to Murine Cytomegalovirus Linked to the Major Histocompatibility Complex of the Mouse. *J. Gen. Virol.* 37: 107–114.
75. Dighe, A., M. Rodriguez, P. Sabastian, X. Xie, M. McVoy, and M. G. Brown. 2005. Requisite H2k Role in NK Cell-Mediated Resistance in Acute Murine Cytomegalovirus-Infected MA/My Mice. *J. Immunol.* 175: 6820–6828.
76. Xie, X., A. Dighe, P. Clark, P. Sabastian, S. Buss, and M. G. Brown. 2007. Deficient Major Histocompatibility Complex-Linked Innate Murine Cytomegalovirus Immunity in MA/My.L-H2b Mice and Viral Downregulation of H-2k Class I Proteins. *J. Virol.* 81: 229–236.
77. Xie, X., M. D. Stadnisky, and M. G. Brown. 2009. MHC Class I Dk Locus and Ly49G2+ NK Cells Confer H-2k Resistance to Murine Cytomegalovirus. *J. Immunol.* 182: 7163–7171.
78. Xie, X., M. D. Stadnisky, E. R. Coats, M. M. A. Rahim, A. Lundgren, W. Xu, A. P. Makrigiannis, and M. G. Brown. 2010. MHC class I Dk expression in hematopoietic and nonhematopoietic cells confers natural killer cell resistance to murine cytomegalovirus. *Proc. Natl. Acad. Sci.* 107: 8754–8759.
79. Yokoyama, W. M., and S. Kim. 2006. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol. Rev.* 214: 143–154.
80. Nash, W. T., J. Teoh, H. Wei, A. Gamache, and M. G. Brown. 2014. Know thyself: NK-cell inhibitory receptors prompt self-tolerance, education, and viral control. *Mol. Innate Immun.* 5: 175.
81. Brodin, P., K. Kärre, and P. Höglund. 2009. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol.* 30: 143–149.
82. Anandasabapathy, N., R. Feder, S. Mollah, S.-W. Tse, M. P. Longhi, S. Mehandru, I. Matos, C. Cheong, D. Ruane, L. Brane, A. Teixeira, J. Dobrin, O. Mizenina, C. G. Park, M. Meredith, B. E. Clausen, M. C. Nussenzweig, and R. M. Steinman. 2014. Classical Flt3L-dependent dendritic cells control immunity to protein vaccine. *J. Exp. Med.* 211: 1875–1891.
83. Apostolopoulos, V., T. Thalhammer, A. G. Tzakos, and L. Stojanovska. 2013. Targeting Antigens to Dendritic Cell Receptors for Vaccine Development. *J. Drug Deliv.* 2013: e869718.
84. Elssen, C. H. M. J. V., T. Oth, W. T. V. Germeraad, G. M. J. Bos, and J. Vanderlocht. 2014. Natural Killer Cells: The Secret Weapon in Dendritic Cell Vaccination Strategies. *Clin. Cancer Res.* 20: 1095–1103.
85. Ginhoux, F., and A. Schlitzer. 2014. CD11b+ DCs rediscovered: implications for vaccination. *Expert Rev. Vaccines* 13: 445–447.
86. Hömberg, N., C. Adam, T. Riedel, C. Brenner, A. Flatley, M. Röcken, and R. Mocikat. 2014. CD40-independent natural killer-cell help promotes dendritic cell vaccine-induced T-cell immunity against endogenous B-cell lymphoma. *Int. J. Cancer* n/a–n/a.
87. Kastenmüller, W., K. Kastenmüller, C. Kurts, and R. A. Seder. 2014. Dendritic cell-targeted vaccines — hope or hype? *Nat. Rev. Immunol.* 14: 705–711.

88. Lion, E., E. L. J. M. Smits, Z. N. Berneman, and V. F. I. V. Tendeloo. 2012. NK Cells: Key to Success of DC-Based Cancer Vaccines? *The Oncologist* 17: 1256–1270.
89. Pampena, M. B., and E. M. Levy. 2015. Natural killer cells as helper cells in dendritic cell cancer vaccines. *Immunother. Vaccines* 6: 13.
90. Wakeland, E., L. Morel, K. Achey, M. Yui, and J. Longmate. 1997. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol. Today* 18: 472–477.
91. Newton, K., X. Sun, and V. M. Dixit. 2004. Kinase RIP3 Is Dispensable for Normal NF-κBs, Signaling by the B-Cell and T-Cell Receptors, Tumor Necrosis Factor Receptor 1, and Toll-Like Receptors 2 and 4. *Mol. Cell. Biol.* 24: 1464–1469.
92. Salmena, L., B. Lemmers, A. Hakem, E. Matysiak-Zablocki, K. Murakami, P. Y. B. Au, D. M. Berry, L. Tamblyn, A. Shehabeldin, E. Migon, A. Wakeham, D. Bouchard, W. C. Yeh, J. C. McGlade, P. S. Ohashi, and R. Hakem. 2003. Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev.* 17: 883–895.
93. Weinlich, R., A. Oberst, C. P. Dillon, L. J. Janke, S. Milasta, J. R. Lukens, D. A. Rodriguez, P. Gurung, C. Savage, T. D. Kanneganti, and D. R. Green. 2013. Protective Roles for Caspase-8 and cFLIP in Adult Homeostasis. *Cell Rep.* 5: 340–348.
94. Seillet, C., S. Laffont, F. Trémollières, N. Rouquié, C. Ribot, J.-F. Arnal, V. Douin-Echinard, P. Gourdy, and J.-C. Guéry. 2012. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor α signaling. *Blood* 119: 454–464.
95. Wilson, N. S., G. M. N. Behrens, R. J. Lundie, C. M. Smith, J. Waithman, L. Young, S. P. Forehan, A. Mount, R. J. Steptoe, K. D. Shortman, T. F. de Koning-Ward, G. T. Belz, F. R. Carbone, B. S. Crabb, W. R. Heath, and J. A. Villadangos. 2006. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat. Immunol.* 7: 165–172.
96. Zhang, A. J. X., C. Li, K. K. W. To, H.-S. Zhu, A. C. Y. Lee, C.-G. Li, J. F. W. Chan, I. F. N. Hung, and K.-Y. Yuen. 2014. Toll-Like Receptor 7 Agonist Imiquimod in Combination with Influenza Vaccine Expedites and Augments Humoral Immune Responses against Influenza A(H1N1)pdm09 Virus Infection in BALB/c Mice. *Clin. Vaccine Immunol.* 21: 570–579.
97. Pawar, R. D., P. S. Patole, D. Zecher, S. Segerer, M. Kretzler, D. Schlöndorff, and H.-J. Anders. 2006. Toll-Like Receptor-7 Modulates Immune Complex Glomerulonephritis. *J. Am. Soc. Nephrol.* 17: 141–149.
98. Fuertes Marraco, S. A., C. L. Scott, P. Bouillet, A. Ives, S. Masina, D. Vremec, E. S. Jansen, L. A. O'Reilly, P. Schneider, N. Fasel, K. Shortman, A. Strasser, and H. Acha-Orbea. 2011. Type I Interferon Drives Dendritic Cell Apoptosis via Multiple BH3-Only Proteins following Activation by PolyIC In Vivo. *PLoS ONE* 6: e20189.
99. Démoulins, T., M.-L. Baron, N. Kettaf, A. Abdallah, E. Sharif-Askari, and R.-P. Sékaly. 2009. Poly (I:C) induced immune response in lymphoid tissues involves three sequential waves of type I IFN expression. *Virology* 386: 225–236.
100. Wei, H., W. T. Nash, A. P. Makrigiannis, and M. G. Brown. 2014. Impaired NK-cell education diminishes resistance to murine CMV infection. *Eur. J. Immunol.* 44: 3273–3282.

101. Inaba, K., W. J. Swiggard, R. M. Steinman, N. Romani, G. Schuler, and C. Brinster. 2001. Isolation of Dendritic Cells. In *Current Protocols in Immunology* John Wiley & Sons, Inc.
102. Wheat, R. L., P. Y. Clark, and M. G. Brown. 2003. Quantitative measurement of infectious murine cytomegalovirus genomes in real-time PCR. *J. Virol. Methods* 112: 107–113.
103. Ong, M. L., M. E. Wikstrom, P. Fleming, M. J. Estcourt, P. J. Hertzog, G. R. Hill, C. E. Andoniou, and M. A. Degli-Esposti. 2013. CpG pretreatment enhances antiviral T-cell immunity against cytomegalovirus. *Blood* 122: 55–60.
104. Smedt, T. D., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. D. Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* 184: 1413–1424.
105. Montoya, M., M. J. Edwards, D. M. Reid, and P. Borrow. 2005. Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN. *J. Immunol. Baltim. Md 1950* 174: 1851–1861.
106. Andrews, D. M., A. A. Scalzo, W. M. Yokoyama, M. J. Smyth, and M. A. Degli-Esposti. 2003. Functional interactions between dendritic cells and NK cells during viral infection. *Nat. Immunol.* 4: 175–181.
107. Mattei, F., L. Bracci, D. F. Tough, F. Belardelli, and G. Schiavoni. 2009. Type I IFN regulate DC turnover in vivo. *Eur. J. Immunol.* 39: 1807–1818.
108. Makrigiannis, A. P., A. T. Pau, A. Saleh, R. Winkler-Pickett, J. R. Ortaldo, and S. K. Anderson. 2001. Class I MHC-Binding Characteristics of the 129/J Ly49 Repertoire. *J. Immunol.* 166: 5034–5043.
109. Stadnisky, M. D., X. Xie, E. R. Coats, T. N. Bullock, and M. G. Brown. 2011. Self MHC class I–licensed NK cells enhance adaptive CD8 T-cell viral immunity. *Blood* 117: 5133–5141.
110. Mitrović, M., J. Arapović, S. Jordan, N. Fodil-Cornu, S. Ebert, S. M. Vidal, A. Krmpotić, M. J. Reddehase, and S. Jonjić. 2012. The NK Cell Response to Mouse Cytomegalovirus Infection Affects the Level and Kinetics of the Early CD8+ T-Cell Response. *J. Virol.* 86: 2165–2175.
111. Sheehan, K. C. F., K. S. Lai, G. P. Dunn, A. T. Bruce, M. S. Diamond, J. D. Heutel, C. Dungo-Arthur, J. A. Carrero, J. M. White, P. J. Hertzog, and R. D. Schreiber. 2006. Blocking Monoclonal Antibodies Specific for Mouse IFN-α/β Receptor Subunit 1 (IFNAR-1) from Mice Immunized by In Vivo Hydrodynamic Transfection. *J. Interferon Cytokine Res.* 26: 804–819.
112. Fischer, J. A. A. 2008. Identification and functional characterization of mPDCA-1 as a novel antigen-uptake receptor on murine plasmacytoid dendritic cells enabling (cross-) priming of naïve CD4+ and CD8+ T cells. .
113. Andrew, A., and K. Strebel. 2010. The Interferon-Inducible Host Factor Bone Marrow Stromal Antigen 2/Tetherin Restricts Virion Release, but Is It Actually a Viral Restriction Factor? *J. Interferon Cytokine Res.* 31: 137–144.

114. Naik, S. H., D. Metcalf, A. van Nieuwenhuijze, I. Wicks, L. Wu, M. O'Keeffe, and K. Shortman. 2006. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* 7: 663–671.
115. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175: 1157–1167.
116. del Hoyo, G. M., P. Martín, H. H. Vargas, S. Ruiz, C. F. Arias, and C. Ardavín. 2002. Characterization of a common precursor population for dendritic cells. *Nature* 415: 1043–1047.
117. Garcin, G., Y. Bordat, P. Chuchana, D. Monneron, H. K. W. Law, J. Piehler, and G. Uzé. 2013. Differential Activity of Type I Interferon Subtypes for Dendritic Cell Differentiation. *PLoS ONE* 8.
118. Gigante, M., M. Mandic, A. K. Wesa, E. Cavalcanti, M. Dambrosio, V. Mancini, M. Battaglia, L. Gesualdo, W. J. Storkus, and E. Ranieri. 2008. Interferon-alpha (IFN-α)–conditioned DC Preferentially Stimulate Type-1 and Limit Treg-type In Vitro T-cell Responses From RCC Patients: *J. Immunother.* 31: 254–262.
119. Papewalis, C., B. Jacobs, M. Wuttke, E. Ullrich, T. Baehring, R. Fenk, H. S. Willenberg, S. Schinner, M. Cohnen, J. Seissler, K. Zacharowski, W. A. Scherbaum, and M. Schott. 2008. IFN-α Skews Monocytes into CD56+-Expressing Dendritic Cells with Potent Functional Activities In Vitro and In Vivo. *J. Immunol.* 180: 1462–1470.
120. Dalod, M., T. Hamilton, R. Salomon, T. P. Salazar-Mather, S. C. Henry, J. D. Hamilton, and C. A. Biron. 2003. Dendritic Cell Responses to Early Murine Cytomegalovirus Infection Subset Functional Specialization and Differential Regulation by Interferon α/β. *J. Exp. Med.* 197: 885–898.
121. Longhi, M. P., C. Trumpfheller, J. Idoyaga, M. Caskey, I. Matos, C. Kluger, A. M. Salazar, M. Colonna, and R. M. Steinman. 2009. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J. Exp. Med.* 206: 1589–1602.
122. Simmons, D. P., P. A. Wearsch, D. H. Canaday, H. J. Meyerson, Y. C. Liu, Y. Wang, W. H. Boom, and C. V. Harding. 2012. Type I IFN Drives a Distinctive Dendritic Cell Maturation Phenotype That Allows Continued Class II MHC Synthesis and Antigen Processing. *J. Immunol.* 188: 3116–3126.
123. Pantel, A., A. Teixeira, E. Haddad, E. G. Wood, R. M. Steinman, and M. P. Longhi. 2014. Direct Type I IFN but Not MDA5/TLR3 Activation of Dendritic Cells Is Required for Maturation and Metabolic Shift to Glycolysis after Poly IC Stimulation. *PLoS Biol* 12: e1001759.
124. Lorenzi, S., F. Mattei, A. Sistigu, L. Bracci, F. Spadaro, M. Sanchez, M. Spada, F. Belardelli, L. Gabriele, and G. Schiavoni. 2011. Type I IFNs Control Antigen Retention and Survival of CD8α+ Dendritic Cells after Uptake of Tumor Apoptotic Cells Leading to Cross-Priming. *J. Immunol.* 186: 5142–5150.
125. Diamond, M. S., M. Kinder, H. Matsushita, M. Mashayekhi, G. P. Dunn, J. M. Archambault, H. Lee, C. D. Arthur, J. M. White, U. Kalinke, K. M. Murphy, and R. D. Schreiber. 2011. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. *J. Exp. Med.* 208: 1989–2003.

126. Parlato, S., G. Romagnoli, F. Spadaro, I. Canini, P. Sirabella, P. Borghi, C. Ramoni, I. Filesi, S. Biocca, L. Gabriele, and F. Belardelli. 2010. LOX-1 as a natural IFN-α–mediated signal for apoptotic cell uptake and antigen presentation in dendritic cells. *Blood* 115: 1554–1563.
127. Cucak, H., U. Yrlid, B. Reizis, U. Kalinke, and B. Johansson-Lindbom. 2009. Type I Interferon Signaling in Dendritic Cells Stimulates the Development of Lymph-Node-Resident T Follicular Helper Cells. *Immunity* 31: 491–501.
128. Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough. 2001. Type I Interferons Potently Enhance Humoral Immunity and Can Promote Isotype Switching by Stimulating Dendritic Cells In Vivo. *Immunity* 14: 461–470.
129. Yen, J.-H., and D. Ganea. 2009. Interferon β induces mature dendritic cell apoptosis through caspase-11/caspase-3 activation. *Blood* 114: 1344–1354.
130. Lehner, M., T. Felzmann, K. Clodi, and W. Holter. 2001. Type I interferons in combination with bacterial stimuli induce apoptosis of monocyte-derived dendritic cells. *Blood* 98: 736–742.
131. Seth, S., A.-M. Georgoudaki, B. J. Chambers, Q. Qiu, E. Kremmer, M. K. Maier, N. Czeloth, I. Ravens, R. Foerster, and G. Bernhardt. 2009. Heterogeneous expression of the adhesion receptor CD226 on murine NK and T cells and its function in NK-mediated killing of immature dendritic cells. *J. Leukoc. Biol.* 86: 91–101.
132. Hayakawa, Y., V. Screpanti, H. Yagita, A. Grandien, H.-G. Ljunggren, M. J. Smyth, and B. J. Chambers. 2004. NK Cell TRAIL Eliminates Immature Dendritic Cells In Vivo and Limits Dendritic Cell Vaccination Efficacy. *J. Immunol.* 172: 123–129.
133. Ferlazzo, G., B. Morandi, A. D'Agostino, R. Meazza, G. Melioli, A. Moretta, and L. Moretta. 2003. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur. J. Immunol.* 33: 306–313.
134. Ferlazzo, G., C. Semino, and G. Melioli. 2001. HLA Class I molecule expression is up-regulated during maturation of dendritic cells, protecting them from natural killer cell-mediated lysis. *Immunol. Lett.* 76: 37–41.
135. Morandi, B., L. Mortara, L. Chiossone, R. S. Accolla, M. C. Mingari, L. Moretta, A. Moretta, and G. Ferlazzo. 2012. Dendritic Cell Editing by Activated Natural Killer Cells Results in a More Protective Cancer-Specific Immune Response. *PLOS ONE* 7: e39170.
136. Andrews, D. M., C. E. Andoniou, F. Granucci, P. Ricciardi-Castagnoli, and M. A. Degli-Esposti. 2001. Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. *Nat. Immunol.* 2: 1077–1084.
137. Gautier, E. L., T. Huby, F. Saint-Charles, B. Ouzilleau, M. J. Chapman, and P. Lesnik. 2008. Enhanced Dendritic Cell Survival Attenuates Lipopolysaccharide-Induced Immunosuppression and Increases Resistance to Lethal Endotoxic Shock. *J. Immunol.* 180: 6941–6946.
138. Idoyaga, J., N. Suda, K. Suda, C. G. Park, and R. M. Steinman. 2009. Antibody to Langerin/CD207 localizes large numbers of CD8α+ dendritic cells to the marginal zone of mouse spleen. *Proc. Natl. Acad. Sci.* 106: 1524–1529.

139. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
140. Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11: 373–384.
141. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell, and B. Beutler. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. U. S. A.* 101: 3516–3521.
142. Guiducci, C., G. Ott, J. H. Chan, E. Damon, C. Calacsan, T. Matray, K.-D. Lee, R. L. Coffman, and F. J. Barrat. 2006. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J. Exp. Med.* 203: 1999–2008.
143. Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya, and T. Taniguchi. 2005. Spatiotemporal regulation of MyD88–IRF-7 signalling for robust type-I interferon induction. *Nature* 434: 1035–1040.
144. Krieg, A. M. 2002. Cpg Motifs in Bacterial Dna and Their Immune Effects. *Annu. Rev. Immunol.* 20: 709–760.
145. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdörfer, S. Blackwell, Z. K. Ballas, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. *Eur. J. Immunol.* 31: 2154–2163.
146. CpG ODNs - TLR9 Agonists - Toll-Like receptor 9 ligands - single-stranded DNA molecules. .
147. Jordan, S., Z. Ruzsics, M. Mitrović, T. Baranek, J. Arapović, A. Krmpotić, E. Vivier, M. Dalod, S. Jonjić, L. Dölken, and U. H. Koszinowski. 2013. Natural Killer Cells Are Required for Extramedullary Hematopoiesis following Murine Cytomegalovirus Infection. *Cell Host Microbe* 13: 535–545.
148. Kurokawa, M., and S. Kornbluth. 2009. Caspases and Kinases in a Death Grip. *Cell* 138: 838–854.
149. McIlwain, D. R., T. Berger, and T. W. Mak. 2013. Caspase Functions in Cell Death and Disease. *Cold Spring Harb. Perspect. Biol.* 5: a008656.
150. Chen, X., W. Li, J. Ren, D. Huang, W. He, Y. Song, C. Yang, W. Li, X. Zheng, P. Chen, and J. Han. 2014. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. *Cell Res.* 24: 105–121.
151. Wu, J., Z. Huang, J. Ren, Z. Zhang, P. He, Y. Li, J. Ma, W. Chen, Y. Zhang, X. Zhou, Z. Yang, S.-Q. Wu, L. Chen, and J. Han. 2013. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res.* 23: 994–1006.
152. Wu, Y.-T., H.-L. Tan, Q. Huang, X.-J. Sun, X. Zhu, and H.-M. Shen. 2011. zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFα mediated by the PKC–MAPKs–AP-1 pathway. *Cell Death Differ.* 18: 26–37.
153. Banks, T. A., S. Rickert, C. A. Benedict, L. Ma, M. Ko, J. Meier, W. Ha, K. Schneider, S. W. Granger, O. Turovskaya, D. Elewaut, D. Otero, A. R. French, S. C. Henry, J. D. Hamilton, S. Scheu, K. Pfeffer, and C. F. Ware. 2005. A Lymphotoxin-IFN-β Axis

Essential for Lymphocyte Survival Revealed during Cytomegalovirus Infection. *J. Immunol.* 174: 7217–7225.
154. Yoshida, H., H. Sumichika, S. Hamano, X. He, Y. Minamishima, G. Kimura, and K. Nomoto. 1995. Induction of apoptosis of T cells by infecting mice with murine cytomegalovirus. *J. Virol.* 69: 4769–4775.
155. Bahl, K., A. Hüebner, R. J. Davis, and R. M. Welsh. 2010. Analysis of Apoptosis of Memory T Cells and Dendritic Cells during the Early Stages of Viral Infection or Exposure to Toll-Like Receptor Agonists. *J. Virol.* 84: 4866–4877.
156. Munier, M., S. Jubeau, A. Wijaya, M. Morançais, J. Dumay, L. Marchal, P. Jaouen, and J. Fleurence. 2014. Physicochemical factors affecting the stability of two pigments: R-phycoerythrin of Grateloupia turuturu and B-phycoerythrin of Porphyridium cruentum. *Food Chem.* 150: 400–407.
157. Daley-Bauer, L. P., L. J. Roback, G. M. Wynn, and E. S. Mocarski. 2014. Cytomegalovirus Hijacks CX3CR1hi Patrolling Monocytes as Immune-Privileged Vehicles for Dissemination in Mice. *Cell Host Microbe* 15: 351–362.
158. Nabekura, T., M. Kanaya, A. Shibuya, G. Fu, N. R. J. Gascoigne, and L. L. Lanier. 2014. Costimulatory Molecule DNAM-1 Is Essential for Optimal Differentiation of Memory Natural Killer Cells during Mouse Cytomegalovirus Infection. *Immunity* 40: 225–234.
159. Benedict, C. A., C. D. Trez, K. Schneider, S. Ha, G. Patterson, and C. F. Ware. 2006. Specific Remodeling of Splenic Architecture by Cytomegalovirus. *PLOS Pathog* 2: e16.
160. Kamath, A. T., J. Pooley, M. A. O'Keeffe, D. Vremec, Y. Zhan, A. M. Lew, A. D'Amico, L. Wu, D. F. Tough, and K. Shortman. 2000. The Development, Maturation, and Turnover Rate of Mouse Spleen Dendritic Cell Populations. *J. Immunol.* 165: 6762–6770.
161. Varanasi, V., A. A. Khan, and A. V. Chervonsky. 2014. Loss of the death receptor CD95 (Fas) expression by dendritic cells protects from a chronic viral infection. *Proc. Natl. Acad. Sci. U. S. A.* 111: 8559–8564.
162. Ashany, D., A. Savir, N. Bhardwaj, and K. B. Elkon. 1999. Dendritic cells are resistant to apoptosis through the Fas (CD95/APO-1) pathway. *J. Immunol. Baltim. Md 1950* 163: 5303–5311.
163. 2000. Differential susceptibility to CD95 (Apo-1/Fas) and MHC class II-induced apoptosis during murine dendritic cell development. *Publ. Online 28 Sept. 2000 Doi101038sjcdd4400734* 7.
164. Maney, N. J., G. Reynolds, A. Krippner-Heidenreich, and C. M. U. Hilkens. 2014. Dendritic Cell Maturation and Survival Are Differentially Regulated by TNFR1 and TNFR2. *J. Immunol.* 193: 4914–4923.
165. Leverkus, M., A. D. McLellan, M. Heldmann, A. O. Eggert, E.-B. Bröcker, N. Koch, and E. Kämpgen. 2003. MHC class II-mediated apoptosis in dendritic cells: a role for membrane-associated and mitochondrial signaling pathways. *Int. Immunol.* 15: 993–1006.
166. Drénou, B., V. Blancheteau, D. H. Burgess, R. Fauchet, D. J. Charron, and N. A. Mooney. 1999. A Caspase-Independent Pathway of MHC Class II Antigen-Mediated Apoptosis of Human B Lymphocytes. *J. Immunol.* 163: 4115–4124.

167. Zang, W., S. Kalache, M. Lin, B. Schroppel, and B. Murphy. 2005. MHC Class II–Mediated Apoptosis by a Nonpolymorphic MHC Class II Peptide Proceeds by Activation of Protein Kinase C. *J. Am. Soc. Nephrol.* 16: 3661–3668.
168. Newell, M. K., J. VanderWall, K. S. Beard, and J. H. Freed. 1993. Ligation of major histocompatibility complex class II molecules mediates apoptotic cell death in resting B lymphocytes. *Proc. Natl. Acad. Sci.* 90: 10459–10463.
169. Welsh, R. M., and S. N. Waggoner. 2013. NK cells controlling virus-specific T cells: Rheostats for acute vs. persistent infections. *Virology* 435: 37–45.
170. Xu, H. C., M. Grusdat, A. A. Pandyra, R. Polz, J. Huang, P. Sharma, R. Deenen, K. Köhrer, R. Rahbar, A. Diefenbach, K. Gibbert, M. Löhning, L. Höcker, Z. Waibler, D. Häussinger, T. W. Mak, P. S. Ohashi, K. S. Lang, and P. A. Lang. Type I Interferon Protects Antiviral CD8+ T Cells from NK Cell Cytotoxicity. *Immunity* .
171. Waggoner, S. N., K. A. Daniels, and R. M. Welsh. 2014. Therapeutic Depletion of Natural Killer Cells Controls Persistent Infection. *J. Virol.* 88: 1953–1960.
172. Waggoner, S. N., R. T. Taniguchi, P. A. Mathew, V. Kumar, and R. M. Welsh. 2010. Absence of mouse 2B4 promotes NK cell–mediated killing of activated CD8+ T cells, leading to prolonged viral persistence and altered pathogenesis. *J. Clin. Invest.* 120: 1925–1938.
173. Caumartin, J., B. Favier, M. Daouya, C. Guillard, P. Moreau, E. D. Carosella, and J. LeMaoult. 2007. Trogocytosis-based generation of suppressive NK cells. *EMBO J.* 26: 1423–1433.
174. Cho, F.-N., T.-H. Chang, C.-W. Shu, M.-C. Ko, S.-K. Liao, K.-H. Wu, M.-S. Yu, S.-J. Lin, Y.-C. Hong, C.-H. Chen, C.-H. Hung, and Y.-H. Chang. 2014. Enhanced Cytotoxicity of Natural Killer Cells following the Acquisition of Chimeric Antigen Receptors through Trogocytosis. *PLOS ONE* 9: e109352.
175. HoWangYin, K.-Y., E. D. Carosella, and J. LeMaoult. 2010. Trogocytosis and NK Cells in Mouse and Man. In *Natural Killer Cells* J. Zimmer, ed. Springer Berlin Heidelberg. 109–123.
176. Miner, C. A., T. K. Giri, C. E. Meyer, M. Shabsovich, and S. K. Tripathy. 2015. Acquisition of Activation Receptor Ligand by Trogocytosis Renders NK Cells Hyporesponsive. *J. Immunol.* 1402408.
177. Fernandez, N. C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, and L. Zitvogel. 1999. Dendritic cells directly trigger NK cell functions: Cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat. Med.* 5: 405–411.
178. Mocikat, R., H. Braumüller, A. Gumy, O. Egeter, H. Ziegler, U. Reusch, A. Bubeck, J. Louis, R. Mailhammer, G. Riethmüller, U. Koszinowski, and M. Röcken. 2003. Natural Killer Cells Activated by MHC Class ILow Targets Prime Dendritic Cells to Induce Protective CD8 T Cell Responses. *Immunity* 19: 561–569.
179. Palucka, K., and J. Banchereau. 2013. Dendritic-Cell-Based Therapeutic Cancer Vaccines. *Immunity* 39: 38–48.

180. Mac Keon, S., M. S. Ruiz, S. Gazzaniga, and R. Wainstok. 2015. Dendritic cell-based vaccination in cancer: therapeutic implications emerging from murine models. *Immunother. Vaccines* 243.
181. Langers, I., V. Renoux, A. Reschner, A. Touzé, P. Coursaget, J. Boniver, J. Koch, P. Delvenne, and N. Jacobs. 2014. Natural killer and dendritic cells collaborate in the immune response induced by the vaccine against uterine cervical cancer. *Eur. J. Immunol.* n/a–n/a.
182. Miloud, T., N. Fiegler, J. Suffner, G. J. Hämmerling, and N. Garbi. 2012. Organ-Specific Cellular Requirements for In Vivo Dendritic Cell Generation. *J. Immunol.* 188: 1125–1135.
183. Eidenschenk, C., K. Crozat, P. Krebs, R. Arens, D. Popkin, C. N. Arnold, A. L. Blasius, C. A. Benedict, E. M. Y. Moresco, Y. Xia, and B. Beutler. 2010. Flt3 permits survival during infection by rendering dendritic cells competent to activate NK cells. *Proc. Natl. Acad. Sci.* 107: 9759–9764.
184. Torti, N., S. M. Walton, K. M. Murphy, and A. Oxenius. 2011. Batf3 transcription factor-dependent DC subsets in murine CMV infection: Differential impact on T-cell priming and memory inflation. *Eur. J. Immunol.* 41: 2612–2618.
185. Fainaru, O., D. Shseyov, S. Hantisteanu, and Y. Groner. 2005. Accelerated chemokine receptor 7-mediated dendritic cell migration in Runx3 knockout mice and the spontaneous development of asthma-like disease. *Proc. Natl. Acad. Sci. U. S. A.* 102: 10598–10603.
186. R N Germain, A. B. 1982. A single monoclonal anti-Ia antibody inhibits antigen-specific T cell proliferation controlled by distinct Ir genes mapping in different H-2 I subregions. *J. Immunol. Baltim. Md 1950* 128: 1409–13.
187. Sadhu, C., H. J. Ting, B. Lipsky, K. Hensley, L. F. Garcia-Martinez, S. I. Simon, and D. E. Staunton. 2007. CD11c/CD18: novel ligands and a role in delayed-type hypersensitivity. *J. Leukoc. Biol.* 81: 1395–1403.
188. Snyder, C. M., K. S. Cho, E. L. Bonnett, J. E. Allan, and A. B. Hill. 2011. Sustained CD8+ T Cell Memory Inflation after Infection with a Single-Cycle Cytomegalovirus. *PLOS Pathog* 7: e1002295.
189. Holmes, T. D., E. B. Wilson, E. V. I. Black, A. V. Benest, C. Vaz, B. Tan, V. M. Tanavde, and G. P. Cook. 2014. Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proc. Natl. Acad. Sci.* 111: E5688–E5696.
190. Nopora, A., and T. Brocker. 2002. Bcl-2 Controls Dendritic Cell Longevity In Vivo. *J. Immunol.* 169: 3006–3014.
191. Chen, M., Y.-H. Wang, Y. Wang, L. Huang, H. Sandoval, Y.-J. Liu, and J. Wang. 2006. Dendritic Cell Apoptosis in the Maintenance of Immune Tolerance. *Science* 311: 1160–1164.
192. Chen, M., K. Felix, and J. Wang. 2011. Immune Regulation through Mitochondrion-Dependent Dendritic Cell Death Induced by T Regulatory Cells. *J. Immunol.* 187: 5684–5692.