Summer 2010

Plasmacytoid and respiratory dendritic cells control the magnitude of the virus-specific CD8 T cell response to lethal dose influenza virus infections

Ryan Andrew Langlois
University of Iowa

PLASMACYTOID AND RESPIRATORY DENDRITIC CELLS CONTROL THE MAGNITUDE OF THE VIRUS-SPECIFIC CD8 T CELL RESPONSE TO LETHAL DOSE INFLUENZA VIRUS INFECTIONS

by

Ryan Andrew Langlois

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Immunology
in the Graduate College of
The University of Iowa

July 2010

Thesis Supervisor: Associate Professor Kevin L. Legge

ABSTRACT

CD8 T cells have been demonstrated to be critical in the resolution of acute influenza A virus (IAV) infections. Previously our laboratory has demonstrated that the magnitude of the IAV-specific CD8 T cell response is inversely proportional to the initial IAV inoculum. The decrease in CD8 T cells observed during lethal dose IAV infections was shown tobe a result of apoptosis driven by FasL expressed on lymph node dendritic cells (LNDC) during lethal, but not sublethal, dose IAV infections. However the specific LNDC subset(s) responsible for FasL:Fas mediated apoptosis of IAV-specific CD8 T cells remains to be identified. The existence of multiple subsets of dendritic cells (DC) within the lymph node (LN) with distinct functions during viral infections suggest the possibility that a specific subset(s) may be responsible for this effect. Furthermore, the regulation of FasL expression on LNDC during lethal versus sublethal dose IAV infections was shown to be dependent on the levels of IL-12p40. However, the specific IL-12p40 containing cytokine, as well as which cells produce this cytokine within the LN remain unknown. Finally, whether or not the expression of FasL expression induced during infections is unique to IAV or if other pulmonary insults can mediate this effect is as of yet undetermined.

Here we demonstrate that plasmacytoid DC (pDC), which accumulate in the lung draining LN, are the only LNDC subset eliminating IAV-specific CD8 T cells through FasL:Fas dependent mechanism both in vitro and in vivo during lethal dose IAV infections despite FasL expression by all LNDC subsets. Further we demonstrate that this disparity in LNDC FasL induced apoptosis is related to the individual DC subsets ability to present IAV antigen as pDC are the only LNDC subset incapable of viral antigen presentation via MHC class I during IAV infections.

This dissertation further demonstrates that IL-12p40 homodimer ($p40_2$), which is produced by respiratory DC (rDC) and LNDC, and not IL-12p40 monomer controls FasL

expression on LNDC during lethal dose IAV infections. Additionally I also demonstrate that IL-12Rβ1, which binds IL-12p40, is important for $p40_2$ mediated LNDC FasL expression. We further go on to demonstrate that rDC migration from the lungs to the LN is required for both LNDC $p40_2$ production and FasL expression. However, LNDC in isolation are unable maintain FasL expression suggesting their production of $p40_2$ may not me sufficient for FasL expression. Conversely, rDC are sufficient to induce FasL expression on LNDC from IL-12p40 deficient LN. Together these data suggest that rDC are a critical component of $p40_2$ mediated LNDC FasL expression. Finally, we demonstrate that the differential production of $p40_2$, and downstream LNDC FasL expression, observed during lethal versus sublethal dose IAV infections is not unique to IAV as intranasal stimulation with diverse TLR agonists also results in differential rDC $p40_2$ production and LNDC FasL expression. These data suggest that in addition to IAV, a multitude of pulmonary pathogens may regulate antigen-specific CD8 T cells through this pathway.

Taken together the results present herein detail a mechanism of CD8 T cell regulation during IAV infections mediated through $p40_2$ induction of FasL expression on pDC within the LN. FasL expressing pDC then induce apoptosis of activated Fas^+ IAV-specific CD8 T cells within the LN during lethal a IAV infections leading to a reduction in the number of IAV-specific CD8 T cells that reach the lung and as a result death of the host.

Abstract Approved: _____
Thesis Supervisor

_____
Title and Department

_____
Date

PLASMACYTOID AND RESPIRATORY DENDRITIC CELLS CONTROL THE MAGNITUDE OF THE VIRUS-SPECIFIC CD8 T CELL RESPONSE TO LETHAL DOSE INFLUENZA VIRUS INFECTIONS

by

Ryan Andrew Langlois

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Immunology
in the Graduate College of
The University of Iowa

July 2010

Thesis Supervisor: Associate Professor Kevin L. Legge

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

PH.D. THESIS

_____

This is to certify that the Ph.D. thesis of

Ryan Andrew Langlois

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Immunology at the July 2010 graduation.

Thesis Committee: _____
Kevin L. Legge, Thesis Supervisor

_____
Thomas S. Griffith

_____
John T. Harty

_____
Stanley Perlman

_____
Richard Roller

To my parents for their continuous support

ACKNOWLEDGMENTS

This work was made possible through the efforts of many people who generously donate their time and resources to assist me in completion of this dissertation.

I would first like to thank my advisor Dr. Legge for his continuous support, encouragement, patience and confidence throughout my time in the laboratory. Further, I would like to thank all members of my thesis committee; Dr. Griffith, Dr. Harty, Dr. Perlman and Dr. Roller for their time and insight throughout this process.

I would also like to thank the members of the Legge laboratory not only for providing me with technical and theoretical assistance but also for creating and enjoyable workplace especially; Dr. Jodi McGill, Dr. Becky van Oosten, Emily Hemann and Michelle Edsen.

Many people and laboratories have provided technical assistance and reagents throughout the course of my graduate career: Dr. Nhat-Long Pham in Dr. Harty's Laboratory, Dr. Matthew Olson in Dr. Varga's Laboratory, Dr. Thomas Griffith, Dr. David Meyerholz, Dr. Kalipada Pahan (Rush University) and Dr. Paul Allen (Washington University)

Finally, I would like to thank my parents, Dick and Jan, and sister, Kelsey, for their unending support with out which this dissertation would not be possible.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

Ag: antigen

APC: antigen presenting cell

cDC: conventional dendritic cell

DC: dendritic cell

HA: hemagglutinin

IAV: influenza A virus

iBALT: inducible bronchus associated lymphoid tissue

IFN: interferon

LN: lymph node

LNDC: lymph node dendritic cell

MFI: mean fluorescence intensity

NA: neuraminidase

NP: nucleocapsid portein

NK cell: natural killer cell

pDC: plasmacytoid dendritic cell

p.i.: post infection

PRR: pattern recognition receptors

$p40_2$: IL-12p40 homodimer

rDC: respiratory dendritic cell

RSV: respiratory syncitial virus

TCR: T cell receptor

TLR: toll like receptors

TRAIL: tumor necrosis factor apoptosis inducing ligand

CHAPTER 1 GENERAL INTRODUCTION

Dendritic Cells

Dendritic cells (DC) are specialized cells capable of bridging the innate and adaptive arms of the immune system. These cells act as sentinels for the immune system as they are uniquely positioned throughout the body at points of potential pathogen invasion (i.e. skin, lungs and intestines) and in sites where adaptive immune responses are initiated (i.e. spleen, lymph nodes [LN] and peyers patches). DC are able to recognize and engulf pathogenic organisms and subsequently produce cytokines and chemokines as well as present antigens from these pathogens to both CD4 and CD8 T cells. Given DC position within the body, their ability to produce cytokines and chemokines as well as activate naïve CD4 and CD8 T cells, DC are integral in the orchestration of the immune response to pathogenic infections.

DC are a heterogeneous cell population classified into two major subtypes, conventional and plasmacytoid, based in part on the precursor populations they arise from and their phenotypic characteristics. Both conventional DC (cDC) and plasmacytoid DC (pDC) can be derived from common myeloid and lymphoid progenitors (1), and a single precursor DC can give rise to both plasmacytoid and conventional lineages (2). However, cDC can also be selectively generated from a lineage$^-$ CD11c$^+$ precursor originating in the bone marrow that is incapable of generating pDC (3) suggesting that the signals and precursors required for the generation of cDC may be more stringent than for pDC. cDC that seed both the lymphoid organs as well as the periphery are further subdivided into multiple subsets. Within lymphoid organs cDC are divided into CD8α^+, CD4$^+$ or CD8α^-CD4$^-$ (ie double negative [DN]) subsets (4, 5). While these subsets are thought to be stable there is evidence that DN DC are capable of differentiating into CD8α^+DC, through Flt3L signaling or during viral infections (6). The exact function of DN DC during infections remains unclear and given the above findings this subset may

simply represent an immature form of CD8α$^+$DC. During multiple infections, CD8α$^+$ DC within the LN are the primary cell that present antigens to naïve CD8 T cells (7) and are specialized to allow cross presentation of antigens in addition to direct presentation through the classical MHC class I pathway (8, 9). In this connection, CD8α$^+$ DC acquire antigens from pathogens either from DC that migrate into the LN from the site of infection (10, 11) or through direct uptake of antigen from antigen drainage into the LN from the lymphatics (12).

In addition to CD8α$^+$DC antigen presentation to naïve CD8 T cells, DC that migrate from the periphery into LN also present antigen to naïve CD8 T cells (10, 11, 13, 14). Migratory DC represent a heterologous group of cDC that exist at sites throughout the periphery and take up exogenous antigen for cross presentation and/or can become directly infected with pathogens (10, 11, 15, 16). Furthermore, in addition to migration to the draining LN to aid in the activation of naïve CD8 T cells, rDC can also remain in the periphery to assist in infection clearance through the production of type I IFN and pro-inflammatory cytokines (17-19).

As described above pDC can arise from either the common myeloid or lymphoid progenitor. However a unique precursor for pDC, that is incapable of also differentiating into other cDC subtypes remains elusive (1), suggesting that pDC may comprise a heterogenous population of cells derived from multiple sources. pDC are predominantly thought to produce type I IFN following virus infections and were originally described and named interferon producing cells (IPC) (20); however, these cells also have antigen presenting function and might be capable of cross-presenting antigen to CD8 T cells (21). Together these data suggest a multifaceted role for pDC in immune responses.

Both cDC and pDC must undergo maturation in order to orchestrate immune responses. Maturation of quiescent DC occurs after exposure to pathogenic, but not non-pathogenic material (22). Pathogens entering the body express many byproducts that are recognized by DC through expression of pattern recognition receptors (PRR). DC utilize

multiple PRR (i.e. toll like receptors [TLR], and rig I like receptors [RLR], etc) as well as cytokine receptors that permit the DC to either directly (i.e. PRR) or indirectly (i.e. cytokine) sense a dangerous challenge and undergo activation and maturation (23, 24). The TLR4 ligand LPS is among the best-studied examples of TLR-mediated maturation of DC. LPS induces upregulation of co-stimulatory molecules and cytokines by both human and murine cDC (25, 26). This maturation/activation in turn permits DC to drive potent antigen-specific T cell responses.

Individual subsets of DC have been shown to differentially express TLR and it has therefore been proposed that the individual DC subsets may be specialized in recognition of individual classes of pathogens and priming appropriate immune responses to that pathogen (17). For instance, the double stranded RNA mimic polyI:C, a ligand for TLR3, drives cytokine production from human cDC but not pDC (17). In contrast, TLR9 (bacterial DNA mimic, CpG) and TLR7 ligands (influenza ssRNA, imiquimod) promote type I IFN release only from pDC (17, 25, 27). All together these data suggest that during an infection DC subsets may be specialized to perform unique tasks i.e. antigen presentation, production of type I IFN, or drive specific flavors of immune responses.

In addition to direct stimulation by pathogen byproducts, DC can be stimulated to mature (i.e. upregulate costimulatory molecules and produce pro-inflammatory cytokines) via cytokine signals (22, 28). For example, type I IFN induces upregulation of costimulatory molecules and enhances antigen presentation by cDC (29). Therefore, TLR stimulation can lead to DC maturation either directly through interaction with the DC or indirectly through TLR-induced cytokine production by other cells. Furthermore, TNFα and type I IFN drive the maturation of DC populations independent of pathogen exposure (29). These data suggest that rDC can mature and subsequently migrate to the draining LN in response to indirect infection stimuli (i.e. TLR induced cytokines) (30, 31). Since these cytokines can be critical to DC maturation, many pathogens have evolved mechanisms to avoid DC maturation. For example, influenza A virus (IAV), respiratory

syncytial virus and measles virus block type I IFN production or signaling in mDC and/or pDC (32, 33) allowing the virus to evade detection and destruction by the immune system within the host.

DC within the periphery are critical for detection and subsequent migration to the LN during pathogen invasion. Following acquisition of antigen and activation through PRR or cytokines as described above, these DC subsequently migrate to lymphoid organs and present antigen to naïve CD4 and CD8 T cells. DC cell migration from the periphery was initially studied either by transferring dye labeled cells into the lungs or by using fluorescent labels given either on the skin or intranasally (30, 34). These studies demonstrated that DC within the skin and lung utilize CCR7 and its ligands CCL19/21 to migrate into their respective draining LN not only during inflammatory responses but also during the steady state (30). Furthermore given the steady state DC migration these studies suggest that DC within the periphery are not only critical for the initiation of anti-pathogen responses but likely also play a role in the induction of tolerance to innocuous antigens (34-36).

In addition to induction of early innate responses and the activation of adaptive T cell responses, DC can act as both effector and regulatory cells. Multiple DC subsets have been identified that can directly eliminate target cells using a variety of mechanisms. Splenic CD8α^+ DC, langerhans DC and LNDC have all been implicated in the elimination of T cells through the utilization of FasL as an effector molecule (37-39). Additionally pDC, interferon-producing killer DC, and cDC can employ tumor necrosis factor apoptosis inducing ligand (TRAIL) to eliminate cancer cells (40-42). DC have also been shown to affect the immune response through the production of indoleamine 2,3-dioxygenase leading to decreased T cell proliferation and the generation of tolerance (43). These data demonstrate that DC not only participate in the generation of effector responses but also regulate the developing T cell response either by direct elimination or through indoleamine 2,3-dioxygenase mediated control of expansion. All together these

studies demonstrate that, given DC location within the body and their ability to recognize and present antigens from pathogenic challenges, DC are critical in the induction and regulation of adaptive immune responses.

Influenza virus

The influenza A virus (IAV) is an enveloped single stranded, negative sense RNA virus with a segmented genome that contains 8 gene segments that encode 11 proteins (44, 45). IAV are classified by their two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are also the targets for neutralizing antibody responses. There are currently 16 different HA and 9 NA subunits that have been described (46). Each year IAV infections cause significant morbidity and mortality throughout the world resulting in approximately 250,000 to 500,000 deaths (47). Despite likely exposure to IAV yearly as well as the generation of a potent acute B and T cell response, sterilizing immunity to subsequent seasonal IAV infections is not generated. This is largely due to IAV ability to subvert the immune system by undergoing processes known as antigenic drift and antigenic shift. Antigenic drift results from minor changes in the two surface glycoproteins HA and NA which occur during viral transcription due to a lack of proof reading capabilities in IAV's RNA-dependent RNA polymerase, ultimately leading to an inability of neutralizing antibodies to recognize and bind IAV particles (48). Additionally, because IAV has a segmented genome, it is capable of undergoing reassortment, potentially resulting in pandemic strains of IAV. When two antigenically distinct IAV (usually an avian and human virus) infect the same cell in a host animal, gene segments from the two different virus can reassort leading to a new, immunologically distinct virus that may be capable of evading neutralizing antibody responses as well as protective memory CD4 and CD8 T cell responses (49) potentially leading to pandemic strains. Recently the novel 2009 H1N1 virus arose through reassortment between two swine IAV and rapidly spread throughout the human

population (50). Additionally the most notable IAV pandemic occurred in 1918 through the introduction of an avian IAV strain into the human population ultimately leading to between 20 and 50 million death worldwide (47). Together these data highlight how the lack of immunologic memory to new immunologically distinct strains of IAV can lead to severe pandemic strains.

Much of our understanding of the immune response as well as pathogenesis of IAV infections has come from small animal models. Small animal models of IAV infections, specifically mice, faithfully mimic many aspects of human disease including; infection of similar airway epithelial cells and dendritic cells, increased pulmonary edema and airway occlusion, and predisposition to secondary bacterial infections (51-56). Importantly small animal models also mimic many aspects of the immune response including type I IFN induction and strong B and T cell responses (57-59). This has allowed for a detailed examination of many aspects of the host response to IAV from the induction of type I IFN to the generation and regulation of adaptive immunity.

IAV enters the body via inhalation through the respiratory tract (47). The virus HA is cleaved by host proteases within the respiratory tract resulting in HA_1 and HA_2 subunits. These HA subunits bind to sialic acid residues on the surface of pulmonary epithelial cells and the virus is then internalized via clathrin-coated pits, caveolae or by macropinocytosis (60, 61). Once in the endosome, exposure to low pH results in a conformation change in the HA_2 subunit exposing the fusion domain allowing for fusion of the viral and host membranes (62). After fusion has occurred, the M2 ion channel protein acidifies the viral capsid disrupting protein-protein interactions causing the virus to disassemble and viral RNA and proteins to be shuttled into the cytoplasm (63). The nucleocapsid protein (NP), associates with the viral RNA dependent RNA polymerase (consisting of PB1, PB2 and PA proteins). This complex is bound to the viral RNA gene segments, and the NP within this complex facilitates in the shuttling of this complex into the nucleus (64). Once in the nucleus the PB2 protein steals 5’ methylguanosine caps

from host mRNA, and these caps then serve as primers for the RNA dependent RNA polymerase to transcribe complementary strands of the viral genome (65, 66). Newly transcribed positive strands of IAV that are polyadenylated are transported to the cytoplasm and translated into IAV proteins (67). Transcription of IAV genes is aided by IAV non-structural protein 1 (NS1) which binds to the 5'UTR of viral RNA and recruits eIF4GI, enhancing translation of viral mRNA over host RNAs (68). Non-polyadenylated complete strands of RNA remain in the nucleus and serve as templates for the 8 IAV gene segments. The NS1 gene aids in nuclear egresss of the viral RNA into the cytoplasm. Influenza virions are then packaged in the cytoplasm out the plasma membrane and released from the cell through cleavage of sialic acids bound to HA by NA. Initially it was thought that the different segments of IAV were packaged into the virion at random; however, recent evidence suggests that incorporation of RNA segments occurs in a selective and organized manner through pairing with neighboring segments. Such ordered packaging likely decreases the number of defective viral particle that are produced (69). NS1, in addition to its role in transcription of IAV genes, also plays a critical role in mediating evasion of the virus from the immune response, specifically the type I IFN response. This occurs through 2 distinct mechanisms: 1) NS1 binds to viral RNA or to viral RNA-RIG I complexes and prevents initiation of IFN production (70, 71) and 2) by blocking cellular mRNA processing and IFNβ synthesis through inhibiting cellular pre-mRNA processing (72). Importantly, NS1 is capable of inhibiting rDC maturation during IAV infection leading to decreased ability to activate naïve IAV-specific CD8 T cell responses (73, 74). Furthermore infection with IAV deficient in NS1 leads to enhanced type I interferon production and viral clearance highlighting the importance of NS1 in IAV evasion of the immune response (75). Together these studies demonstrate that NS1 is critical to the success of IAV infections through both transcription of IAV gene products as well as mediating evasion from type I IFN responses.

The immune response to influenza A virus infections

Given IAV's ability to evade the type I IFN response through NS1 and to avoid neutralizing antibody responses generated during previous IAV infections, coordinated responses by both arms of the immune system are required to mediate clearance of IAV infections. As such, immunity to acute IAV infections is multi-factorial beginning with the induction of a type I IFN response followed by an innate immune response mediated by NK cells, pulmonary macrophages and DC. At the same time pulmonary DC migrate to the LN where they, and LNDC, initiate adaptive immune responses that mediate clearance of virally infected cells and confer protection to infection with the same IAV strain.

Recognition of IAV infection, type I IFN response and NK cells

Following infection within the respiratory tract IAV is recognized by a variety of patter recognition receptors including TLR, RLR and the inflammasome. The IAV replication intermediate double stranded RNA is recognized by TLR3 within endosomes of respiratory epithelial cells and DC (76). In addition to detection by TLR3 IAV is also recognized by RIG I within the cytoplasm through IAV RNA containing 5' triphosphates (70, 77). Additionally IAV is recognized by pDC through TLR7 and the detection of the single stranded viral RNA (27). Detection of IAV through TLR3, 7 and RIG I pathways leads to the production of type I IFN from both DC and pulmonary epithelial cells (27, 70, 76, 77). Additionally recently the detection of IAV through the inflammasome complex, specifically the nod like receptor 3 complex, within macrophages has been demonstrated to be critical to early innate responses. This detection is mediated through IL-1β as well as induction of downstream CD4 and CD8 T cell responses (78, 79). The recognition of IAV by the inflammasome is mediated through binding of IAV M2 protein to the nod like receptor 3 complex leading to activation of caspase 1 and IL-1β (80). Loss

of this activation pathway leads to increased pulmonary viral titers as well as enhanced mortality. Together these results demonstrate that IAV is detected through multiple distinct mechanisms leading to the production of cytokines and ultimately the initiation of innate and adaptive immune responses.

Type I IFN is critical in the immune response to viral infections. Type I IFN serves as a signal to infected and neighboring uninfected cells to induce an anti-viral state thereby preventing the spread of the virus within the host. Additionally Type I IFN also serves as an activation signal for DC to undergo maturation enhancing antigen presentation (81). The importance of type I IFN during IAV infections was highlighted by studies using either gene targeted knockouts of either type I IFN or downstream IFN induced-genes. These animals displayed delayed viral clearance and enhanced mortality during IAV infections (82, 83). During the initial stages of IAV infection pDC recognize the virus through TLR7 and produce large amounts of type I interferon (84). However, Lambrecht and colleagues have demonstrated that depletion of pDC during IAV infections did not result in a reduction of pulmonary type I IFN (15). These data suggest that during IAV infections another cell(s) within the lung is capable of compensating for the loss of pDC and produce type I IFN. One cell that may be capable of compensating for the loss of pDC-produced type I IFN is the alveolar macrophage as depletion of these cells prior to infection, but not at later time points, results in the inability to control viral replication (85). Furthermore during Newcastle disease virus infection alveolar macrophages, and not pDC, were found to be the primary producer of type I IFN (86). These data suggest that alveolar macrophages may be playing a critical role early in controlling the early spread of the virus within the host IAV infections.

Type I IFN not only serves as an activation signal for DC but for NK cells as well. Importantly type I IFN was demonstrated to be critical for NK cytotoxicity in vitro (87). NK cell responses during IAV infections are critical as in vivo depletion of NK cells or infection of mice that lack NCR1, a receptor for IAV HA on NK cells, results in failure to

control early IAV viral replication leading to death of the host (88). Together these data suggest that early control of IAV infections are critically dependent on type I IFN and NK cell responses.

rDC migration and CD8 T cell activation

Respiratory DC (rDC) within the lungs play a critical role during IAV infections. rDC acquire IAV antigens likely through either direct infection (VanOosten and Legge, manuscript submitted) (89-91) or through phagocytosis of dead and dying IAV-infected epithelial cells (92-95). Following acquisition of IAV antigen these rDC then mature, upregulate co-stimulatory markers and migrate to the regional LN (11, 30). Interestingly, TLR mediators as well as TNFα administered alone intranasally also induced a rapid maturation and migration of rDC from the lungs to the LN suggesting that direct or indirect TLR signaling triggered during IAV infections is sufficient to induce rDC migration to the LN (30). However unexpectedly, rDC migration from the lungs to the draining LN was only enhanced for the first ~24-48 hours post-infection (30). This result suggested that IAV-induced rDC migration was transient and limited to the early stages of infection. Interestingly, the establishment of the DC migratory refractory state profoundly affected subsequent respiratory challenges as the lack of rDC migration led to inhibited adaptive immune responses to subsequent pathogenic challenges (30). Furthermore, blockade of rDC migration ((11, 30), and Katewa and Legge, unpublished results) or deletion of rDC ((15), Boeding and Legge, unpublished results) prior to IAV infection inhibits the activation of naive CD8 T cells. These results suggest that migration of the rDC from the lungs to the LN is a necessary component in the activation of the naïve T cells within the LN. However, in contrast to the above results the early kinetics of rDC migration during IAV infections remain controversial, as multiple groups have demonstrated that DC can emigrate from the lungs as late as 7 days post infection (96, 97). Interestingly, these studies demonstrated that early and late migration was mediated

by different rDC subsets (97). The consequence of late rDC migration as well as differential function of rDC subsets remains unclear.

Recently, studies have demonstrated that rDC may not solely be responsible for presenting virus antigen to naïve CD8 T cells. Belz et al demonstrated that both the migratory rDC as well as a LN resident DC population (i.e. the CD8α^+DC) had the ability to activate naïve IAV-specific CD8 T cells following IAV infections (10, 11). However, when rDC migration from the lungs to the LN was blocked, CD8α^+ DC did not present IAV antigens suggesting that rDC are critical for the transport of IAV antigens to the LN and may transfer these antigens to LN resident DC. Similar transfer of antigen from migratory DC to LNDC has been described in herpes simplex virus-1, vaccinia virus and Lysteria monocytogenes infections (13, 98). Together these results suggest that rDC are most likely responsible for transport of IAV antigens into the LN and then they may share that antigen, as well as the responsibility to activate naïve T cells, with endogenous CD8α^+ LNDC. Interestingly, in contradiction with the previously published literature, one recent report demonstrated that CD8α^+ DC were unable to present antigen within the LN and that only migratory DC were able to activate naïve CD8 T cells (96). These differences in antigen presentation within the LN could represent differences in viral strain, viral dose, route of administration or strain of animal used. Therefore given the above caveats the DC population(s) within the LN responsible for antigen presentation during IAV infections remains controversial. However, what is clear is that not all LN resident DC subsets or migratory subsets are capable of presenting IAV antigens to naïve CD8 T cells as pDC isolated from IAV infected LN were unable to stimulate naïve CD8 T cell responses despite the presence of intracellular IAV antigens (10, 11, 15, 97). In this regard, depletion of pDC during IAV infection resulted in no diminution of the CD8 T cell response suggesting that in vivo these cells are not critical for the induction of IAV-specific CD8 T cell responses (15). However, when pulsed with IAV peptide ex vivo or directly infected in vitro pDC gained the ability to activate naïve

CD8 T cells (97). These data suggest that while in vivo pDC may not be able to efficiently process IAV antigens within the LN during IAV infection they could remain inherently capable of antigen presentation to naïve CD8 T cells if the IAV peptides reached the cell surface independently (97). Together these data suggest that pDC, despite their ability to acquire IAV antigen (15), are unable to efficiently process and present IAV antigens via MHC I and do not play a role in activating CD8 T cells within the LN in vivo.

The cooperation between DC subsets within the LN in generating an IAV-specific T cell response is not just limited to antigen presentation. Recently, our laboratory has described a novel regulatory axis where LNDC expression of FasL is differentially regulated following lethal and sublethal IAV infections (Figure 1) (38). In naive mice, the LNDC within the lung draining LN normally express FasL. Following sublethal dose IAV infections, however, the emigrated rDC and LNDC down regulate IL-12 p40 expression (38). This loss of IL-12p40 from the LN in turn down regulates FasL expression on the LNDC, through a yet to be determined mechanism. In contrast, during lethal dose IAV infections IL-12 p40 expression by rDC and LNDC is maintained allowing LNDC FasL expression to remain intact (38). At this time the specific cell type responsible for IL-12p40 production as well as the IL-12p40 isoform required for IL-12p40-dependent LNDC FasL regulation remains unknown. As the naïve IAV-specific CD8 T cell repertoire is activated by rDC and CD8α^+ DC within the LN, these CD8 T cells upregulate Fas expression and become susceptible to FasL-mediated apoptosis (38). In the case of lethal IAV infections, the burgeoning activated IAV-specific CD8 T cells are therefore eliminated by the FasL$^+$ LNDC. This loss of IAV-specific CD8 T cells leads to delayed virus clearance in the lungs and increased mortality. Importantly, blockade of FasL expression during lethal IAV infections or deficiency in functional FasL rescues CD8 T cell immunity and protects mice from IAV-associated mortality during lethal dose IAV infections (38). At this time the LNDC subset(s) responsible for FasL mediated

elimination of IAV-specific CD8 T cells during lethal dose IAV infections have yet to be identified. Further any additional requirements for LNDC mediated elimination of IAV-specific CD8 T cells have not been defined. Taken together these results suggest that the magnitude of the IAV-specific CD8 T cell response is intimately dependent upon, and regulated by, rDC and LNDC within the LN.

CD8 and CD4 T cell responses within the lung

Initiation of an effector CD8 T cell response is critical for the resolution of acute IAV infections (99-101). Following presentation of IAV-antigens by DC within the LN, CD8 T cells rapidly divide and migrate into the lungs beginning at day 4 p.i and peaking at approximately day 10 p.i. (18, 96). These CD8 T cells then recognize IAV infected cells through viral peptide-MHC I complexes and subsequently eliminate these cells using a variety of effector mechanisms including the perforin and granzyme pathways as well as TRAIL and FasL (100, 102). Following resolution of IAV infection, effector IAV-specific CD8 T cells contract leaving a long-lived memory CD8 T cell pool capable of responding to, and mediating some protection from, secondary IAV infections (103-105).

Like IAV-specific CD8 T cells, CD4 T cells are activated within the lung draining LN, migrate into the lungs and play an important role during IAV infections. In the absence of CD4 T cells viral clearance is delayed ~2-4 days. However CD4 T cells alone, i.e. in the absence of CD8 T cells and B cells, are not sufficient to clear IAV infections suggesting the major role for CD4 T cells is to provide help to other immune cells(58, 106). Within the lungs IAV-specific CD4 T cells produce IFNγ, IL-2 and TNFα that help to sustain effector CD8 T cell responses as well as activate innate cells to aid in clearance of the IAV infection (107, 108). Finally memory CD4 T cell responses are also critical in the generation and maintenance of memory CD8 T cell and B cell responses allowing for enhanced protection from secondary IAV infections (108, 109).

In addition to the role DC play in shaping the CD8 T cell response within the LN during IAV infections, DC also participate locally (i.e. at the site of infection). During IAV infections, DC are recruited into the lungs for at least 6 days p.i. (15, 30, 110, 111). While it is still unclear exactly where all of these cells localize, it is thought that they repopulate areas vacated by LN emigrant rDC. Additionally some of these recruited DC may localize in the inducible bronchus associated lymphoid tissue (iBALT) (112, 113) as DC are critical for iBALT formation within the lung of IAV infected mice (114). The importance of iBALT in the immune response to IAV is highlighted by studies demonstrating that even in the absence of secondary lymph organs an effective adaptive immune response can be generated in the lungs within the iBALT (71, 72). These results suggest that the DC that are recruited into and remain in the lungs during IAV infections may also play an important role in generating and/or regulating the effector CD8 T cell response to IAV infections. Indeed, the regulation of CD8 T cell responses by DC following initial antigen encounter are not limited to DC within the LN as recent studies in our laboratory suggest that IAV-specific CD8 T cells require a second hit within the periphery, i.e. in addition to the initial MHC I: IAV antigen encounter within the LN, from rDC and LNDC. In addition to presenting MHC I: IAV antigen complexes these rDC also trans-present IL-15 (110, 111). If the newly emigrated CD8 T cells do not see both IL-15 and MHC I: IAV antigen complexes on DC within the lungs they undergo increased levels of apoptosis and are unable to accumulate to the levels required to eliminate virally infected cells (110, 111). The above data demonstrate a complex regulatory system for CD8 T cells activation that is mediated by DC both in the LN and lungs during IAV infection. Heavy regulation of the CD8 T cell response to IAV likely arose to prevent CD8 T cell mediated immunopathology within the lungs, which can be lethal to the host (115). All together these data demonstrate that for effective clearance of IAV infections DC within the lungs and the LN must coordinate with CD8 T cells to generate an effective immune response capable of eliminating the infection.

FasL

FasL was first described by Nagata et al as a molecule involved in lymphoproliferative disease (116) and has been subsequently demonstrated to be critical for maintenance of immune privilege (117, 118), the prevention of autoimmunity by elimination of self reactive cells (119, 120) and the clearance of viral, bacterial and parasite infected cells (100, 121, 122). During immune responses against pathogens, FasL is generally thought of as an effector molecule capable of eliminating infected target cells. Specifically, FasL is critical in the clearance of IAV infections as mice deficient in Fas (i.e. FasL receptor) have delayed viral clearance (100). However, as described above, expression of FasL by LNDC can play an aberrant role during lethal dose IAV infections by eliminating IAV-specific CD8 T cells in the LN (38).

Expression of FasL, a type II transmembrane protein, is heavily regulated. The FasL promoter is activated by a variety of transcription factors including; NFAT, NFkB, cMYC, STAT1, IRF-1, Ap-1 etc (123-126), and is negatively regulated by the transcription factor c-fos (127). Following translation, FasL is expressed in the secretory pathway or at the cell surface. Once on the cell surface FasL expression can be regulated through cleavage mediated by matrix metalloproteases, ADAM10 and plasminogen, reducing the ability of the effector cell to induce apoptosis of its target through cell:cell contract mechanisms (125, 126). A role for secretory FasL, generated either through cleavage from the cell surface or by insertion into the secretory pathway following translation, remains controversial. Studies have demonstrated that FasL in multivesicular bodies that are secreted are capable of inducing apoptosis of Fas^{+} target cells (128, 129). However, recently it was demonstrated that secretion of FasL by CD8 T cells was not required for the induction of apoptosis in target cells or for the induction of activation induced cell death suggesting that membrane, not soluble, FasL is sufficient for effector function (130).

The receptor for FasL, Fas is expressed on a wide variety of cells including lymphocytes (131-133). Fas is upregulated following cellular activation and cell stress and in certain situations, is constitutively expressed. Expression of Fas on the cell surface makes the cell susceptible to FasL:Fas mediated apoptosis (131-133). For apoptosis to be induced a trimeric Fas complex must form allowing the death domain on the intracellular tail of Fas to associate with the Fas associated dead domain (FADD). This leads to the recruitment of pro-caspase 8 forming the death inducing signaling complex, which subsequently leads to cell death by either the type I or type II pathway (Figure 2A) (131, 133).

Furthermore in addition to regulation of FasL:Fas interactions on the effector cell surface and therefore regulation of apoptosis, apoptosis can be inhibited down-stream of Fas receptor signaling. This can occur through upregulation of NFκB, which protects T cells, macrophages and B cells from Fas mediated apoptosis (134-137). NFκB is upregulated in T cells following T cell receptor (TCR) stimulation (138) suggesting that TCR induced NFκB may be capable of protecting T cells from Fas mediated apoptosis. Similarly signals through the B cell receptor, which upregulate NFκB, result in a transient (less than 24h) protection from Fas-mediated apoptosis (Figure 2B) (135). One potential mechanism for NFkB induced protection is upregulation of cFLIP. cFlip contains a death domain and is capable of decreasing caspase 8 activation by binding and blocking processing of caspase 8 into its active form (Figure 2B) (139). Together these data suggest that antigen-specific activation of adaptive immune cells and the subsequent upregulation of NFκB might be able to protect these cells from immediate Fas mediated apoptosis.

In addition to a role for FasL:Fas interactions in apoptosis, stimulation of Fas by FasL can also play a costimulatory role. Interestingly two down-stream signaling components critical for Fas mediated induction of apoptosis, FADD and caspase 8, have been shown to also be important for T cell activation and proliferation (Figure 2C) (131,

133, 140, 141). Further these findings suggest that, under certain conditions, Fas can act as a costimulatory molecule enhancing T cell activation. However, at this time the mechanisms of how Fas signaling leads to death versus costimulation as determined by the cell are poorly understood but may be related to the cell-type or the cellular activation state and additional signals the cell receives from the environment at the time of FasL engagement (133).

IL-12p40

IL-12 is a proinflammatory cytokine that is predominantly produced by antigen presenting cells during infection and plays a critical role as a co-stimulatory signal during antigen presentation (142). IL-12 is made up of two distinct subunits IL-12p40 and IL-12p35 (142). Furthermore the IL-12p40 subunit also heterodimerizes with an additional subunit IL-23p19 to form IL-23 (142). Finally, IL-12p40 can exist in monomeric form or alternatively pairs with itself forming IL-12p40 homodimer ($p40_2$). The IL-12 subunits p40 and p35 are located on separate chromosomes, 3 and 11 respectively, and are differentially regulated at the transcriptional level (143, 144). In naïve animals IL-12p40 is made in 5-500 fold excess over IL-12p35 or IL-12p19, and $p40_2$ represents approximately 20-40% of the total IL-12p40 found in the serum of LPS treated mice (145). Therefore the levels of IL-12p35, which preferentially pairs with IL-12p40, can dictate not only the amount of IL-12 produced but also the level of p40 monomer and $p40_2$. Interestingly direct TLR stimulation leads to preferential production of IL-12p40 over the other IL-12p40 binding partners (146). In this connection, while signaling through TLR7 and TLR9 have been demonstrated to induce strong $p40_2$ production from DC, signaling through TLR4 induces only transient production and is rapidly shut off (146). Additionally PGE_2 (a pro-inflammatory lipid compound) selectively stimulates production of $p40_2$ but not p70 in DC (147). In addition to regulated transcription of the various IL-12p40 binding partners, IL-12p40 is also post transcriptionally modified.

Following translation p40 monomer subunits require chaperone protein disulfide isomerase (148) for formation of disulfide bonds that hold the homodimer together. All together these studies suggest that various TLR stimulations may differentially regulate the production of $p40_2$ and therein potentially the down-stream immune response that is generated.

Similar to $p40_2$ production and regulation, $p40_2$ signaling is also complex. Early studies demonstrated that because IL-12p40 bound with high affinity to IL-12Rβ1 that $p40_2$ and IL-12p40 monomer could act as IL-12 signaling antagonists (145, 149-151). However, more recent reports have demonstrated that $p40_2$ administered to mice deficient in IL-12p35 and p40 protected these animals from Bacillus Calmette-Guerin in the absence of IL-12 (152, 153), enhanced macrophage chemotaxis through IL-12Rβ1 (154), induced DC migration from the lung to the draining LN, and enhanced CD8 T cell priming during mycobacterium tuberculosis infections (155). These data suggest that $p40_2$ can mediate its own novel agonist signaling, in addition to the previously described antagonist function of blocking IL-12 signaling. One major hurdle in further elucidating a role for $p40_2$ in the immune system was a lack of $p40_2$ specific reagents as well as a limited understanding of $p40_2$ receptor/signaling properties. Recent data has demonstrated that $p40_2$ signals directly through IL-12Rβ1 mediating the production of nitrous oxide and IL-16 from microglia as well as lymphotoxin α and nitrous oxide from splenocytes (156-159). This $p40_2$ signaling through IL-12Rβ1 was demonstrated to require p38 MAPK and ERK (156). However the full signaling cascade as well as the potential for an additional receptor remain uncharacterized. All together these data suggest that $p40_2$ signaling represents a complex balance between not only the formation of IL-12 and IL-23 but also in blockade of IL-12 signaling and $p40_2$ generated agonist signaling.

Rationale for project

CD8 T cell responses have been demonstrated to be critical for the resolution of acute IAV infections (100, 101, 160). However, during lethal dose IAV infection, DC within the LN induce apoptosis of these CD8 T cells though IL-12p40 regulated FasL:Fas interactions (38). Currently, the LNDC population that induces apoptosis of IAV-specific CD8 T cells as well as the cell/cells that produce IL-12p40 during lethal dose IAV infections remains unknown. This dissertation has been undertaken to answer the following questions:

1) Which LNDC subset(s) induce apoptosis of IAV-specific CD8 T cells during lethal dose IAV infections, and are DC mediated MHCI:IAV-Antigen complexes capable of protecting IAV-specific CD8 T cells from LNDC FasL mediated apoptosis.
2) Which IL-12p40 cytokine controls LNDC FasL expression, and what cell within the LN produces this cytokine during lethal dose IAV infections?
3) What role does migration of rDC from the lungs to the LN play in the regulation of FasL expression by LNDC during lethal dose IAV infections?

Figure 1. Model of LNDC mediated elimination of IAV-specific CD8 T cells during lethal dose IAV infections. (A) During lethal dose IAV infections there is an increased level of IL-12p40 monomer and/or homodimer resulting in subsequent increases in LNDC FasL expression (blue cylinders). CD8 T cells that have become activated and upregulate Fas (green arrows) are eliminated by FasL:Fas (DC:T cell) mediated apoptosis leading to decreased numbers of IAV-specific CD8 T cells that reach the lung ultimately leading to the death of the host. (B) In contrast, during sublethal dose IAV infections there is a decrease in the total amount of IL-12 p40 monomer and/or homodimer resulting in reduced LNDC FasL expression. This reduced FasL expression by LNDC allows Fas+ activated CD8 T cells to escape FasL:DC mediated apoptosis leading to an increased accumulation of IAV-specific CD8 T cells in the lungs and clearance of the infection.

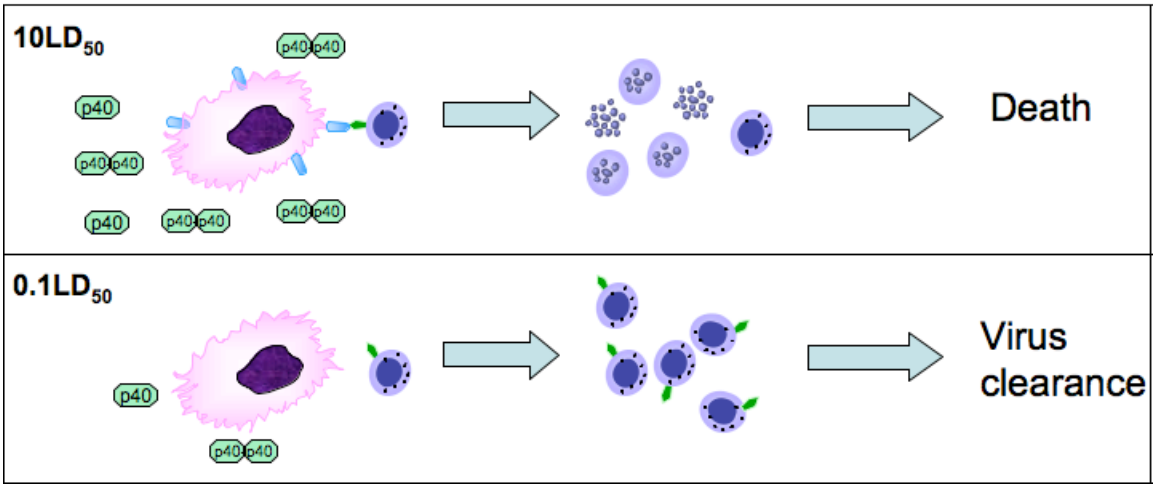
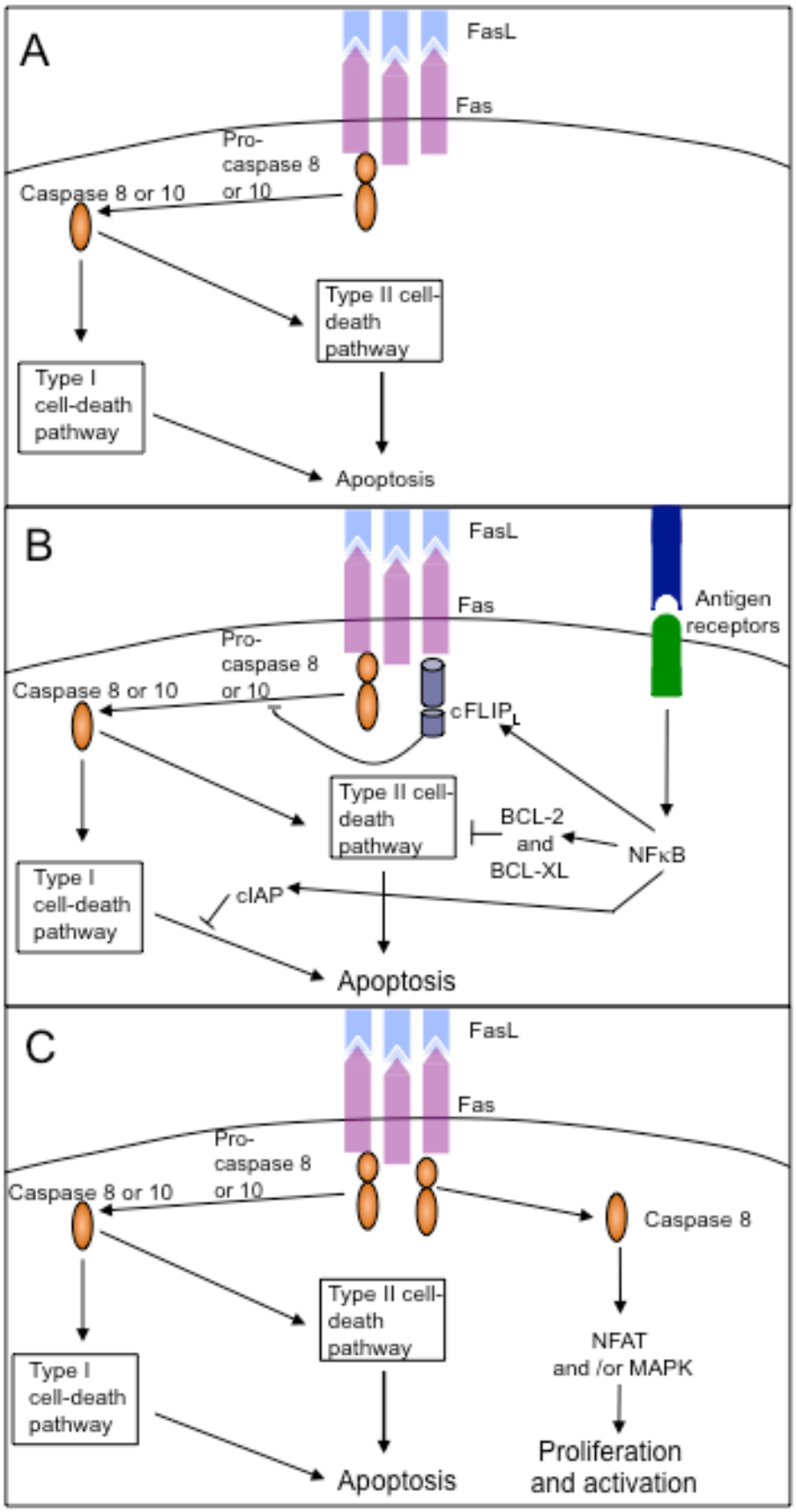

10LD50
p40
Death
0.1LD50
Virus
clearance

Figure 2. Models of Fas signaling. (A) Following ligation of Fas by FasL a trimeric Fas complex forms leading to recruitment of procaspase 8. Caspase 8 then mediates apoptosis through either type I or type 2 cell death pathways. (B) Fas mediated apoptosis is regulated at many points within the signaling cascade. Increased NFκB generated from antigen specific stimulation can lead to the upregulation of cFLIP preventing conversion of procaspase 8 into active caspase 8. Further, NFκB upregulation of cIAP inhibits type I cell death and increases Bcl-2 and Bcl-X_L transcription therein inhibiting type II cell death. (C) In addition to apoptosis, Fas and downstream caspase 8 can mediate enhanced activation and proliferation in CD8 T cells.


A
FasL
Fas
Pro-
caspase 8
or 10
Caspase 8 or 10
Type II cell-
death
pathway
Type I
cell-death
pathway
Apoptosis
B
FasL
Fas
Antigen
receptors
Pro-
caspase 8
or 10
Caspase 8 or 10
cFLIP$_L$
Type II cell-
death
pathway
BCL-2
and
BCL-XL
NFκB
Type I
cell-death
pathway
cIAP
Apoptosis
C
FasL
Fas
Pro-
caspase 8
or 10
Caspase 8 or 10
Caspase 8
Type II cell-
death
pathway
NFAT
and /or MAPK
Type I
cell-death
pathway
Apoptosis
Proliferation
and activation

CHAPTER II PLASMACYTOID DENDRITIC CELLS ENHANCE MORTALITY DURING LETHAL DOSE INFLUENZA INFECTIONS BY ELIMINATING VIRUS-SPECIFIC CD8 T CELLS

Introduction

Clearance of primary influenza virus (IAV) infections is greatly enhanced by the generation of an effector CD8 T cell response (99-101). Initially, these CD8 T cells are primed by DC within lung draining lymph nodes (LN) (11, 15, 30, 97) and then subsequently traffic to the lung where they eliminate virally infected cells via effector mechanisms including; perforin, FasL and tumor necrosis factor related apoptosis inducing ligand (TRAIL) (100, 102). Previously, we have demonstrated a novel regulatory mechanism whereby lymph node resident dendritic cells (LNDC) directly drive apoptosis of IAV-specific CD8 T cells within the LN during lethal, but not sublethal, IAV infections (38) (Figure 1). This loss of IAV-specific CD8 T cells leads to decreased numbers of CTLs that enter the lungs, resulting in a failure to clear the infection and ultimately the death of the host. Such elimination of Fas^+ IAV-specific CD8 T cells occurs subsequent to the initial priming of CD8 T cells on days three and four post-infection (p.i.). In contrast to lethal dose IAV infections, during sublethal infections LNDC downregulate FasL expression allowing the developing Fas^+ IAV-specific CD8 T cells to escape apoptosis and traffic into the lungs in sufficient numbers to clear the infection (38).

At least six distinct populations of DC have been described within the lung-draining LN. There are four LN resident DC subsets that can be identified phenotypically as $CD4^+$ DC, $CD8\alpha^+$ DC and $CD4^-CD8\alpha^-$ DC, also known as double negative (DN) DC (161-163). Furthermore, in response to infection plasmacytoid DC (pDC) are recruited into the LN from the blood (164, 165). In addition to these LNDC, there are also at least

two respiratory DC (rDC) populations that migrate from the lung into the draining LN during infections (11, 15, 30, 97). Once they enter the LN these migratory rDC are thought to share antigen with LNDC, particularly with CD8α^+ DC, allowing this LNDC subset to also participate in activation of naïve CD8 T cells (10, 11). Interestingly, although Ag may be shared with all LNDC subsets, not all LNDC subsets are able to present IAV antigens to naïve CD8 T cells as pDC purified from the LN of IAV infected mice are unable to activate CD8 T cells directly ex vivo (10, 11, 15, 97). In support of the idea that pDC do not participate in the activation of naïve CD8 T cells, when pDC are depleted in vivo during sublethal IAV infections there is no diminution of the CD8 T cell response (15, 166). However, in contrast to the above ex vivo/in vivo results, when pDC are IAV-infected or pulsed with IAV peptides in vitro they are now able to activate naïve CD8 T cells (21, 167). Together these results suggest that while inherently capable of presenting viral antigens to naïve CD8 T cells, pDC may not present IAV-antigens via MHC class I in the LN due to inefficient processing of acquired viral proteins in vivo (15, 166).

While our previous work has demonstrated that LNDC eliminate IAV-specific CD8 T cells through a FasL-mediated pathway during lethal dose infections, it did not pinpoint the individual DC subset(s) that directly eliminate IAV-specific CD8 T cells. Further, it remains unknown what role MHC class I presentation of viral antigens by these DC subsets might play in the LNDC FasL:Fas-mediated elimination of IAV-specific CD8 T cells. The data presented in this chapter of my thesis demonstrates that whereas all LN resident DC subsets were FasL$^+$ during lethal dose IAV infection, only pDC were able to eliminate activated IAV-specific CD8 T cells. In contrast, pDC isolated from sublethal dose IAV infected mice downregulated FasL and were therefore unable to eliminate activated Fas$^+$ CD8 T cells. Interestingly, our findings also demonstrate that the recruitment of pDC into the lung draining LN was increased during lethal versus sublethal dose IAV infections elevating the putative in vivo E:T ratio (i.e. pDC:T cell)

and that transfer of $FasL^+$ pDC into mice deficient in functional FasL (i.e. gld mice) was sufficient to reverse the previously described protection of gld mice from lethal dose IAV infections (38). Finally, this chapter demonstrates that lethal dose pDC elimination of IAV-specific CD8 T cells occurs in the absence of direct viral-peptide MHC class I presentation. Taken together these data suggest that pDC are the cell type responsible for dampening the CD8 T cell response during lethal IAV infection in vivo and that such elimination occurs in the absence of cognate IAV antigen presentation.

Materials and Methods

Mice

Wild-type BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). BALB/c CD90.1 congenic mice were kind gifts from Dr. Richard Enelow (Dartmouth College, Hanover, NH) and Dr. John T. Harty (University of Iowa, Iowa City, IA). Clone-4 (CL-4) TCR transgenic mice specific for the HA_{533}/HA_{529} epitope of H1 and H2 IAV viruses, respectively were a kind gift from Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA). BALB/c gld mice ($CPt.C3\text{-}Tnfsf6^{gld}/J$) were obtained from the Jackson Laboratory (Bar Habor, ME). DUC18 TCR transgenic mice specific for mutated $tERK_{136\text{-}144}$ were kindly provided by Dr. Paul Allen (Washington University, St. Louis, MO). All experiments were performed in accordance with federal and institutional guidelines approved by the University of Iowa Animal Care and Use Committee.

Virus Infection

6-10 week old BALB/c mice were anesthetized by halothane or isofluorane and infected i.n. with either a $10LD_{50}$ or a $0.1LD_{50}$ dose of mouse-adapted A/JAPAN/305/57 in 50μl of Iscoves media. Viruses were grown and stored as previously described (38).

MHC I Tetramers

Tetramers HA_{204} (H-2K(d)/LYQNVGTYV), HA_{529} (H-2K(d)/IYATVAGSL), and NP_{147} (H2K(d)/TYQRTRALV) were obtained from National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility (Atlanta, GA).

Flow Cytometry

LN cells were stained with the following monoclonal antibodies: rat anti-mouse CD8α (53-6.7), hamster anti-mouse CD11c (HL3), rat anti-mouse CD3ε (145-2C11), rat anti-mouse CD4 (CT-CD4), and rat anti-mouse CD43 (S7) purchased from Becton Dickinson; mouse anti-mouse CD90.2 (5a-8), rat anti-mouse CD45R (RA3-6B2), rat anti-mouse DX5, and rat anti-mouse CD19 (6D5) purchased from Caltag (Invtrogen). Anti-FasL CD95L (MFL3) was purchased from eBioscience. For FasL staining, cells were blocked with 1:100 rat serum, 1:100 hamster serum and 1:400 free streptavadin (Molecular Probes) on ice for 25 mins. Cells were then washed twice and stained with 2x (i.e. 1:50) biotin-congugated anti-FasL (MFL3) followed by streptavadin-PE purchased from Becton Dickinson. For surface staining, isolated cells (10^6) were stained with antibody, and then fixed using BD FACS Lysing Solution (BD Biosciences). All flow cytometry data were acquired on a BD FACS Calibur or BD FACS Canto II (BD Immunocytometry Systems) and analyzed using FlowJo software (TreeStar, Ashland, OR).

CD8 CL-4 T cell purification and adoptive transfer

Spleens from CL-4 mice were removed and processed into single-cell suspensions. Cells were then labeled with anti-CD8α Microbeads and purified according to manufacturer's instructions (Miltenyi Biotec). The purified $CD90.2^+$ CL-4 cells (2×10^6) were then adoptively transferred i.v. into BALB/c $CD90.1^+$ mice. 24 hrs post-transfer the host mice were infected i.n. with a $0.1LD_{50}$ of IAV as described above. For isolation of activated CL-4 CD8 T cells lung draining LN from IAV-infected CD90.1-

CL-4 transferred host mice were removed on day 3 p.i. and digested as described above. CD8 T cells were enriched using anti-CD8α beads according to manufacturer's instructions (Miltenyi Biotech). $CD8\alpha^+$ cells were then stained with antibodies to CD90.2 and CD43 and activated $CD90.2^+CD43^+$ CL-4 cells were sort purified using a FACS DIVA.

Lymph Node Dendritic Cell Purification

Lung draining LN (peribronchiolar and mediastinal) were removed and digested with 4000 units of type IV collagenase (Worthington) and 600 units of DNase 1 (Sigma) in Iscoves media for 10 mins at room temperature. LN were then processed into a single-cell suspension and red blood cells lysed using NH_4Cl-Tris. Cells were then stained with anti-CD3ε-PE and anti-CD19-PE mAb followed by anti-PE microbeads according to manufacture's instructions (Miltenyi Biotech). Labeled cells were isolated using an autoMACS. The negative fraction was saved, stained with antibodies to CD11c, CD45R (B220), CD8α, CD4 and sorted into $CD11c^{mod}CD45R^+CD8^+$ cells (i.e. pDC); $CD11c^+CD45R^-CD8^+$ cells (i.e. $CD8\alpha^+$ DC); $CD11c^+CD45R^-CD8^-CD4^-$cells (i.e. DN DC); and $CD11c^+CD45R^-CD8^-CD4^+$ cells (i.e. $CD4^+$DC) using a FACS DIVA.

Ex vivo LNDC killing assay

Purified LNDC subsets (10^4) were co-incubated with 10^4 activated CL-4 cells and 2.5μg of rmFas-human Fc (R&D Systems), 1μM HA_{529} peptide (Biosyn), or control media for 18 hours at 37°C. To determine viability, cells were resuspended in 1X annexin binding buffer and stained with Annexin-V-APC and 7-AAD (40% as per BD recommendation for 10^6 cells). The degree of DC killing of T cells was determined by measuring the fraction of CD8 T cells that were non-apoptotic (i.e. Annexin-V^- 7-AAD^-, live cells) and normalizing this value to CD8 T cells cultured alone.

In vivo pDC transfer studies

Spleens from naïve wild-type or gld mice were removed, and single cell suspensions stained with anti-PDCA-1 microbeads and purified according to manufacturer's instructions (Miltenyi Biotec). 2 x 10^6 wild-type or gld pDC were then adoptively transferred i.v. into gld mice 18 hours post lethal IAV infection. Some groups of pDC were also pulsed with 1μM HA_{529} peptide for 30 mins at 37°C. Mice were monitored daily for weight loss and mortality and on day 4 lung draining LN were removed to determine IAV-specific T cell responses in the LN.

pDC depletion

Mice were infected with a $10LD_{50}$ of IAV and treated intravenously at 0 and 24 hours post infection with 125μg of anti-PDCA-1 (Miltenyi Biotech). Depletion was assessed within the lung draining LN using antibodies against CD11c and B220.

Statistical Analysis

Statistical analysis between 2 data sets was performed using a one tailed student's T test. Differences were considered to be statistically significant at p values at or below 0.05. Statistical analysis for mortality experiments was performed using Kaplan-Meier survival analysis.

Results

CD8α$^-$ DC and pDC downregulate FasL expression during lethal dose IAV infections

Our previous studies have demonstrated that the reduction in IAV-specific CD8 T cell numbers in lethal dose IAV infections (Figure 3) is linked to FasL expression on DC within the LN (38). This FasL expression on LNDC decreases during sublethal, compared to lethal dose IAV infection, therein allowing activated effector IAV-specific

CD8 T cells to leave the LN and traffic to the lungs to fight the infection. However the decrease in FasL expression on LNDC during sublethal IAV infections was not uniform with some LNDC maintaining high levels of FasL expression. This finding suggested that a unique LNDC subset(s) may be triggering the elimination of CD8 T cells during lethal dose IAV infection (38). In order to determine which LNDC subset(s) differentially modulate FasL expression during IAV infections, mice were infected with either a lethal or sublethal dose of IAV and the level of FasL on individual LNDC subsets determined on day 3 p.i., the time-point where LNDC-mediated elimination of IAV-specific CD8 T cells begins (38). Interestingly, while both $CD8\alpha^+$ DC and pDC decreased FasL expression during sublethal compared to lethal dose IAV infection, $CD8\alpha^-$ DC, including both the $CD4^+$ and DN DC subsets, did not modulate FasL expression between the two IAV infection doses and had increased FasL expression compared to both $CD8\alpha^+$ DC and pDC (Figure 4). Further, the rDC subsets, which have migrated from the lungs to the LN during IAV infection, remained $FasL^-$ during both lethal and sublethal IAV infection (Legge and Braciale, unpublished results). Given that the pDC and $CD8\alpha^+$ DC subsets are the only LNDC populations to down-modulate FasL expression during sublethal dose IAV infections, and IAV-specific CD8 T cell responses are rescued at this dose of infection (Figure 3) despite the remaining $CD8\alpha^-$DC FasL expression (Figure 4) (38), it suggests that $CD8\alpha^-$DC are not the cells responsible for the elimination of CD8 T cells during lethal dose IAV infections. Further these results importantly indicate that either the pDC and/or $CD8\alpha^+$ DC are likely responsible for mediating FasL driven CD8 T cell apoptosis during lethal dose IAV infections.

pDC accumulate in the lung draining LN in greater numbers during lethal dose IAV infections

Since our above results suggested the involvement of CD8α$^+$DC and/or pDC in the induction of CD8 T cell apoptosis during lethal dose IAV infections, we next determined the kinetics and magnitude of their recruitment/expansion within the lung draining LN during lethal and sublethal dose IAV infections. Our results show that the number of both pDC and CD8α$^+$DC increases within the LN between day 2 and 4 (Figure 5), i.e. the time-point during LNDC-mediated induction of CD8 T cell apoptosis occurs within the LN (38). Specifically, the number of pDC substantially increased in the LN during lethal versus sublethal IAV infections between day 2 and 4 reaching significant differences by day 3 p.i. (Figure 5A). Importantly, the number of pDC present in the LN was approximately six times greater than the number of CD8α$^+$DC present during lethal dose IAV infections. Therefore these results together with results demonstrating that there are approximately 6500 activated CD43$^+$CD8 T cells present in the LN on day 4 p.i. during lethal dose IAV infection (data not shown) the putative in vivo E:T (i.e. DC:activated T cell) ratio for pDC is conservatively ~1:1 compared to ~1:6 for CD8α$^+$DC. Given that pDC exhibited enhanced LN recruitment during lethal dose IAV infections (Figure 5) and that FasL expression on pDC was dependent on the dose of IAV infection (Figure 4), these data suggest that pDC may be the predominant LNDC population responsible for elimination of IAV-specific CD8 T cells during lethal IAV infections.

pDC directly kill IAV-specific CD8 T cells during lethal dose IAV infections

To directly determine the LNDC subset responsible for driving apoptosis of IAV-specific CD8 T cells during lethal dose IAV infections we utilized an ex vivo apoptosis

assay. Briefly, transgenic CD90.2$^+$ Clone-4 T cells (CL-4), which are specific for the HA_{529} epitope of IAV, were transferred into CD90.1 hosts that were then infected with a sublethal dose of IAV. Activated donor CD90.2$^+$ CL-4 cells, which express higher levels of Fas (Figure 6), were then purified on day 3 p.i. (i.e. the time directly before T cell apoptosis occurs during lethal dose IAV infections) (38). These activated CD90.2$^+$ CL-4 T cells were then subsequently incubated with CD90.1$^+$ LNDC subsets purified from day 3 lethal dose IAV infected mice (Figure 7A). Subsequent CL-4 CD8 T cell apoptosis was measured following 18 hours of coculture (Figure 7B). Interestingly, despite the fact that all lethal dose LNDC subsets express FasL at this time-point (Figure 4), only pDC induced statistically significant levels of apoptosis of the IAV-specific CL-4 CD8 T cells after coculture. The T cell apoptosis induced by pDC was FasL:Fas dependent as coculture in the presence of Fas-Fc, which blocks FasL-mediated apoptosis, abrogated the ability of pDC to drive T cell apoptosis (Figure 7C). Given that pDC during sublethal IAV infection downregulate FasL expression (Figure 4) we next determined if pDC from sublethal dose IAV-infected mice showed a similar ability to induce apoptosis of activated IAV-specific CD8 T cells. Consistent with their downregulation of FasL during sublethal IAV infection, pDC from sublethal dose IAV-infected mice were unable to induce apoptosis of IAV-specific CD8 T cells (Figure 7D).

pDC contribute to increased mortality during lethal dose IAV infection

Our previous studies have shown that gld mice (i.e. mice lacking functional FasL) exhibit increased IAV-specific CD8 T cell expansion and protection during lethal dose IAV infections (38). Therefore we used gld mice as hosts for pDC in order to directly determine if wild-type pDC (i.e. FasL$^+$) could mediate a similar reduction in CD8 T cell responses in vivo. Indeed when wild-type pDC were adoptively transferred into gld mice,

the number of CL-4 T cells within the LN on day 4 p.i. was significantly reduced (~65%) (Figure 8A). In fact this reduction mirrored the number of IAV-specific T cells found in the LN of lethal dose infected wild-type mice. Further when wild-type pDC were transferred into lethal dose IAV infected gld mice it resulted in an enhanced mortality that was statistically similar to lethal dose IAV-infected wild-type mice (Figure 8B). This increase in disease severity was FasL-dependent as adoptive transfer of gld pDC into lethal dose IAV-infected gld mice did not increase the severity of disease. Importantly in these latter experiments, the transferred donor pDC are the only cells expressing FasL and therefore the only LNDC population able to induce FasL:Fas-mediated apoptosis of IAV-specific CD8 T cells. In preliminary studies to further demonstrate the detrimental role of pDC during lethal dose IAV infection, pDC were depleted using anti-PDCA-1, which reduces pDC numbers within the LN during lethal dose IAV infections (Figure 9A). Consistent with the above results mice that were pDC depleted displayed significantly delayed progression to mortality than mice treated with control antibody (Figure 9B) supporting the idea that pDC are enhancing mortality during lethal dose IAV infections by eliminating IAV-specific CD8 T cells within the LN. Taken together these data suggest that pDC are sufficient to dampen in vivo the magnitude of the IAV-specific CD8 T cell response during lethal dose IAV infections.

pDC eliminate IAV-specific CD8 T cells through FasL:Fas interactions in the absence of cognate IAV-antigen presentation

Given that both CD8α^+DC and pDC downregulate FasL expression in sublethal dose IAV infected mice, it was surprising that only the pDC eliminated IAV-specific CD8 T cells directly ex vivo. Further, both CD4 and DN DC expressed FasL during lethal dose IAV infections yet did not lead to any detectable apoptosis. We therefore next

undertook experiments to determine the mechanism regulating this differential killing. Previous studies have demonstrated that pDC isolated directly ex vivo from IAV-infected mice are unable to stimulate naïve CD8 T cells suggesting that pDC do not present IAV antigens via MHC class I in vivo (10, 11, 15, 97). In contrast to pDC, CD8α^+ DC and CD8α^-DC purified from the LN of IAV-infected mice are able to induce proliferation of naïve CD8 T cells directly ex vivo (10, 11, 97). Given this differential ability of LNDC subsets to present IAV-antigen to CD8 T cells, we hypothesized that IAV-peptide-MHC I presentation might rescue the IAV-specific CD8 T cells from FasL-mediated apoptosis. To test this hypothesis activated CL-4 T cells were incubated with pDC from the LN of day 3 lethal dose IAV-infected mice in the presence or absence of exogenous IAV-peptide and the ability of pDC to drive apoptosis of IAV-specific CD8 T cells was determined. Strikingly, culturing pDC with IAV-peptide abrogated their ability to eliminate IAV-specific CD8 T cells (Figure 10A). To determine if antigen presentation by pDC ablates their ability to eliminate IAV-specific CD8 T cells in vivo, pDC were pulsed with IAV peptide, transferred into gld mice and the number of transferred CL-4 cells measured following IAV-infection on day 4 p.i.. Unlike unpulsed pDC (Figure 8A and Figure 10B), pDC pulsed with cognate a IAV peptide epitope did not eliminate IAV-specific CD8 T cells leading to a T cell response similar to that observed in gld mice that did not receive pDC (Figure 8B). Importantly, peptide pulsing of pDC did not increase pDC apoptosis (Figure 11) suggesting that in vitro pDC that express IAV peptides are not eliminated by IAV-specific CD8 T cells and that the decrease in CD8 T cell apoptosis does not relate to a loss of effector cells (i.e. pDC). Taken together these data suggest that concomitant viral antigen peptide:MHC I presentation overrides the ability of pDC to induce FasL-mediated apoptosis in vitro and in vivo and suggest that pDC elimination of IAV-specific CD8 T cells is critically tied to a lack of IAV-antigen presentation. Therefore the lack of apoptosis induction by CD8α^+ and CD8α^- DC may relate to their presentation of IAV-antigens. Consistent with this idea, pDC from lethal dose IAV

infected mice were also able to mediate the apoptosis of in vitro activated DUC18 CD8 T cells (i.e. a non-IAV-specific CD8 T cell that display an activation phenotype similar to in vivo activated CL-4 T cells (Figure 12)) (Figure 13) similar to their elimination of in vitro activated CL-4 cells (Figure 13A). Furthermore while CD8α^+DC purified from IAV infected mice were unable to eliminate IAV-specific transgenic T cells (Figure 7B and 13A), they were capable of eliminating non-IAV-specific transgenic T cells (Figure 13B) suggesting that CD8α^+DC can mediate elimination of activated T cells in the absence of antigen presentation. Together these data support the idea that elimination of IAV-specific T cells during lethal IAV infection does not require engagement of T cell receptors and in fact TCR engagement may inhibit such apoptosis. Therefore CD8 T cell apoptosis may instead relate to the overall T cell activation state and Fas expression.

Discussion

DC elimination of T cells through FasL:Fas interactions has been previously described by multiple investigators (39, 168, 169). Suss and Shortman demonstrated that CD8α^+ splenic DC expressing FasL were able to eliminate CD4 T cells during a mixed leukocyte reaction. Additionally, multiple groups have used adoptive transfer of DC transfected with FasL to control T cell numbers in a variety of disease settings including autoimmunity, cancer and viral infection (168-170). Herein we have shown that LN resident and/or recruited pDC can mediate the elimination of IAV-specific CD8 T cells during lethal dose IAV infections. Together, our results, suggest that elimination of activated Fas$^+$ T cells by FasL$^+$pDC may represent an integral mechanism for dampening T cell numbers.

While DC-mediated reduction of the effector T cell response in autoimmune reactions or at the conclusion of an immune response would be beneficial, the loss of effector CD8 T cells here is clearly detrimental to survival during a high dose IAV

infection (38). Indeed our previous studies have shown that in the absence of functional FasL, sufficient numbers of CD8 T cells develop to control the high dose IAV inoculum (Figure 8) (38). Thus, the enhanced recruitment of pDC into the LN observed during lethal dose IAV infections (Figure 5), coupled with pDC-mediated elimination of the IAV-specific CD8 T cell response (Figures 7 and 8), allows the virus to escape adaptive immune control leading to death of the infected host. In this regard, recent studies have shown that individuals infected with highly pathogenic Avian (H5N1) IAV have dampened or reduced CD4 and CD8 T cell responses (171). Similarly, mice and monkeys infected with highly pathogenic H5N1 IAV develop T cell lymphopenia (172-174) with the loss of CD8 T cells in the lungs and lymph nodes associated with enhanced levels of apoptosis (172). The exact pathway(s) and cell type mediating the apoptosis responsible for T cell lymphopenia remain poorly understood at this time. However, given our results, it will be important to determine what role FasL expression by pDC plays in the lymphopenia associated with high pathogenic avian H5N1 IAV infections.

Surprisingly, LNDC elimination of IAV-specific CD8 T cells does not require cognate MHCI:antigen presentation. In fact the rescue of these T cells from $FasL^+$ DC induced apoptosis during recognition of viral peptide-MHC I complexes may be due in part to TCR mediated upregulation of NFκB, which has been shown to protect T cells, macrophages and B cells from Fas mediated apoptosis (134-137). Interestingly signals through the B cell receptor, which upregulate NFκB, result in a transient (less than 24h) protection from Fas-mediated apoptosis (135). In our studies, pDC driven apoptosis is induced in the LN at a time point concomitant with DC-mediated antigen presentation. Additionally, signaling through Fas on T cells may act as a co-stimulatory molecule and in the presence of TCR stimulation has been demonstrated to enhance proliferation and activation (132, 138, 175, 176). Together these data suggest the possibility that during a narrow window immediately following the activation of naive T cells within the LN cognate MHCI:antigen TCR interactions the presence of additional FasL:Fas interactions

results in enhanced activation and proliferation of the T cells rather than apoptosis. Conversely, FasL:Fas engagement alone in the LN or after egress from the LN would mediate death. Consistent with our results showing that co-culturing of pDC and CL-4 T cells with IAV-peptide epitopes reverses the pDC-mediated loss of the T cells (Figure 10A), a recent report has demonstrated that antigen-pulsed FasL-transfected DC enhance antigen-specific CD8 T cell responses rather than induce apoptosis. This finding suggests that the presentation of antigen by these transfected DC inhibits their ability to drive elimination of cognate T cells (177). In contrast to this report, and our own findings, other groups have suggested that DC FasL-mediated elimination of CD4 and CD8 T cells can occur in an antigen-dependent manner (168, 169, 178). The reason for these differences is not clear at this time; however, in contrast to our own studies, these latter experiments utilized conventional bone-marrow derived DC or DC cell lines rather than pDC obtained from the LN. In addition, these studies used effector DC transfected with FasL cDNA resulting in constitutively high levels of FasL.

Our results suggest that pDC-mediated induction of apoptosis in activated T cells during lethal dose IAV infections occurs independent of concurrent IAV-antigen presentation (Figure 10 and 13). In agreement with this idea recent reports have demonstrated that although LN resident pDC contain IAV-proteins following infection, they are unable to stimulate naïve or memory CD8 T cells and may instead regulate B cell responses (15). Unlike pDC, LN resident CD8α^+ DC acquire IAV-antigen (likely from migratory rDC) and cross-present this antigen during IAV infections (10). Interestingly while our results show that CD8α^+ DC are also able to regulate FasL expression in an IAV dose-dependent manner (see Figure 4), they do not eliminate IAV-specific CD8 T cells during lethal dose IAV infections (Figure 7). Together these results along with those demonstrating that pDC from lethal-dose IAV infected mice kill both activated non-IAV specific and IAV-specific CD8 T cells with the same efficiency (Figure 13A) suggests that pDC elimination of effector CD8 T cells during lethal dose

IAV infections is independent of concurrent T cell receptor engagement. Rather, pDC-mediated elimination of the T cells is associated with the T cells activation state and Fas expression. Thus Fas expressing activated or memory CD8 T cells of any specificity might be susceptible to pDC-mediated apoptosis during lethal dose IAV infections.

In addition to the differential FasL expression on pDC from lethal and sublethal dose IAV-infected mice, our results demonstrate an enhanced recruitment of pDC into the LN of lethal-dose infected mice (Figure 5). pDC recruitment into LN through high endothelial venules (HEV) is thought to be mediated in part in response to CXCL12 and CXCL9 expression (164, 165). Interestingly, CXCL9 is upregulated in response to IL-1 and IFNγ expression (179). Consistent with these findings, preliminary experiments from our laboratory have demonstrated increased secretion of IFNγ, IL-1α, and IL1β from in vitro cultured LN obtained from lethal compared with sublethal dose IAV infected mice (Legge and Braciale; unpublished results). Importantly, as pDC leave the HEV and enter the into LN, the increased recruitment of pDC during lethal IAV infections will also raise the local in vivo E:T ratio. Even without factoring in a localization of these recruited pDC into areas of the LN that accumulate newly activated Fas^+ T cells (84), our data suggest that in vivo there is ~1 pDC available for every activated CD8 T cell. In our ex vivo analysis (Figure 7), pDC cultured at a 1:1 ratio with T cells were able to reduce a static number of T cells by ~45% in the span of 18 hours. Therefore the ~80-95% reduction of the endogenous T cell response observed in vivo on days 5 and 6 p.i. (see (38) and Figure 3) may relate to either the fact that elimination of activated T cells would also reduce the subsequent burst size of the total response and/or an increased local LN in vivo E:T ratio.

All together the data presented herein describe a novel role for pDC during lethal dose IAV-infection- namely the elimination of activated CD8 T cells leading to enhanced mortality. Given the emerging threat of highly pathogenic pandemic IAV and the detrimental role $FasL^+$ pDC can play during a lethal IAV infection our findings suggest

that pDC and FasL may be strong candidates for therapeutic blockade during highly virulent IAV infections.

Figure 3. IAV-specific CD8 T cells are reduced in the lymph nodes of lethal dose IAV infected mice. Mice were infected with either $10LD_{50}$ or $0.1LD_{50}$ of IAV and on day 5 p.i. the number of HA_{529}, HA_{204} and NP_{147} tetramer^{+} $CD8^{+}CD3^{+}$ cells were enumerated. LN were pooled from 3-5 mice per group. Data are representative of 3 independent experiments.

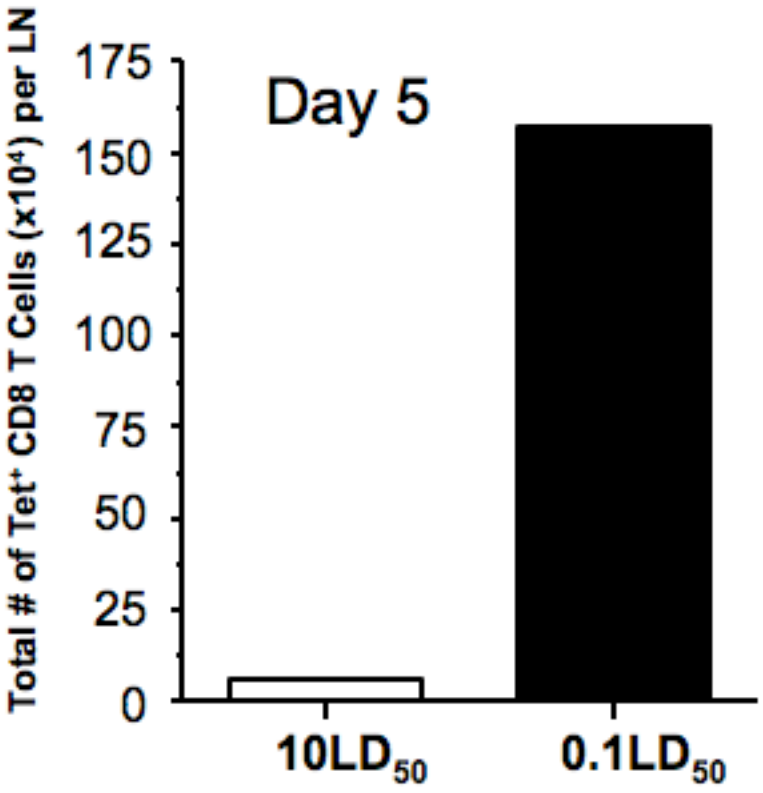

Day 5
Total # of Tet+ CD8 T Cells (x10⁴) per LN
175
150
125
100
75
50
25
0
10LD50
0.1LD50

Figure 4**.** pDC and CD8α^+ DC modulate FasL expression during IAV infection. Mice were infected with either a $10LD_{50}$ (black line) or $0.1LD_{50}$ (shaded histogram) of IAV and on day 3 p.i. cells from draining LN (pooled) from each group examined for FasL expression. Dotted line represents staining with an isotype control mAb. (A) FasL expression on $CD11c^{mod}CD45R^+CD8^+$ cells (i.e. pDC); $10LD_{50}$ M.F.I.=60.5 and $0.1LD_{50}$ M.F.I.=28.3. (B) FasL expression on $CD11c^+CD45R^-CD8^+$ cells (i.e. CD8α^+ DC); $10LD_{50}$ M.F.I.=139 and $0.1LD_{50}$ M.F.I.=118. (C) FasL expression on $CD11c^+CD45R^-CD8^-$ cells (i.e. CD8α^- DC); $10LD_{50}$ M.F.I.=618 and $0.1LD_{50}$ M.F.I.=496.2. The MFI of staining with isotype control mAb has been subtracted from the FasL MFI for the DC subsets to yield the MFI reported above. Data are representative of 5 independent experiments.

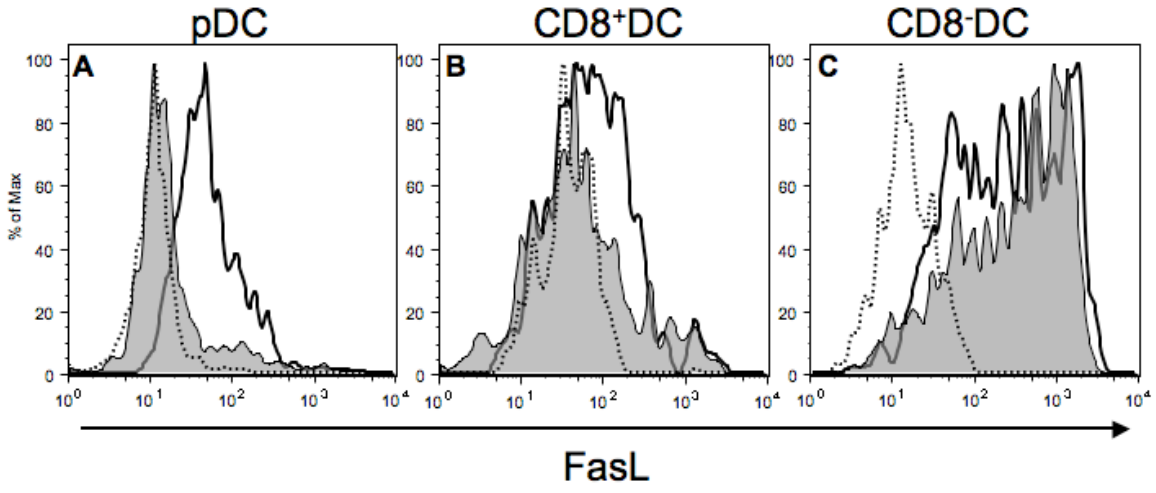

pDC
CD8+DC
CD8-DC
A
B
C
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
FasL

Figure 5**.** pDC preferentially accumulate in the lung draining LN of lethal dose IAV infected mice. Mice were infected with IAV as in Figure 1 and on day 1-4 p.i. lung draining LN from each group were pooled and the number of (A) $CD11c^{mod}CD45R^{+}$ cells (i.e. pDC) and (B) $CD11c^{+}CD45R^{-}CD8^{+}$ cells (i.e. $CD8\alpha^{+}$ DC) in the LN was determined. Data are representative of 3 independent experiments with 2-3 mice per group.

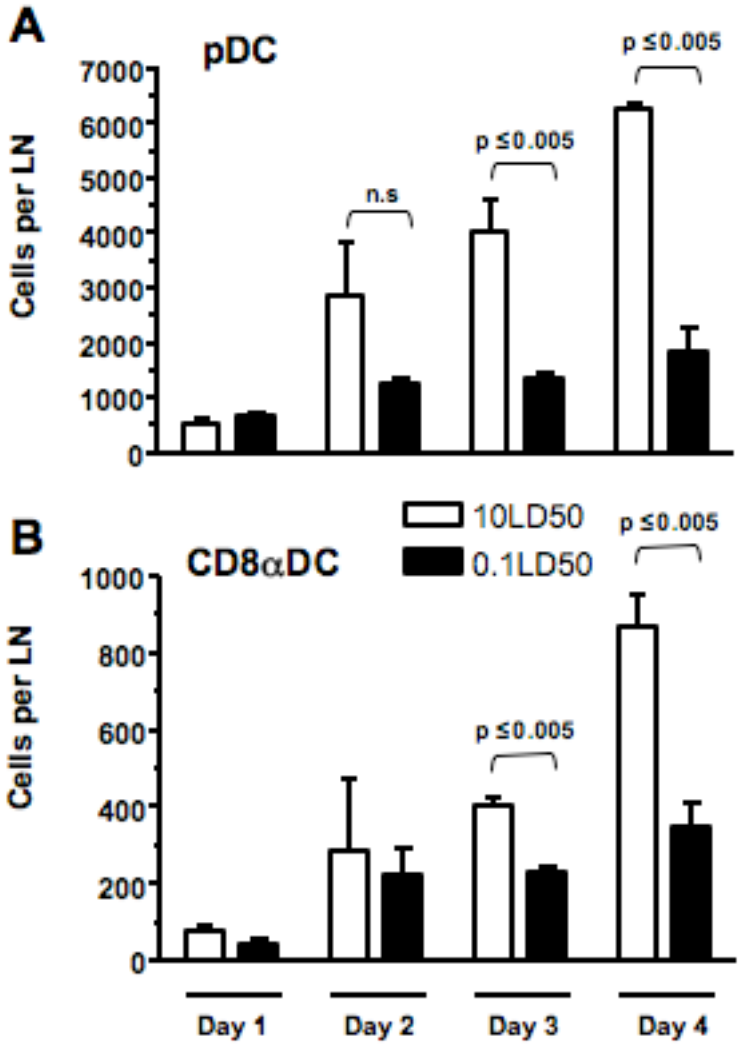

A
pDC
Cells per LN
7000
6000
5000
4000
3000
2000
1000
0
n.s
p ≤0.005
p ≤0.005
B
CD8αDC
10LD50
0.1LD50
Cells per LN
1000
800
600
400
200
0
p ≤0.005
p ≤0.005
Day 1
Day 2
Day 3
Day 4

Figure 6**.** Dividing $CD43^+$ CD8 T cells express Fas during IAV infections. CD90.2 CL-4 T cells were purified by magnetic activated cell sorting and labeled with 25μm CFSE and then transferred into CD90.1 wild-type mice. On day 3 p.i. LN were removed and Fas expression on $CD8^+CD90.2^+CFSE^-CD43^+$ and $CD8^+CD90.2^+CFSE^+CD43^-$ CL-4 T cells was measured.

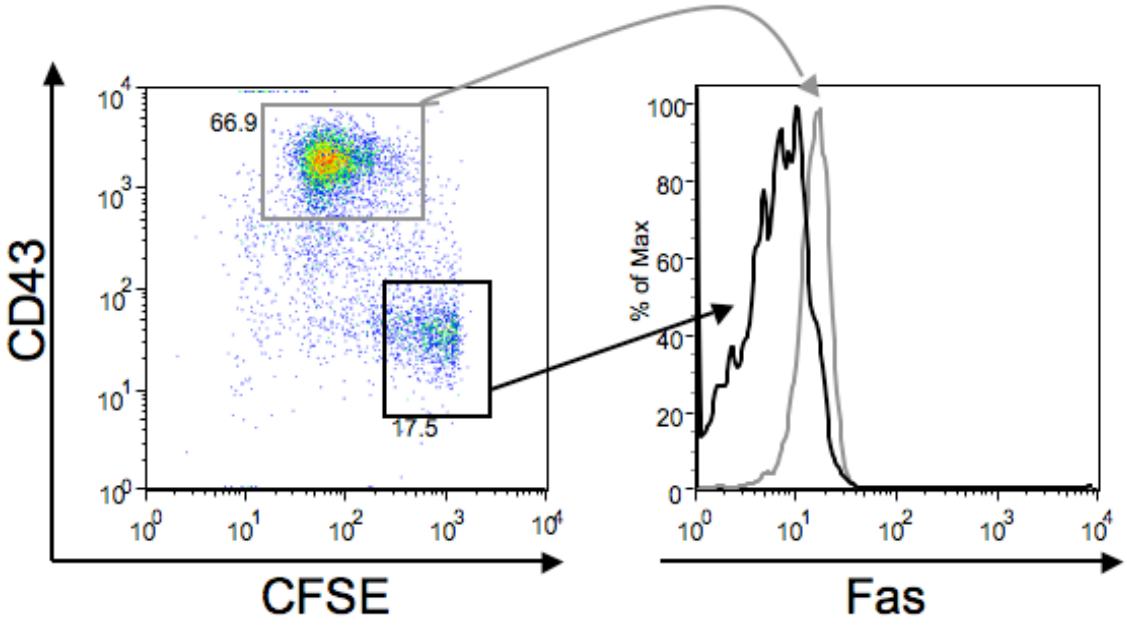

CD43
CFSE
66.9
17.5
% of Max
Fas

Figure 7. pDC from lethal dose IAV infected mice kill activated IAV-specific CD8 T cells. (A) Experimental setup for LNDC:CD8 T cell ex vivo apoptosis assay. (B) 10^4 of the indicated purified LNDC subsets were incubated with 10^4 activated CL-4 T cells and incubated at 37°C for 18 hours. After incubation the percentage of $CD90.2^+$Annexin V^-7-AAD^- live CL-4 T cells was determined and normalized to CD8 T cells incubated alone (~70% live). Data are representative of 3 independent experiments. (C) pDC and CL-4 T cells were purified and incubated +/- Fas-Fc and the percentage of live CD8 T cells was determined as described above. Data are representative of 2 independent experiments. (D) 10^4 pDC from $0.1LD_{50}$ IAV-infected mice were incubated with 10^4 activated CL-4 T cells and apoptosis measured as in B. Data are representative of 3 independent experiments. n.s. = not significant.

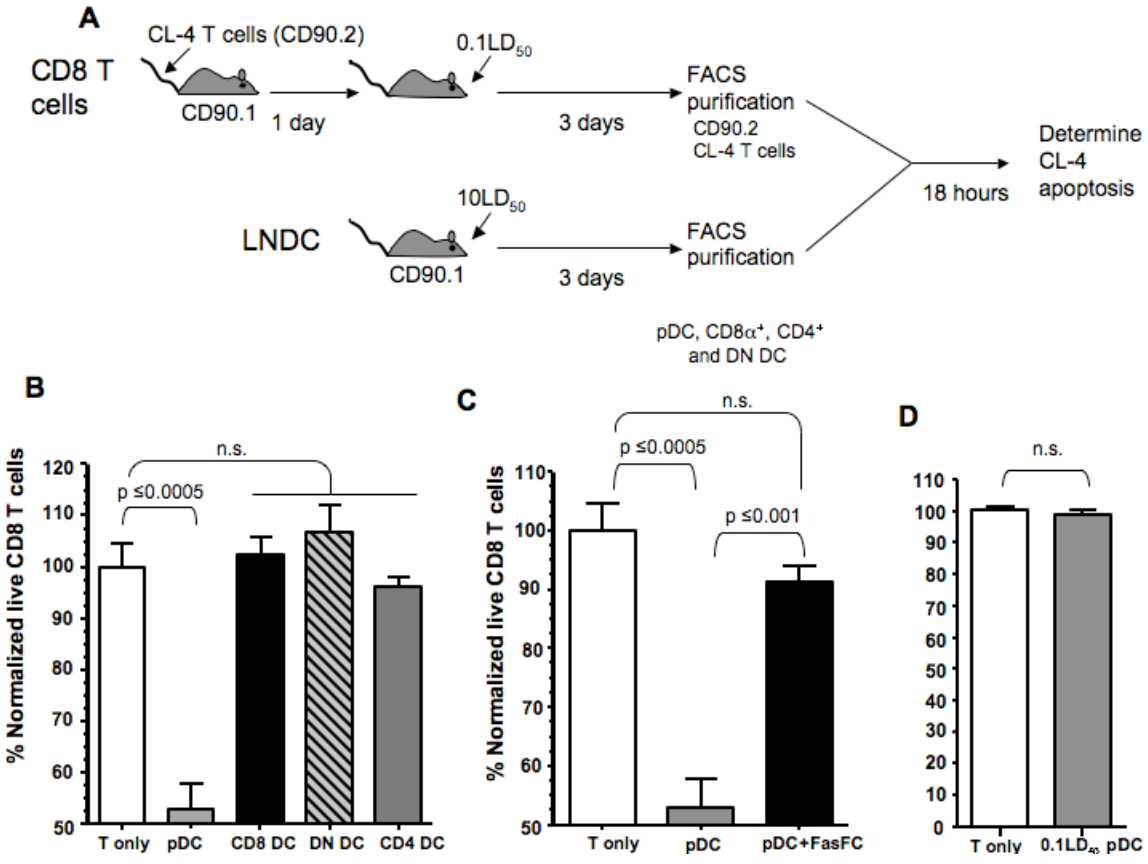

A
CD8 T cells
CL-4 T cells (CD90.2)
CD90.1
1 day
0.1LD50
3 days
FACS purification
CD90.2
CL-4 T cells
18 hours
Determine CL-4 apoptosis
LNDC
10LD50
CD90.1
3 days
FACS purification
B
% Normalized live CD8 T cells
n.s.
p ≤0.0005
T only
pDC
CD8 DC
DN DC
CD4 DC
pDC, CD8α+, CD4+ and DN DC
C
% Normalized live CD8 T cells
n.s.
p ≤0.0005
p ≤0.001
T only
pDC
pDC+FasFC
D
n.s.
T only
0.1LD50 pDC

Figure 8. Donor wild-type pDC reduce IAV-specific T cell numbers and cause enhanced mortality in mice deficient in functional FasL. (A) CD90.2 wild type (Wt) and gld mice received $2x10^6$ CD90.1 CL-4 T cells and 24 hours post transfer were infected with a $10LD_{50}$ dose of IAV. 24 hours p.i. one group of gld mice received $2x10^6$ Wt pDC i.v.. On day 4 p.i. lung draining LN were removed and $CD3^+CD8^+CD90.1^+$ Cl-4 T cells enumerated. Data are pooled from 2 independent experiments with 8-10 mice per group. (B) gld or wild type mice were infected with a $2.5LD_{50}$ of IAV. 24 hours p.i. pDC were purified from naïve wild-type or gld mice and then transferred ($2x10^6$) into IAV-infected gld mice. Mortality was then monitored for 10 days p.i.. p values are as follows: Wt vs GLD=0.0285, Wt vs (GLD+ Wt pDC) =0.7013, GLD vs. (GLD+Wt pDC) =0.0157, (GLD+ Wt pDC) vs (GLD+ GLD pDC) =0.0103 and (GLD+ GLD pDC) vs GLD=0.5722. Data are pooled from 3 independent experiments with n values equaling; Wt=15, GLD+Wt pDC =14, GLD+ GLD pDC=9, GLD=18.

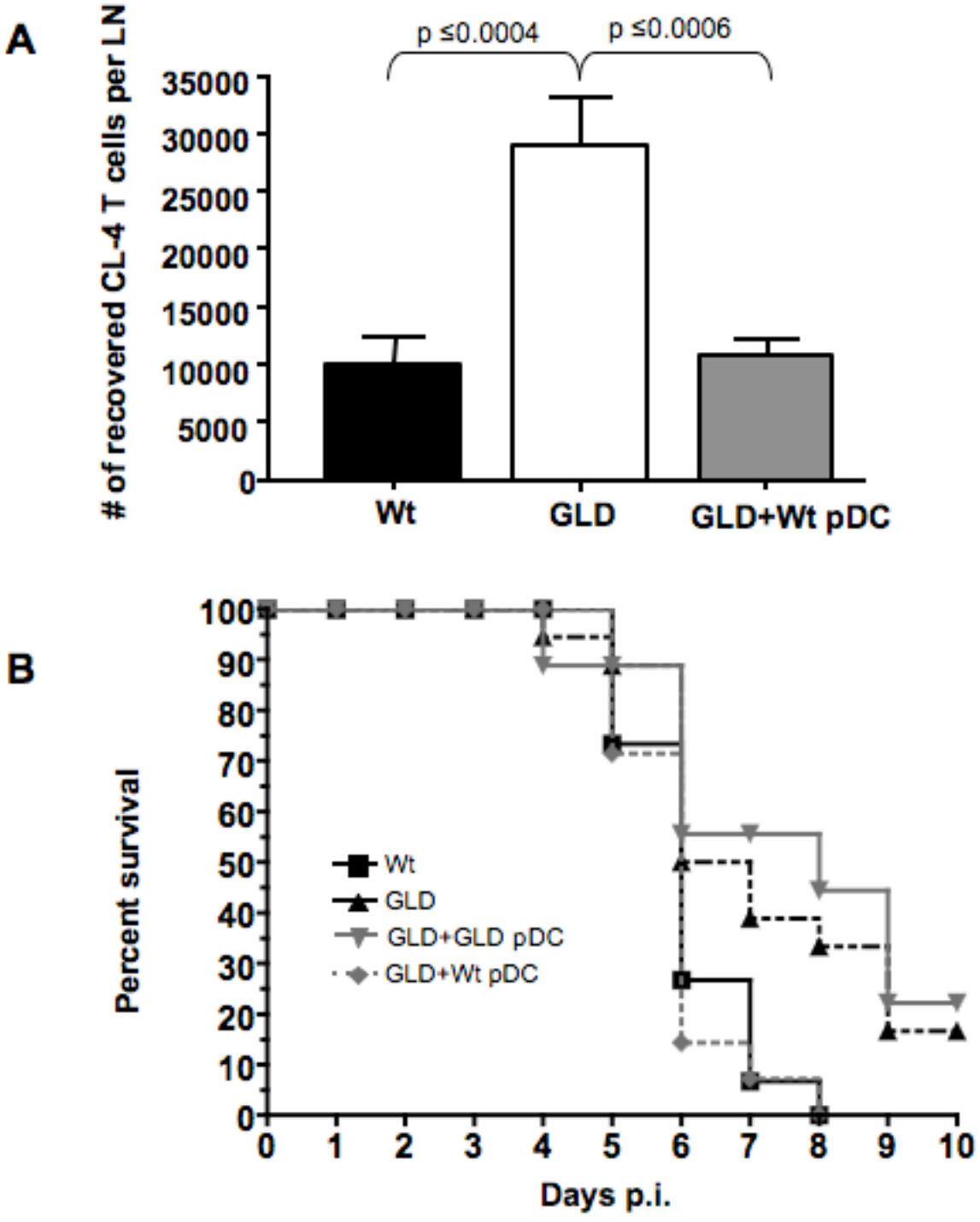

A
p ≤0.0004
p ≤0.0006
# of recovered CL-4 T cells per LN
35000
30000
25000
20000
15000
10000
5000
0
Wt
GLD
GLD+Wt pDC
B
Percent survival
100
90
80
70
60
50
40
30
20
10
0
Wt
GLD
GLD+GLD pDC
GLD+Wt pDC
0 1 2 3 4 5 6 7 8 9 10
Days p.i.

Figure 9. Depletion of pDC decreases mortality during lethal dose IAV infections. Mice were treated at 0 and 24 hours post lethal dose IAV infection with 125μg of anti-PDCA-1 pDC depleting antibody. (A) On day 3 post infections (i.e. the window directly before LNDC FasL mediated elimination of IAV-specific CD8 T cells) pDC within the spleen were enumerated representative of 3 mice. (B) Progression to mortality was followed during lethal dose IAV infections. Statistical differences were seen between aPDCA-1 treatment and IgG control; $p=0.029$.

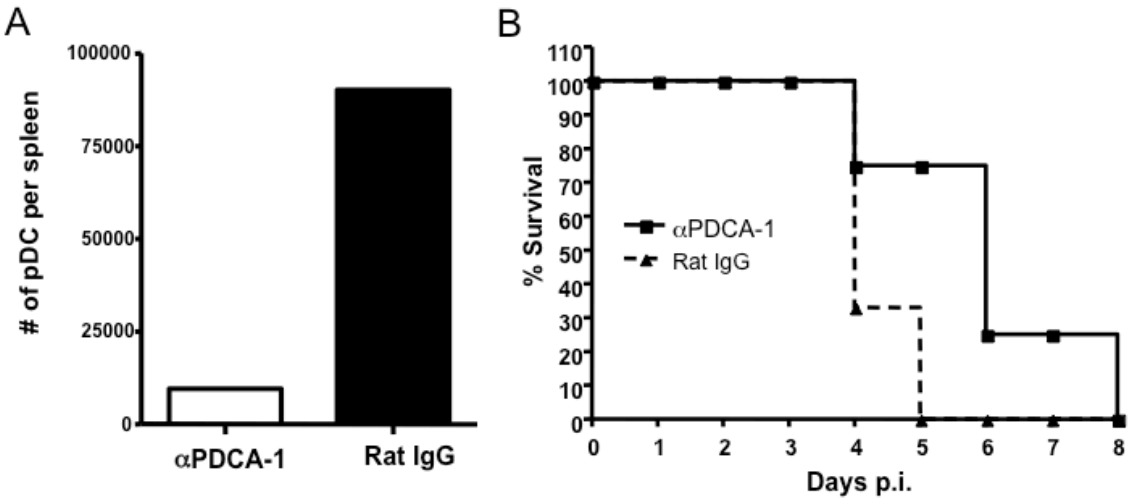

A
# of pDC per spleen
100000
75000
50000
25000
0
αPDCA-1
Rat IgG
B
% Survival
110
100
90
80
70
60
50
40
30
20
10
0
αPDCA-1
Rat IgG
0
1
2
3
4
5
6
7
8
Days p.i.

Figure 10. pDC induction of apoptosis in IAV-specific CD8 T cells is abrogated by cognate viral peptide:MHC I presentation. (A) CL-4 T cells and pDC were purified as described in Figure 8, co-cultured +/- 1μM HA_{529} peptide and then the percentage of live CD8 T cells determined as in Figure 8. (B) pDC were purified as in Figure 7 and pulsed with 1μM HA_{529} peptide and transferred into IAV-infected mice and CL-4 T cell numbers measured as in Figure 8. Data are representative of 2-3 independent experiments. n.s. = not significant.

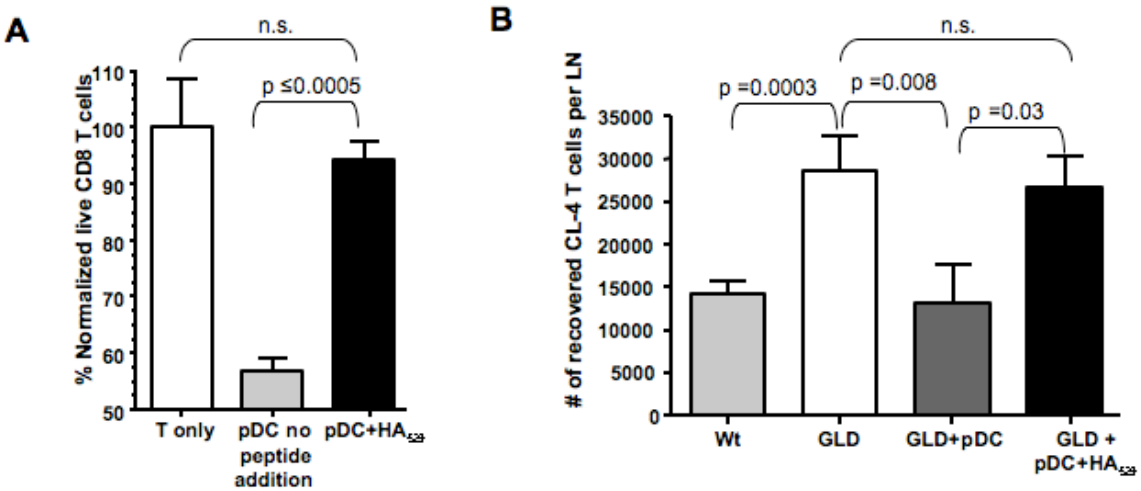

A
n.s.
p ≤0.0005
% Normalized live CD8 T cells
110
100
90
80
70
60
50
T only
pDC no peptide addition
pDC+HA529
B
n.s.
p =0.0003
p =0.008
p =0.03
# of recovered CL-4 T cells per LN
35000
30000
25000
20000
15000
10000
5000
0
Wt
GLD
GLD+pDC
GLD + pDC+HA529

Figure 11. pDC are not eliminated by CL-4 T cells at increased rates after peptide pulsing. CL-4 T cells and pDC were purified as described in Figure 7, co-cultured +/- 1μM HA_{529} peptide and then the percentage of live pDC (i.e. $B220^{+}CD90.2^{-}$) cells was determined as Annexin V and 7-AAD negative. Data are representative of 2 independent experiments. n.s. = not significant.

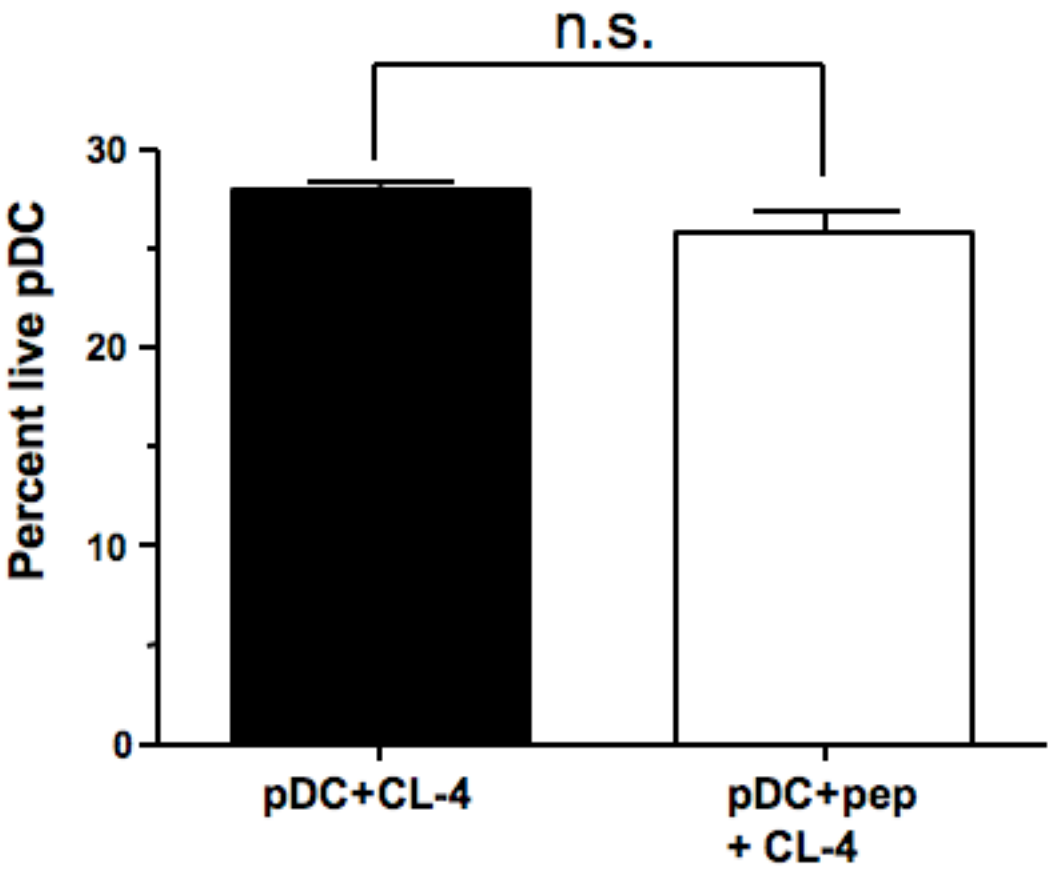

n.s.
Percent live pDC
30
20
10
0
pDC+CL-4
pDC+pep
+ CL-4

Figure 12. In vitro activated IAV-specific and non IAV-specific CD8 T cells express similar levels of CD43 and Fas. CD8 T cells from the spleens of naïve CL-4 and DUC18 transgenic mice were purified and cultured for 3 days in the presence of αCD3/CD28 antibodies in order to activate the T cells. Shown is the level of CD43 expression on $CD8^+$ gated cells (upper panel) or the expression of Fas by $CD8^+CD43^+$ T cells (lower panel). Data are representative of 3 independent experiments.

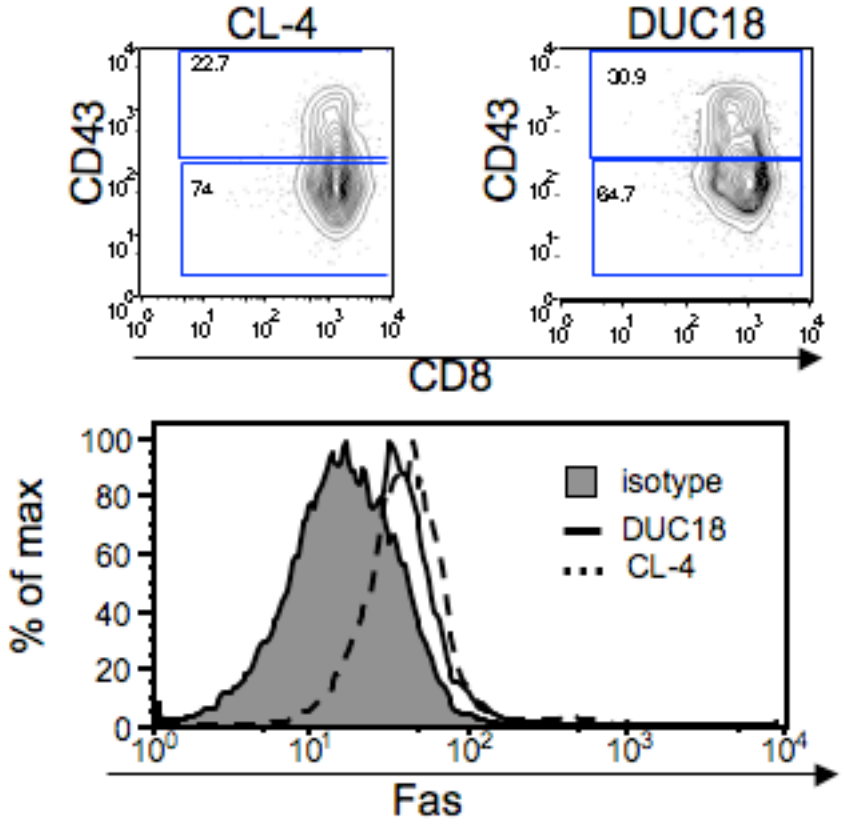
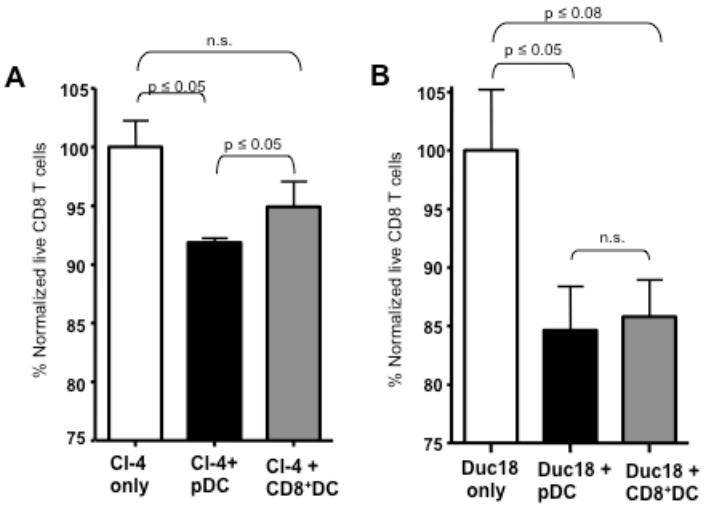

CL-4
DUC18
CD43
CD43
22.7
74
30.9
64.7
CD8
% of max
isotype
DUC18
CL-4
Fas

Figure 13. pDC from lethal dose IAV infected mice eliminate IAV-specific and non IAV-specific activated CD8 T cells. CD8 T cells from the spleens of naïve CL-4 (A) and DUC18 (B) transgenic mice were purified and cultured for 3 days in the presence of αCD3/CD28 antibodies in order to activate the T cells. Activated transgenic T cells (10^4) were incubated with pDC or $CD8\alpha^+$DC (10^4) from lethal dose IAV-infected mice at 37°C for 18 hours as described in Figure 3. After incubation the percentage of $CD90.2^+$Annexin V^-7-AAD^- live transgenic T cells was determined and normalized to CD8 T cells incubated alone. n.s.= $p > 0.1$. Data are representative of 2 independent experiments.


A
n.s.
p ≤ 0.05
p ≤ 0.05
% Normalized live CD8 T cells
105
100
95
90
85
80
75
CI-4 only
CI-4+ pDC
CI-4 + CD8⁺DC
B
p ≤ 0.08
p ≤ 0.05
n.s.
% Normalized live CD8 T cells
105
100
95
90
85
80
75
Duc18 only
Duc18 + pDC
Duc18 + CD8⁺DC

CHAPTER III IL-12P40 HOMODIMER CONTROLS LNDC FASL EXPRESSION DURING LETHAL DOSE IAV INFECTIONS

Introduction

The regulation of FasL on LNDC during lethal dose IAV infections is correlated with the expression of the cytokine IL-12p40 within the lung draining LN (38). However, IL-12p40 is a multimeric cytokine subunit capable of forming multiple distinct cytokines and the specific IL-12p40 dependent cytokine responsible for LNDC FasL expression has not been identified. IL-12p40 preferentially pairs with the IL-12p35 subunit to form the cytokine IL-12p70 but also pairs with IL-23p19 to form IL-23 (142). In addition to pairing with IL-12p35 and IL-23p19 to generate heterodimeric cytokines, IL-12p40 is also found in monomeric or homodimeric forms (142). Importantly, the IL-12p40 monomer is often produced in excess over the IL-12p35 and IL-23p19 subunits allowing the potential for increased levels of IL-12p40 monomer and homodimer ($p40_2$) (145). Surprisingly, in the above study FasL expression did not correlate with either the level of IL-12 and IL-23 within the LN during lethal dose IAV infections (38). Instead, LNDC FasL expression positively correlated with the levels of total $p40_2$ and/or IL-12p40 monomer suggesting that one or both of these cytokines are involved in the regulation of FasL expression during IAV infections (38). However while LNDC FasL expression correlates with the level of $p40_2$ and/or IL-12p40 monomer within the LN it remains unclear if one or both of these cytokines regulates LNDC FasL expression.

The cytokine IL-12p70 signals through the IL-12 receptor complex which is composed of IL-12Rβ1 and IL-12Rβ2. IL-12p40 binds to IL-12Rβ1 and stabilizes the interaction between IL-12p35 and IL-12Rβ2 allowing for the initiation of signaling through IL-12Rβ2 (180). However IL-12Rβ1 alone is not capable of mediating IL-12p70 signals (180, 181). Initially $p40_2$ was shown to bind with high affinity to the IL-12Rβ1

subunit of the IL-12 receptor and was thought to be unable to drive signaling and instead act as an antagonist by blocking access to the IL-12R complex and therefore block IL-12p70/35 signals through IL-12Rβ2 (145, 149-151). However, the role $p40_2$ plays in the immune system is complex as more recent studies have shown that $p40_2$ also has agonistic functions. These studies demonstrated that $p40_2$ could enhance macrophage chemotaxis through IL-12Rβ1(154), DC migration from the lung to the draining LN, as well as enhance T cell priming and IFNγ production during mycobacterium tuberculosis infections (153). These data suggest that $p40_2$, in addition to its well-described antagonistic role, is capable of inducing unique signaling. Recently the development of $p40_2$ specific reagents has allowed for direct dissection of a pathway for $p40_2$ signaling during an immune response. Results from Pahan and colleagues have shown that $p40_2$ signals directly through IL-12Rβ1 to mediate the production of nitrous oxide and IL-16 from microglia as well as lymphotoxin α and nitrous oxide from splenocytes (156-159, 182). These results therefore suggest that $p40_2$ can affect the immune response either directly by signaling through IL-12Rβ1 or indirectly by blocking IL-12p70 and therein antagonize IL-12p35 signaling through IL-12Rβ2. Currently it is unknown what role IL-12Rβ1 plays in the regulation of LNDC FasL during IAV infections.

Previously, we have demonstrated a differential production of IL-12p40, in either monomeric or homodimeric form, during lethal versus sublethal dose IAV infections (38). During lethal dose IAV infections both respiratory DC (rDC) and lymph node resident DC (LNDC) express increased levels of intracellular IL-12p40 in comparison to DC from the LN of sublethal dose IAV infected mice. This increased IL-12p40 in turn correlates with FasL expression on LNDC since neutralization of IL-12p40 results in decreased LNDC FasL expression (38). The consequence of increased LNDC FasL expression, specifically pDC FasL expression, during lethal dose IAV infections is the elimination of IAV-specific CD8 T cells through a FasL:Fas dependent mechanism resulting in a significant decrease in the magnitude of the CD8 T cell response and

ultimately death of the host (Chapter II). Together these results suggest that either IL-12p40 monomer and/or $p40_2$ is capable of regulating the magnitude of the CD8 T cell response to IAV infections through regulation of pDC FasL expression. However the isoform of IL-12p40 that regulates LNDC FasL expression and the cell(s) that produce IL-12p40 within the LN are currently undefined.

In this chapter we demonstrate that $p40_2$, and not IL-12p40 monomer, controls FasL expression on LNDC, specifically pDC, during lethal dose IAV infections. Additionally we demonstrate that IL-12Rβ1 is important for LNDC FasL expression during lethal dose IAV infections. Finally, our results show that both LNDC and rDC produce $p40_2$ during lethal dose IAV infections. Together the data presented herein suggest that $p40_2$ produced by rDC and/or LNDC is responsible for controlling the level of FasL expression on pDC and therefore the magnitude of the IAV-specific CD8 T cell response and the outcome of IAV infections.

Material and methods

Mice

Wild-type BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Il-12p40$^{-/-}$ (C.129S1-Il12b^{tm1Jm}/J) and IL-12Rβ1$^{-/-}$ (C.129S1-Il12rb1^{tm1Jm}/J) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All experiments were performed in accordance with federal and institutional guidelines approved by the University of Iowa Animal Care and Use Committee.

Virus infection

6-10 week old BALB/c, IL-12p40$^{-/-}$ or IL-12Rβ1$^{-/-}$ mice were anesthetized with isofluorane and infected i.n. with a $10LD_{50}$ or a $0.1LD_{50}$ dose of mouse-adapted

A/JAPAN/305/57 (H2N2) in 50µl of Iscoves media. Viruses were grown and stored as previously described (38).

Intranasal CFSE administration

CFSE (Molecular Probes) was dissolved at 25mM in DMSO and further diluted to 8mM in Iscoves DMEM. Mice were anesthetized with isofluorane and 50ul of 8mM CFSE was then given intranasally.

Flow cytometry

LN cells were stained with the following monoclonal antibodies: rat anti-mouse CD8α (53-6.7), hamster anti-mouse CD11c (HL3), rat anti-mouse IL-12Rb1, rat anti-mouse CD3ε (145-2C11), rat anti-mouse CD103 (M290), rat anti-mouse MHC II (M5/114.15.2) and CD11b (M2/70) purchased from Becton Dickinson; rat anti-mouse CD45R (RA3-6B2), and rat anti-mouse CD19 (6D5) purchased from Caltag (Invtrogen). Armenian hamster anti-mouse FasL CD95L (MFL3) was purchased from eBioscience. For FasL staining, cells were blocked with 1:100 rat serum, 1:100 hamster serum and 1:400 free streptavidin (molecular probes) on ice for 25 mins. Cells were then washed twice and stained with 2x (i.e. 1:50) biotin-conjugated anti-FasL (MFL3) followed by streptavidin-PE purchased from Becton Dickinson. For surface staining, isolated cells (10^6) cells were stained with antibody, and then fixed using BD FACS Lysing Solution (BD Biosciences). For intracellular $p40_2$ detection, cells were fixed using BD FACS Lysing Solution and then permeabilized using buffer containing 0.5% saponin (Acros Organics, NJ) and labeled with 1:50 of Armenian hamster anti-mouse $p40_2$ (a3-1D) kindly provided by Kalipada Pahan (Rush University) for 1 hour at 4° C. All flow cytometry data were acquired on a BD FACS Calibur or BD FACS Canto II (BD Immunocytometry Systems) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Lymph node organ culture

Lung draining LN from day 3 post $10LD_{50}$ IAV infected wild-type or IL-12p40$^{-/-}$ mice were removed and placed in DMEM containing 2x penicillin/streptomycin. 5 LN were then placed into a 96 well plate with 200ul of medium alone, medium containing 200ng recombinant $p40_2$, 200ng of recombinant IL-12p40 monomer (R and D systems), or 25μg of anti-$p40_2$ ((a3-1D) kindly provided by Kalipada Pahan (Rush University)) or Armenian hamster IgG isotype control. LN were then placed into a modular incubator chamber (Billups-Rothenberg) 95% O_2 and 5% CO_2 at 37° C for 24 hours. Following incubation LN were removed and processed into a single cell suspension for flow cytometry staining.

Statistical analysis

Statistical differences between IL-12p40$^{-/-}$ and wild-type IL-12p40$^{+/+}$ mice for survival was determined by Kaplan-Meier mean survival analysis.

Results

IL-12p40 homodimer controls FasL expression on LNDC during IAV infections

Previously our laboratory has demonstrated that the levels of IL-12p40 monomer/$p40_2$ within the LN remain high during lethal dose IAV infections but are significantly decreased during sublethal dose IAV infections (38). These changes in IL-12p40 correlate with differential LNDC FasL expression with increasing levels of IL-12p40 leading to increased LNDC FasL expression and decreases in total IL-12p40 leading to decreased LNDC FasL expression (38). Surprisingly, the expression levels of neither IL-12, nor IL-23, correlate with LNDC FasL expression as there were similar levels of IL-12 during both lethal and sublethal dose IAV infections and an increase in

IL-23 during sublethal dose infections. These results further suggests that IL-12p40 monomer/$p40_2$ are responsible for the regulation of LNDC FasL expression during IAV infections (38). Importantly, blockade of total IL-12p40 results in decreased LNDC FasL expression highlighting the importance of total IL-12p40 in the regulation of FasL expression on LNDC during IAV infections (38). Together these data suggest that $p40_2$ and/or momomer may be involved in the IL-12p40 dependent FasL regulation of LNDC (38).

Given that neutralization of total IL-12p40 decreases LNDC FasL expression, LNDC from mice deficient in IL-12p40 would be predicted to exhibit reduced and/or no FasL expression. As expected during lethal dose IAV-infection, LNDC (including pDC) from IL-12p40$^{-/-}$ mice express reduced FasL compared to LNDC from lethal dose IAV-infected IL-12p40$^{+/+}$ mice (Figure 14A). Since IL12p40$^{-/-}$ mice have reduced LNDC FasL we therefore utilized these mice to determine which isoform of IL-12p40 (i.e. IL-12p40 monomer or $p40_2$) regulates FasL expression on LNDC. LN from IL-12p40 deficient mice were cultured intact with either recombinant $p40_2$ or IL-12p40 monomer. As shown in Figure 14B after culture, $p40_2$ induced greater levels of FasL expression on total LNDC and on pDC i.e. the cell type responsible for FasL mediated elimination of IAV-specific CD8 T cells (Chapter II) (Figure 14B). In contrast, IL-12p40 monomer did not upregulate FasL expression on LNDC or pDC (Figure 14B). Together these results suggest that $p40_2$, and not IL-12p40 monomer, is responsible for regulating the levels of LNDC FasL expression during lethal dose IAV infections. To confirm the requirement of $p40_2$ in LNDC FasL expression during lethal dose IAV infection, LN from lethal dose IAV-infected wild-type mice (i.e. mice that express FasL on LNDC) were removed and cultured with a $p40_2$-specific blocking antibody (a3-1d) that does not bind IL-12p40 monomer, IL-12p70 or IL-23 (183). Blocking $p40_2$ resulted in decreased FasL expression on both LNDC and LN pDC (Figure 14C). These data demonstrate that removal of $p40_2$ results in downregulation of FasL expression suggesting that LNDC may need to see

continuous $p40_2$ to maintain FasL expression during lethal dose IAV infections. Surprisingly, culture of wild-type LN from lethal dose IAV infected mice with IL-12p40 monomer also decreased LNDC FasL expression (Figure 14D). These data suggest that instead of signaling, IL-12p40 monomer may instead block $p40_2$ signaling and further that cross linking of IL-12Rβ1 may be required. In this regard the overall ratio of $p40_2$ to IL-12p40 monomer may ultimately dictate LNDC FasL expression.

IL-12Rβ1 is required for LNDC FasL expression

Recent studies from Pahan and colleagues have demonstrated that IL-12Rβ1 is critical for $p40_2$ mediated signaling (156, 159). Importantly all LN resident DC subsets including pDC express IL-12Rβ1 during lethal dose IAV infections suggesting that they may be able to mediate IL-12Rβ1 dependent signals (Figure 15). Given the above results demonstrating that $p40_2$ is critical for LNDC FasL expression we therefore hypothesized that LNDC from mice deficient in IL-12Rβ1 would have reduced FasL expression similar to IL-12p40$^{-/-}$ mice (Figure 14A). Consistent with this idea, LNDC and pDC from lethal dose IAV infected IL-12Rβ1$^{-/-}$ mice have decreased FasL expression (Figure 16). Given that the levels of IL-12 and IL-23 which both signal through IL-12Rβ1 do not correlate with LNDC FasL expression, these data suggest that $p40_2$ signaling through IL-12Rβ1 may be important for LNDC FasL expression during lethal dose IAV infections.

rDC and LNDC produce IL-12p40 homodimer during lethal dose IAV infections

Taken together the above results suggest that $p40_2$ may signal through IL-12Rβ1 to induce FasL expression on LNDC and that the levels of $p40_2$ present within the LN regulate FasL expression on LNDC during IAV infections. Therefore it was important to determine which cell(s) produce this cytokine during lethal dose IAV infections. During IAV infections rDC migrate transiently from the lungs to the draining LN where they present IAV-antigens to naïve CD8 T cells as well as transfer IAV-antigens to LNDC (7,

10, 38). Furthermore our previous studies have demonstrated that these rDC and LNDC both express high levels of total IL-12p40 during lethal dose IAV infections (38). Therefore we hypothesized that DC within the LN, would be the cellular source of $p40_2$ during lethal dose IAV infections. To test this hypothesis mice were infected with either a lethal or sublethal dose of IAV and the level of intracellular $p40_2$ expression by rDC and LNDC within the LN was determined. As expected, rDC (as identified by CD103 expression (Figure 17A) or by i.n. CFSE labeling (Figure 18A)) in LN from lethal dose IAV infected mice expressed higher levels of intracellular $p40_2$ than rDC from sublethal IAV-infected mice (Figures 17A and Figure 18A). Furthermore, non-migratory LN resident DC populations (i.e. $CD103^-$) also produced intracellular $p40_2$ during lethal but not sublethal dose IAV infections (Figures 17B and 18B). Such production was not limited to individual DC subsets as all LNDC subsets; as $CD8\alpha^+$DC, $CD8\alpha^-$DC and pDC produced intracellular $p40_2$ (Figure 19A-C). Non-DC, i.e. $CD11c^-$ LN cells, did not produce $p40_2$ during either lethal or sublethal dose IAV infections (Figure 19D) suggesting that DC within the LN are solely responsible for producing $p40_2$ during IAV infections. However, whether or not the production by individual DC subsets themselves (autocrine signaling) or $p40_2$ from neighboring DC subset (paracrine signaling) is required to control FasL expression remains unknown.

IL-12p40$^{-/-}$ mice exhibit delayed mortality during lethal dose IAV infections

Previously we have demonstrated that mice deficient in functional FasL (i.e. gld mice) have decreased susceptibility to lethal dose IAV infections (Figure 8) (38). Given that IL-12p40 deficient mice have reduced LNDC FasL expression (Figure 14A) we therefore hypothesized that the IL-12p40 deficient animals would also display decreased mortality when challenged with lethal dose IAV infections. To test this hypothesis we infected IL-12p40$^{-/-}$ and IL-12p40$^{+/+}$ wild-type mice with a lethal dose of IAV and

assessed the progression to mortality during infection. Consistent with our hypothesis we observed a significant delay in mortality in IL-12p40$^{-/-}$ mice compared to wild-type mice (Figure 20). These data suggest that the reduction in FasL expression on LNDC, specifically pDC, in IL-12p40$^{-/-}$ LNDC may be sufficient to allow activated IAV-specific CD8 T cells to escape FasL:Fas mediated apoptosis, migrate to the lungs and aid in clearance of the infection.

Discussion

The data presented herein demonstrate a novel role for $p40_2$ in the regulation of LNDC FasL expression. The cytokine $p40_2$, but not IL-12p40 monomer, regulates FasL expression on LNDC during lethal dose IAV infections. Both migratory rDC and LN resident DC express increased levels of intracellular $p40_2$ during lethal versus sublethal dose IAV infections correlating with LNDC FasL expression (Chapter II) (38). Finally, the data presented above demonstrate that the loss of LNDC FasL expression in IL-12p40 deficient mice translates into increased survival to lethal dose IAV inoculums suggesting that CD8 T cells are able to escape pDC:FasL mediated apoptosis allowing for enhanced viral clearance and survival.

Consistent with results from Pahan and colleagues demonstrating that $p40_2$ signals through IL-12Rβ1 and not IL-12Rβ2 (156, 157), we demonstrate that mice deficient in IL-12Rβ1 display a similar LNDC FasL phenotype as mice deficient in IL-12p40 suggesting that IL-12Rβ1 is required for signaling. Despite the requirement for IL-12 and IL23 signaling it is unlikely that the loss of these cytokine signals affects FasL expression given that IL-12 and IL-23 protein levels do not correlate with FasL expression. However because IL-12Rβ1 is required to bind IL-12p40 our experiments do not exclude the possibility that IL-12Rβ1 is required for high affinity binding and that IL-12p40 can mediate low affinity signaling through IL-12Rβ2. The pathways employed by IL-12Rβ1

to mediate downstream signaling as well as the requirement for another receptor remain unknown. Given that the IL-12Rβ1 does not contain any phosphorylatable tyrosines in its cytoplasmic tail to act as docking sites for downstream signaling components (153), it is likely that another receptor may be required to mediate signaling. In the future it will be important to determine the signaling properties required to mediate $p40_2$ signaling and further if IL-12Rβ1 mediates $p40_2$ signaling alone or in tandem with another receptor.

The data presented in Figure 14 show that incubation with IL-12p40 monomer results in decreased LNDC FasL expression suggesting that IL-12p40 monomer may bind to IL-12Rβ1 and block $p40_2$ signaling. Therefore the ratio of $p40_2$ versus p40 monomer in the environment may dictate the levels of FasL expression. In the future it will be important to determine the relative levels of both $p40_2$ and IL-12p40 monomer in the LN during IAV infections. Importantly mice treated with LPS intra-peritoneally have a two fold increase in $p40_2$ over IL-12p40 monomer in the serum suggesting the possibility that lethal dose IAV infections may induced increased production of $p40_2$ over IL-12p40 monomer (145). Additionally to mediate downstream signaling $p40_2$ may cross link the IL-12Rβ1. However IL-12 p40 monomer, which can only bind a single IL-12Rβ1 chain, may be incapable of cross-linking and therefore unable to signal. Another potential explanation for the decreased FasL expression after culture with IL-12p40 monomer is that IL-12p40 monomer sends an inhibitory signal to the cell thereby shutting down FasL production. Consistent with this possibility results from Jana et al demonstrate that IL-12p40 monomer can mediate signals in macrophages leading to TNFα expression similar to IL-12p70 and $p40_2$ (182). These data suggest the possibility that IL-12p40 monomer may also be able to stimulate its own $p40_2$ independent signaling properties. However, the pathway(s)/receptors used by these IL-12p40 cytokines to induce TNFα expression was not determined.

The production of IL-12p40 during infections or stimulation with pathogen by products is generally restricted to DC and macrophages (142). However, despite the

presence of macrophages within the LN during IAV infections (97) only DC produced $p40_2$. Consistent with our results, other studies have shown that macrophages directly infected with IAV do not produce IL-12p40 (184). Together these data suggest that DC may be solely responsible for the production of IL-12p40, and therefore $p40_2$, during IAV infections.

As shown in Figure 20, IL-12p40 deficient mice exhibit a delayed mortality to lethal dose IAV infections compared to IL-12p40 sufficient wild-type mice. This is likely due to loss of $p40_2$. However because IL-12p40$^{-/-}$ mice are also deficient in other cytokines that have been demonstrated to be important in immunity to viral infections, including IL-12 and IL-23 (185), the possibility exists that the differences seen in mortality may instead be due to the loss of one of the additional IL-12p40 cytokines. Importantly mice deficient in IL-12p35 (which are only deficient in IL-12p70) display identical mortality kinetics as wild-type mice during lethal dose IAV infections (186) suggesting that IL-12p70 does not contribute to resistance to lethal dose IAV infection. To date IAV infections of mice deficient in IL-23p19 have yet to be evaluated. Together, these data suggest that the difference in mortality observed in IL-12p40 deficient mice infected with IAV may be due to loss of $p40_2$ and not the absence of IL-12p70.

All together the data presented herein describe a novel role for $p40_2$ during lethal dose IAV-infection through the control of FasL expression on LNDC. Given that FasL$^+$ pDC eliminate IAV-specific CD8 T cells leading to enhanced progression to mortality these data suggest that $p40_2$ produced by DC within the LN may ultimately be capable of regulating the IAV-specific CD8 T cell response. Given the emerging threat of highly pathogenic pandemic IAV infections, which exhibit increased T cell apoptosis (172), and the detrimental role of $p40_2$ in regulation of FasL during a lethal IAV infection; our findings suggest that $p40_2$ may be a strong candidate for therapeutic blockade during highly virulent IAV infections to prevent FasL driven apoptosis of IAV-specific CD8 T cells.

Figure 14. $p40_2$ controls FasL expression on LNDC during lethal dose IAV infections. (A) Wild-type IL-12p40$^{+/+}$ and IL-12p40$^{-/-}$ mice were infected with a $10LD_{50}$ of IAV and the level of FasL expression on total CD11c^{+} LNDC and CD11c^{mod}B220^{+} pDC was determined. (B) Intact LN from $10LD_{50}$ infected IL-12p40$^{-/-}$ mice were incubated with either recombinant $p40_2$ or IL-12p40 monomer ($p40_1$) and the levels of FasL expression on total CD11c^{+} LNDC and CD11c^{mod}B220^{+} pDC was determined. (C) Intact LN from $10LD_{50}$ IAV infected Wt IL-12p40$^{+/+}$ mice were cultured with anti-$p40_2$ (a3-1D) or isotype control, and the levels of FasL expression on total CD11c^{+} LNDC and CD11c^{mod}B220^{+} pDC was determined. (D) Wt IL-12p40$^{+/+}$ mice were infected with a $10LD_{50}$ dose of IAV and intact LN were cultured with either $p40_2$, IL-12p40 monomer ($p40_1$) or media alone. After culture the level of FasL expression on CD11c^{+} LNDC and CD11c^{mod}B220^{+} pDC was determined. LN were pooled from 3-5 mice per group. Data are representative of 2-4 independent experiments.

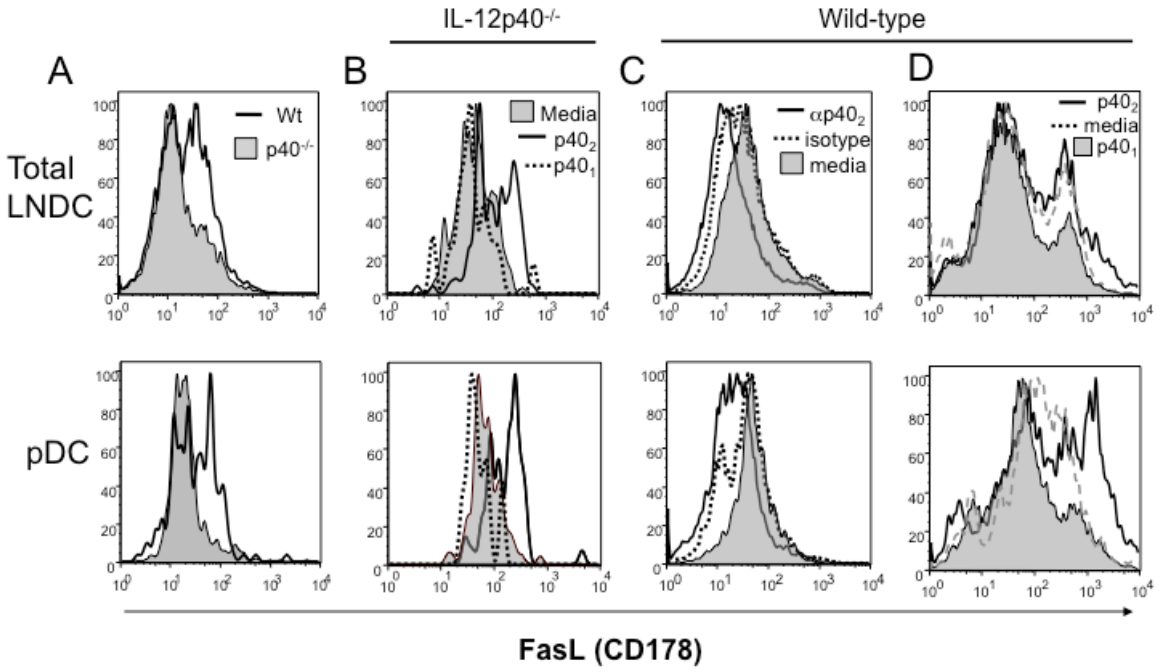

IL-12p40-/-
Wild-type
A
B
C
D
Total LNDC
pDC
Wt
p40-/-
Media
p402
p401
αp402
isotype
media
p402
media
p401
FasL (CD178)

Figure 15. LN resident DC express IL-12Rβ1 during lethal dose IAV infections. Wild-type mice were infected with a $10LD_{50}$ of IAV and on day 3 p.i. LNDC subsets; $CD11c^{+}B220^{-}CD8\alpha^{+}$, $CD8\alpha^{+}$DC; $CD11c^{+}B220^{-}CD8\alpha^{-}$, $CD8\alpha^{-}$DC and $CD11c^{mod}B220^{+}$, pDC were analyzed for FasL expression (open histogram). Isotype control-filled grey histogram. Data are representative of 2 independent experiments with 3-5 mice per group.

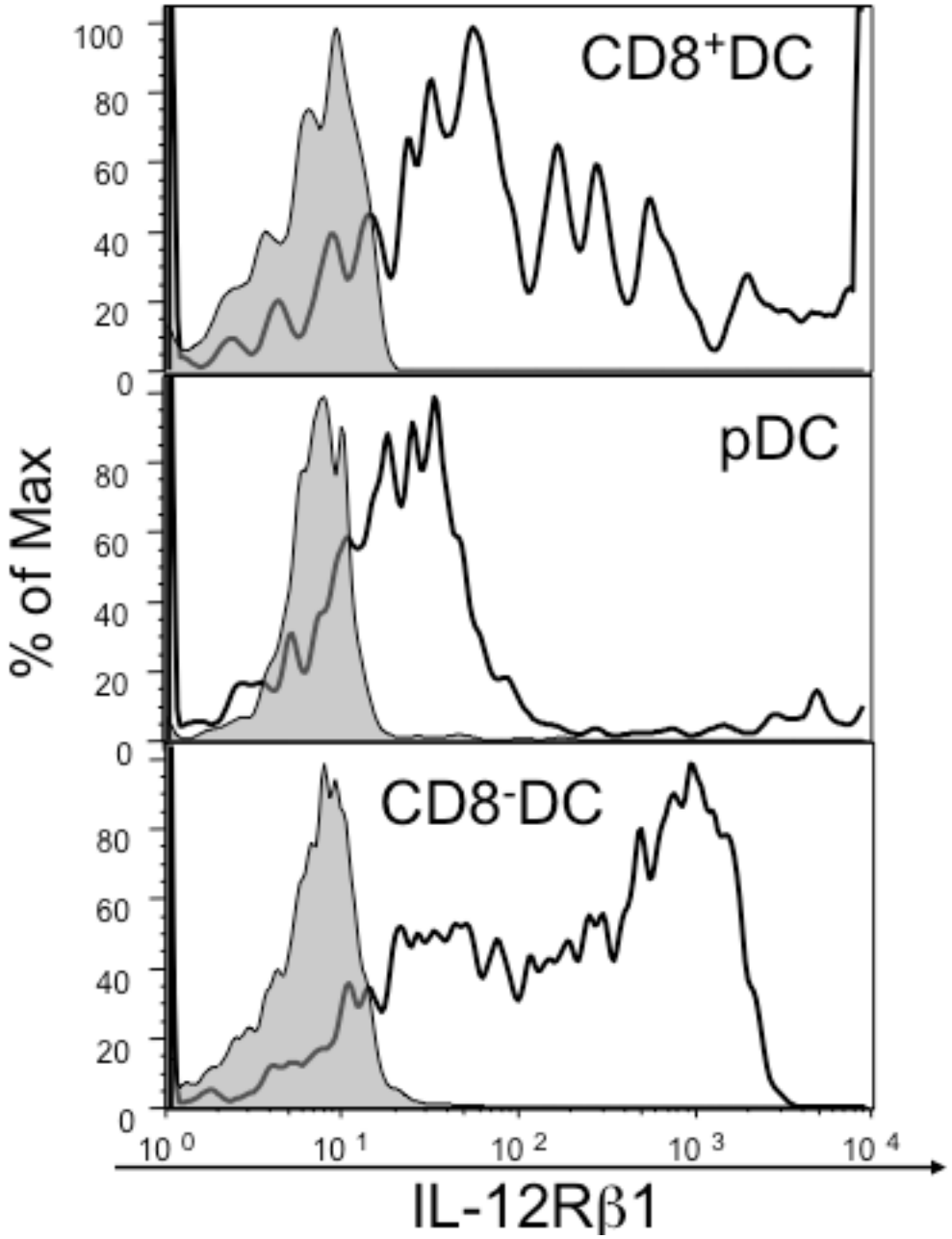

CD8+DC
pDC
CD8-DC
% of Max
IL-12Rβ1
100
80
60
40
20
0
10 0
10 1
10 2
10 3
10 4

Figure 16. IL-12Rβ1 regulates FasL expression on $CD11c^+$ LNDC and pDC. Wild-type and IL-$12R\beta1^{-/-}$ mice were infected with a $10LD_{50}$ of IAV and the level of FasL expression on (A) total $CD11c^+$ LNDC or (B) $CD11c^{mod}B220^+$ pDC was determined. Mean fluorescence intensity (M.FI.) are subtracted from isotype controls. LN were pooled from 3-5 mice per group. Data are representative of 2 independent experiments.

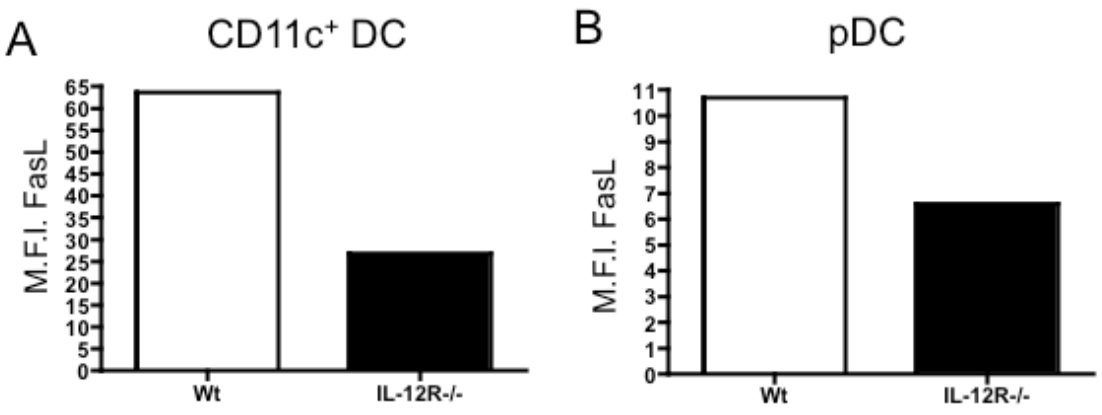

A
CD11c+ DC
M.F.I. FasL
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Wt
IL-12R-/-
B
pDC
M.F.I. FasL
11
10
9
8
7
6
5
4
3
2
1
0
Wt
IL-12R-/-

Figure 17. Both respiratory and LN resident DC produce $p40_2$ during lethal dose IAV infections. Mice were infected with either a $10LD_{50}$ (dotted histograms) or a $0.1LD_{50}$ (open histograms) of IAV and 18 hours post infection (A) $CD11c^+CD103^+$ rDC and (B) $CD11c^+CD103^-$ LN resident DC were analyzed for intracellular $p40_2$ expression. Isotype control-filled grey histograms. LN were pooled from 3-5 mice per group. Data are representative of 3 independent experiments

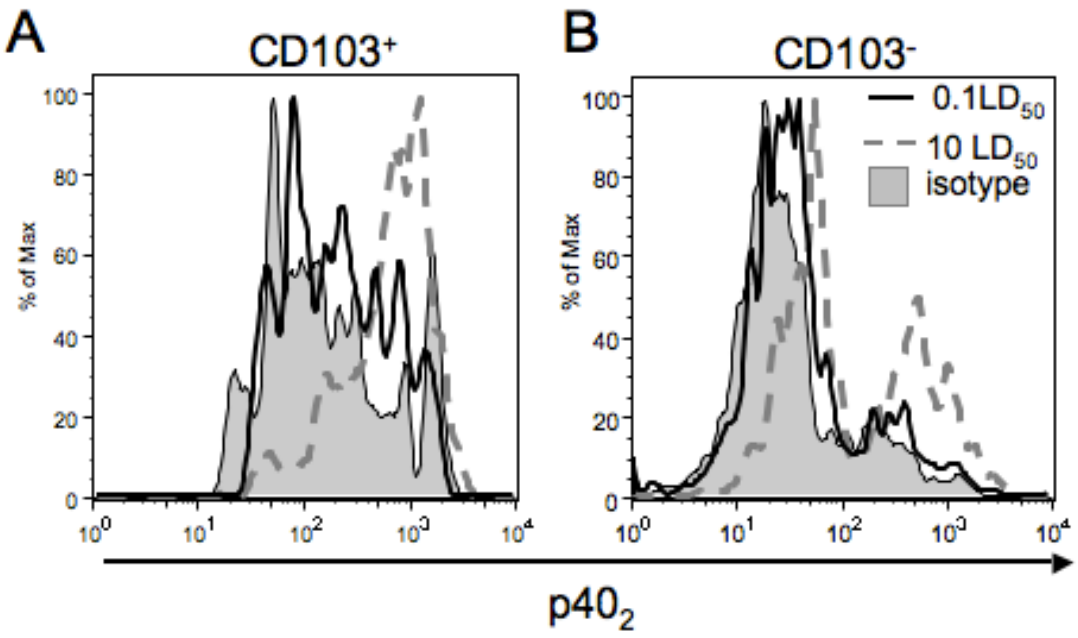

A
CD103+
B
CD103-
0.1LD50
10 LD50
isotype
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
p40_2

Figure 18. Both rDC and LNDC produce intracellular $p40_2$ during lethal dose IAV infections. Mice were treated i.n. with CFSE to label pulmonary DC and 6 hours post-treatment were infected with either a $10LD_{50}$ (dotted histograms) or $0.1LD_{50}$ (open histograms) of IAV. Lung draining LN were removed and the levels of intracellular $p40_2$ production by (A) $CFSE^+CD11c^+$ rDC and (B) $CFSE^-CD11c^+$ LNDC was determined. Isotype control-filled grey histograms. LN were pooled from 3-5 mice per group. Data are representative of 2 independent experiments.

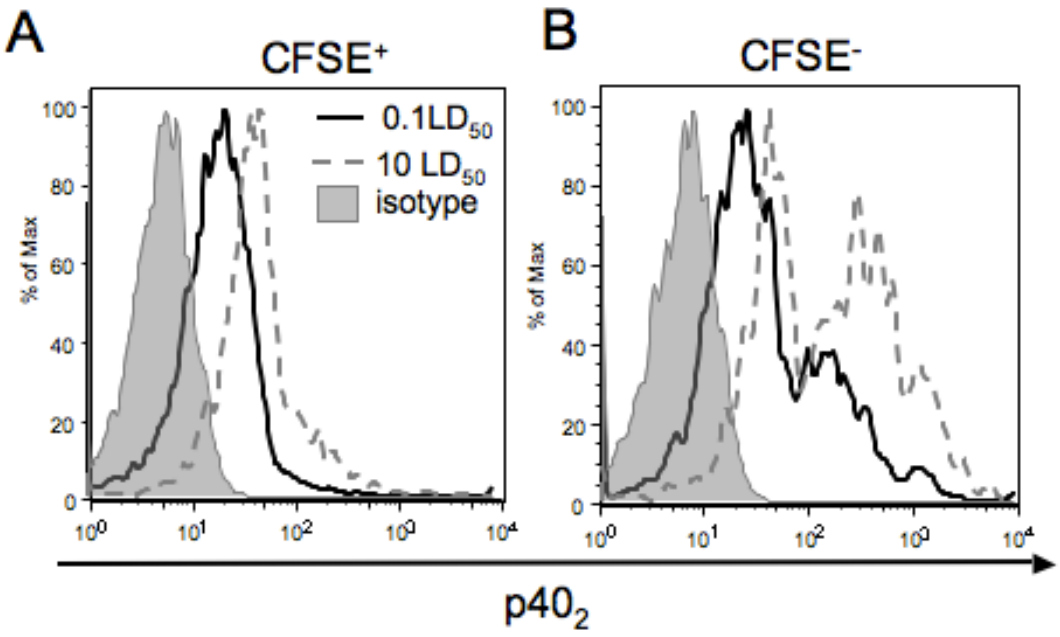

A
CFSE+
B
CFSE-
0.1LD50
10 LD50
isotype
% of Max
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
p40_2

Figure 19. Intracellular $p40_2$ expression by LNDC subsets during lethal dose IAV infections. Mice were infected with either a $10LD_{50}$ (open histogram) or $0.1LD_{50}$ (filled grey histograms) of IAV and the levels of $p40_2$ expression by (A) $CD11c^{mod}B220^+$, pDC; (B) $CD11c^+B220^-CD8^+$, $CD8\alpha^+$DC; (C) $CD11c^+B220^-CD8^-$, $CD8\alpha^-$DC and (D) $CD11c^-$ non-DC was determined. Isotype control-dotted histograms. LN were pooled from 3-5 mice per group. Data are representative of 2 independent experiments.

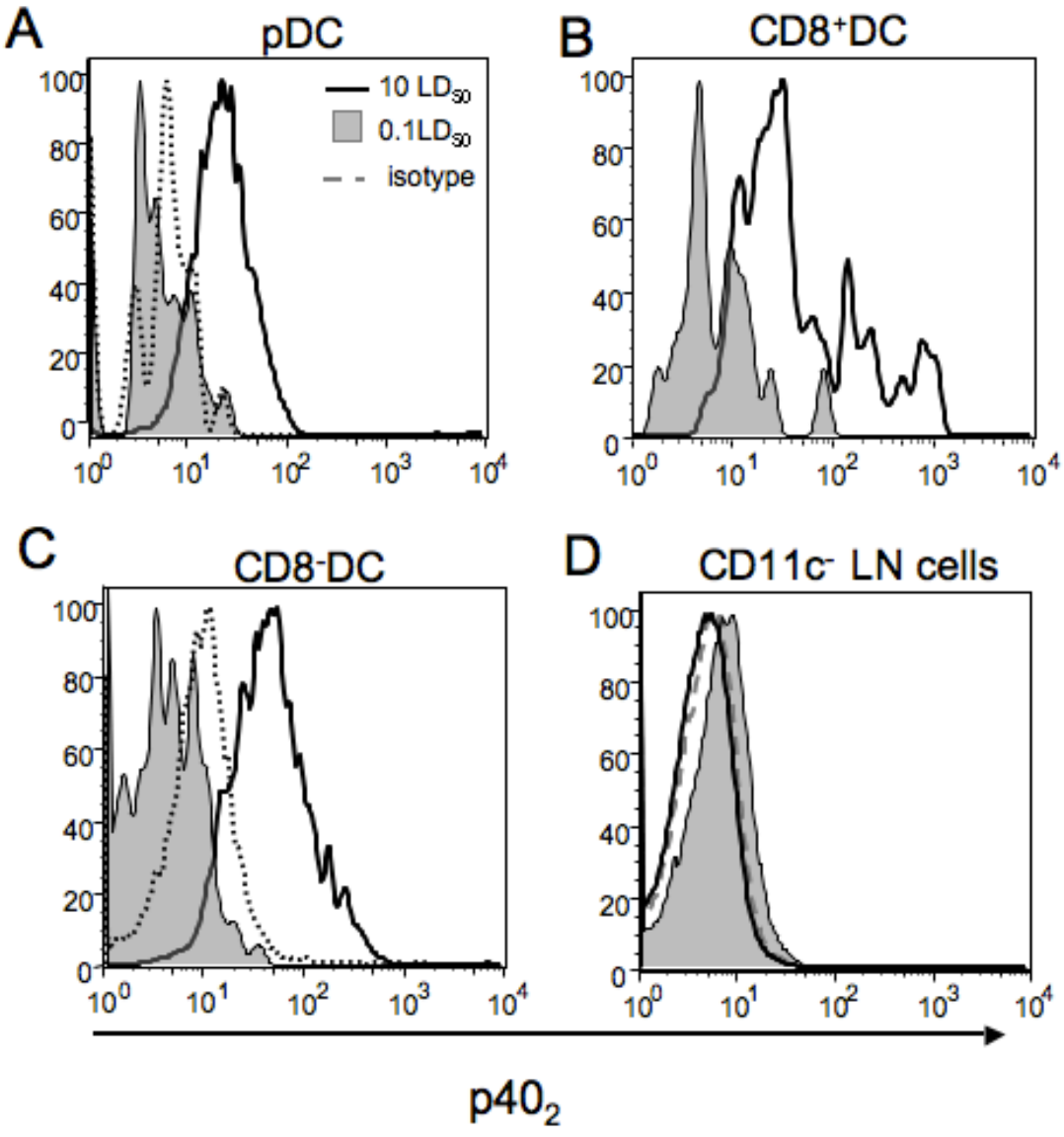
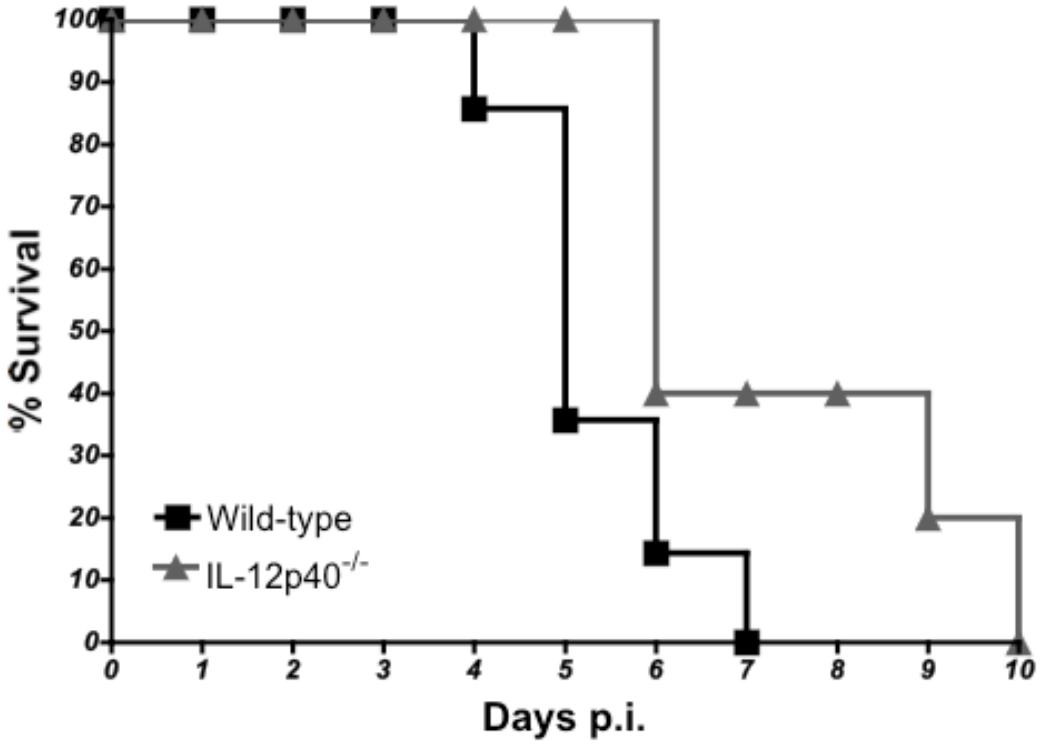

A
pDC
B
CD8+DC
C
CD8-DC
D
CD11c- LN cells
10 LD50
0.1LD50
isotype
p402

Figure 20. IL-12p40 deficient mice have delayed mortality during lethal dose IAV infections. Wild-type IL-12p40$^{+/+}$ and IL-12p40$^{-/-}$ mice were infected with a 10LD$_{50}$ dose of IAV. Mortality was monitored daily post infection. Statistical differences were seen between wild-type and IL-12p40$^{-/-}$ mice, p=0.009 as determined by Kaplan-Meier survival analysis. n=1.


% Survival
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Days p.i.
Wild-type
IL-12p40-/-

CHAPTER IV RESPIRATORY DC CONTROL LNDC FASL EXPRESSION DURING LETHAL DOSE IAV INFECTIONS THROUGH PRODUCTION OF IL-12P40 HOMODIMER

Introduction

Respiratory dendritic cells (rDC) are critical mediators of the immune response to pulmonary viral infections, specifically IAV infections, as they orchestrate the adaptive immune response by acquiring viral antigens within the lungs and migrating into the draining LN (10, 11, 15, 30, 96, 97, 187). Once in the LN these rDC influence the activity of a variety of immune cells through their production of inflammatory cytokines as well as direct presentation of IAV antigen to naïve CD8 and CD4 T cells (10, 11, 15, 30, 97, 187). In addition to cytokine and direct antigen presentation mediated communication with other cells rDC transfer IAV antigen to $CD8\alpha^{+}$DC thereby allowing these DC to activate naïve CD8 T cells (10, 11). Ultimately through all of this communication, rDC are thought to translate the nature of the pulmonary insult into the draining LN allowing for the proper programming of the adaptive immune responses required to resolve the pulmonary insult.

However proper immune induction is not always established following pulmonary insults. Indeed, during lethal dose IAV infections the expression of the cytokine $p40_2$ dictates FasL expression on pDC within the LN (Chapter III). Such pDC FasL expression during lethal dose IAV infections leads to the elimination of IAV-specific CD8 T cells through FasL:Fas interaction and an increased progression to mortality (Chapter II). Further, the data presented in Chapter III demonstrate that both rDC that have migrated from the lungs into the draining LN and LNDC produce increased levels of intracellular $p40_2$ during lethal compared to sublethal dose IAV infections. However whether or not

$p40_2$ production by both rDC and LNDC, or these DC subsets individually, is required for LNDC FasL expression remains unknown.

Previously our laboratory has demonstrated that intranasal stimulation with TLR ligands (CpG, LPS and poly I:C) as well as inflammatory cytokines (TNFα) leads to migration of rDC from the lungs into the lung draining LN similar to that observed during IAV infections (30). In contrast to the similar migration kinetics seen between intranasal TLR stimulators, activation of DC with various TLR agonists and inflammatory cytokines leads to a known differential production of cytokines, including IL-12p40 (146, 147). Importantly while stimulation of TLR4 with LPS drives only transient 6 hour activation of the IL-12p40 gene, stimulation of TLR9 with CpG generates continual long lasting, greater than 48 hour, activation of IL-12p40 (146). Interestingly, there is a similar differential production of intracellular IL-12p40 by rDC within the LN during lethal versus sublethal dose IAV infections (Chapter III) (38). These data suggest that the different inflammatory environments rDC are exposed to during a diverse array of pulmonary pathogen exposures may lead to a differential rDC $p40_2$ expression and hence LNDC FasL expression.

In this chapter we demonstrate that rDC control both LNDC intracellular $p40_2$ production as well as subsequent LNDC FasL expression during lethal dose IAV infections. In addition to differential rDC $p40_2$ production during lethal and sublethal dose IAV infections, stimulation with viral, but not bacterial, derived TLR ligands leads to enhanced rDC intracellular $p40_2$ expression. Further, consistent with levels of intracellular rDC $p40_2$ expression during viral and bacterial derived TLR agonists, these diverse stimulations lead to a differential level of FasL expression on pDC. Therefore, the data presented herein suggest a model where the pulmonary environment initiated by increasing doses of IAV infections as well as diverse pathogen associated molecular patterns leads to differential rDC $p40_2$ production and subsequent pDC FasL expression. This FasL expression then in turn regulates the overall magnitude of the IAV-specific

CD8 T cell responses through pDC FasL mediated elimination of activated CD8 T cells within the LN.

Materials and Methods

Mice

Wild-type BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). IL-12p40$^{-/-}$ (C.129S1-Il12b^{tm1Jm}/J) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All experiments were performed in accordance with federal and institutional guidelines approved by the University of Iowa Animal Care and Use Committee.

Virus infection

6-10 week old mice were anesthetized with isofluorane and infected i.n. with either a 10LD$_{50}$ or a 0.1LD$_{50}$ dose of mouse-adapted A/JAPAN/305/57 (H2N2) in 50µl of Iscoves media. Viruses were grown and stored as previously described (38).

Flow cytometry

LN cells were stained with the following monoclonal antibodies: rat anti-mouse CD8α (53-6.7), hamster anti-mouse CD11c (HL3), rat anti-mouse CD3ε (145-2C11), CD103 (M290), MHC II (M5/114.15.2), rat anti-mouse IL-12Rβ1, and rat anti-mouse CD11b (M2/70) purchased from Becton Dickinson; rat anti-mouse CD45R (RA3-6B2), and rat anti-mouse CD19 (6D5) purchased from Caltag (Invtrogen). Armenian hamster anti-mouse-FasL CD95L (MFL3) was purchased from eBioscience. For FasL staining, cells were blocked with 1:100 rat serum, 1:100 hamster serum and 1:400 free streptavidin (molecular probes) on ice for 25 mins. Cells were then washed twice and stained with 2x (i.e. 1:50) biotin-conjugated anti-FasL (MFL3) followed by streptavidin-PE purchased from Becton Dickinson. For surface staining, isolated cells (10^6) cells were stained with

antibody, and then fixed using BD FACS Lysing Solution (BD Biosciences). For intracellular $p40_2$ detection, cells were fixed using BD FACS Lysing Solution and then permeabilized using buffer containing 0.5% saponin (Acros Organics, NJ) and labeled with 1:50 of Armenian hamster anti-mouse $p40_2$ (a3-1D) kindly provided by Kalipada Pahan (Rush University) for 1 hour at 4° C. All flow cytometry data were acquired on a BD FACS Calibur or BD FACS Canto II (BD Immunocytometry Systems) and analyzed using FlowJo software (TreeStar, Ashland, OR).

rDC depletion

Respiratory DC and macrophages were depleted using liposomes containing dichloromethylene bisphosphonate (Clodronate) which accumulates in phagocytic cells resulting in apoptosis (188). Chlodronate was a kind gift from Roche Diagnostics GmbH, Mannheim, Germany. Clodronate was encapsulated in liposomes as previously described (189). 6 hours prior to infection mice were anesthetized using isofluorane and 75µl of Clodronate containing liposomes were given intranasally.

rDC purification

Mice were treated i.n. with 25mM CFSE suspended in dimethyl sulfoxide (Fisher) and 6 hours post treatment were infected with either a $10LD_{50}$ or a $0.1LD_{50}$ dose of IAV. 18 hours p.i. lung draining LN were removed and digested with 4000 units of type IV collagenase (Worthington) and 600 units of DNase 1 (Sigma) in Iscoves media for 10 mins at room temperature. LN were then processed into a single-cell suspension and red blood cells lysed using NH_4Cl-Tris. Cells were then stained with anti-CD3ε-PE and anti-CD19-PE mAb followed by anti-PE microbeads (Miltenyi Biotech) according to manufacture's instructions. $CD19^+CD3\varepsilon^+$ labeled cells were isolated using an autoMACS. The $CD19^-CD3\varepsilon^-$ fraction was saved, stained with antibodies to CD11c and MHC II, and $CD11c^+MHCII^+CFSE^+$ cells sorted using a FACS DIVA.

Lymph node organ culture

Lung draining LN from day 3 $10LD_{50}$ infected IL-12p40$^{-/-}$ mice were removed and placed in DMEM containing 2x penicillin/streptomycin. 5 LN were then placed into a 96 well plate with 200ul of medium alone or medium containing either 200ng recombinant $p40_2$ (R and D systems), or $2.5x10^4$ purified rDC from $10D_{50}$ IAV infected wild-type mice. LN were then placed into a modular incubator chamber (Billups-Rothenberg) containing 95% O_2 and 5% CO_2 at 37° C for 24 hours. Following incubation LN were removed and processed into a single cell suspension for flow cytometry staining.

Lymph node dendritic cell purification

Lung draining LN (peribronchiolar and mediastinal) were removed and digested with 4000 units of type IV collagenase (Worthington) and 600 units of DNase 1 (Sigma) in Iscoves media for 10 mins at room temperature. LN were then processed into a single-cell suspension and red blood cells lysed using NH_4Cl-Tris. Cells were then stained with anti-CD3ε-PE and anti-CD19-PE mAb followed by anti-PE microbeads according to manufacture's instructions (Miltenyi Biotech). Labeled cells were isolated using an autoMACS. The negative fraction was saved, stained with antibodies to CD11c, CD45R (B220), CD8α, CD4 and sorted into $CD11c^{mod}CD45R^+CD8^+$ cells (i.e. pDC); $CD11c^+CD45R^-CD8^+$ cells (i.e. $CD8\alpha^+$ DC); $CD11c^+CD45R^-CD8^-CD4^-$cells (i.e. DN DC); and $CD11c^+CD45R^-CD8^-CD4^+$ cells (i.e. $CD4^+$DC) using a FACS DIVA. Cells were then cultured in isolation for either 6 or 18 hours.

In vivo TLR stimulation

Wild-type mice were anesthetized with isofluorane and given either 25μg LPS or 25μg of poly I:C. intranasally. Lung draining LN were removed at either 18 or 72 hours post stimulation and processed into a single cell suspension for flow cytometry labeling and analysis.

Results

rDC are required for LNDC $p40_2$ production during lethal dose IAV infections

Previously we have demonstrated that $p40_2$ production either by rDC and/or LNDC may be responsible for regulating LNDC FasL expression during IAV infections (Chapter III). Therefore we next determined if rDC migration from the lungs to the draining LN is required for LNDC $p40_2$ expression or if LNDC production of $p40_2$ is independent of rDC migration. When rDC were depleted within the lungs prior to lethal dose IAV infection, which therein decreases rDC migration into the LN (Figure 21) (111, 188), LN resident DC failed to produce intracellular $p40_2$ (Figure 22). These data suggest that in addition to producing $p40_2$, the presence of rDC is required for LNDC production of $p40_2$.

rDC are required for inducing LNDC FasL expression during lethal dose influenza virus infection.

The above results suggest that rDC may be regulating LNDC FasL expression during lethal dose IAV infections either directly by producing $p40_2$ and/or indirectly by inducing LNDC production of $p40_2$. We next determined if LNDC, after initial exposure to rDC, are programmed to maintain FasL expression or if they need continual rDC stimulation to maintain FasL expression. To test this purified LNDC from day 3 IAV infected mice (i.e. LNDC that have been exposed to migratory rDC for ~3 days) were cultured in isolation and the levels of FasL determined following culture. Surprisingly, despite intracellular $p40_2$ expression by all LN resident DC subsets measured ex vivo in previous experiments (Figure 19), all of these LNDC lost FasL expression when cultured in isolation (Figure 23). These data suggest that the initial ability of LNDC to produce

$p40_2$ is not sufficient to maintain FasL expression and that another cell within the LN, likely another DC subset, given that only DC produce $p40_2$ during lethal dose IAV infections (Chapter II), is required to maintain LNDC FasL expression. Since LNDC expression of FasL is tied to $p40_2$ expression (Figure 14A-C) and LNDC are unable to maintain LNDC FasL expression in isolation following initial rDC encounter, we next determined if rDC are required for LNDC FasL expression, similar to the requirement for $p40_2$ expression (Figure 22). To this end rDC were depleted prior to infection and LNDC FasL expression was assessed following lethal dose IAV challenge. Consistent with the loss of $p40_2$ production in the absence of rDC, LNDC from mice that had been depleted of rDC and infected with a lethal dose of IAV also displayed decreased FasL expression when compared to LNDC from non-rDC depleted mice (Figure 24A-C). Importantly, pDC, the cell responsible for eliminating IAV-specific CD8 T cells (Chapter II), also reduced FasL expression in the absence of rDC migration to the LN (Figure 24C).

Given the above results, we next determined if rDC are sufficient to induce LNDC FasL expression. To address this, rDC were purified from lethal dose IAV infected IL-12p40$^{+/+}$ wild-type mice and then incubated with intact LN from lethal dose IAV infected mice deficient in IL-12p40 whose LNDC are FasL^{low} (Figure 14A). After co-culture IL-12p40$^{-/-}$ LNDC that had been incubated with wild-type IL-12p40$^{+/+}$ rDC from lethal IAV infected mice upregulated FasL expression to a level similar as addition of recombinant $p40_2$ to the culture (Figure 25). These data do not exclude the possibility that autocrine $p40_2$ signaling from LNDC may be capable of boosting FasL expression. However, autocrine $p40_2$ signaling is not required as addition of exogenous $p40_2$ to IL-12p40 deficient LN can mediate upregulation of LNDC FasL expression (Figure 14B and 25). Together these data suggest that migration of rDC from the lungs to the LN during lethal IAV infections is required for LNDC and pDC FasL expression and that LNDC do not self regulate their FasL expression or $p40_2$ expression in the absence of these cells within the LN.

Pulmonary TLR stimulation controls pDC FasL expression within the LN

Given the difference in $p40_2$ production by rDC during lethal versus sublethal dose IAV infections and the subsequent impact on LNDC FasL expression we next determined if this was distinct to IAV infections or if this effect was more global. Previous studies have demonstrated that stimulation of DC with multiple TLR agonists induces differential production of inflammatory cytokines such as IL-12p40 (146, 190). Our laboratory has also demonstrated that i.n. stimulation with TLR agonists results in a level of rDC migration from the lungs to the LN similar to what is observed during IAV infections (30). Therefore to determine if stimulation with TLR ligands likewise modulates $p40_2$ production in vivo, mice were treated i.n. with individual TLR agonists, or infected with either a lethal or sublethal dose of IAV as controls, and the levels of intracellular $p40_2$ expression by rDC within the LN was determined. Interestingly simulation with TLR3, but not TLR4, agonists resulted in higher levels of intracellular $p40_2$ production in rDC (Figure 26A) but not LNDC (Figure 26B). Since $p40_2$ is tied to LNDC FasL expression these data suggested that TLR3 induced $p40_2$ production by rDC may subsequently result in increased FasL expression on LNDC. To test this hypothesis, mice were treated with TLR agonists i.n. and the level of FasL on LNDC was determined. Consistent with the above results stimulation with TLR3 agonists resulted in higher FasL expression on pDC whereas TLR4 agonist did not (Figure 26C). Furthermore our preliminary results have demonstrated that stimulation with another viral derived TLR agonist, R848 which triggers TLR7, also leads to higher levels of LNDC FasL expression similar to stimulation with TLR3 agonists (Langlois and Legge, unpublished resutlts). These data suggest that distinct TLR signaling within the lungs during an array of pathogenic challenges may be able to dictate the levels of $p40_2$ produced by rDC.

Following rDC migration into the lung draining LN altered levels of rDC $p40_2$ production could therein dictate the levels of FasL expression on pDC ultimately leading to either survival or apoptosis of activated Fas^+ CD8 T cells.

Discussion

The data presented in this chapter highlight a novel role for rDC during IAV infections. Herein we demonstrate that rDC are required for the regulation of FasL expression during IAV infections. Furthermore we show that the regulation of rDC $p40_2$ expression as well as the subsequent LNDC FasL expression extends beyond IAV infections as stimulation with diverse TLR agonists leads to differential rDC $p40_2$ production and corresponding LNDC FasL expression.

Previously we have demonstrated that LNDC FasL expression during lethal dose IAV infections is dependent on $p40_2$ expression within the LN (Chapter III). While the production of $p40_2$ within the LN was restricted to DC, which LNDC or migratory DC subset(s) were required for LNDC FasL expression remained unknown. In this chapter we demonstrate that rDC are required for both LNDC $p40_2$ production as well as LNDC FasL expression. Importantly LNDC, which express intracellular $p40_2$ during lethal dose IAV infections, lose FasL expression when cultured in isolation. These data suggest that LNDC require communication with another cell within the LN to maintain FasL expression. Given that neutralization of $p40_2$ leads to decreased LNDC FasL expression (Figure 14C) suggests that $p40_2$ is acting directly upstream of LNDC FasL expression or any other potential additional mediators. In the future it will be important to determine if the regulation of FasL expression by $p40_2$ is direct, or instead if $p40_2$ signals LNDC FasL expression indirectly through the production of another, as yet to be described cytokine. One potential cytokine that could be involved in indirect $p40_2$ mediated LNDC FasL expression is TNFα. TNFα is upregulated by $p40_2$ stimulation and induces FasL

expression in a variety of cell types (182, 191-193). Additionally recent studies have demonstrated that $p40_2$ signals through IL-12Rβ1 to mediate the production of IL-16, which has also been shown to induce FasL expression (157, 194). Importantly preliminary results from our laboratory have demonstrated that TNFα levels within the LN increase during lethal dose IAV infections correlate with IL-12p40, while IL-16 decrease (Legge and Braciale, unpublished). Together these data suggest the possibility that increased TNFα, but likely not IL-16, within the LN during lethal dose IAV infections may be induced by $p40_2$ signaling and may represent an indirect downstream signal that ultimately could mediate LNDC FasL expression.

Our data also demonstrate that regulation of IL-12p40 can also occur in the absence of viral infection as TLR agonists administered i.n. were also capable of altering both rDC $p40_2$ production as well as downstream LNDC FasL expression (Figure 26B-C). Interestingly, our findings demonstrated that a TLR triggered during viral infections (i.e. TLR3) was able to induce both rDC $p40_2$ production as well as subsequent LNDC FasL expression while a TLR most associated with bacterial infections (i.e. TLR4) was unable to mediate this effect. Further our preliminary experiments suggest that a mimic of another viral TLR ligand, R848 (single stranded RNA), behaves similarly to TLR3 ligands when administered intranasally, further suggesting that bacterial and viral infections may lead to differential rDC $p40_2$ production and subsequent LNDC FasL expression. Interestingly given that TLR7 is not expressed on rDC this effect is likely mediated from a downstream signal possible initiated through TLR7 expressing pulmonary pDC (27). In support of this idea a recent report has demonstrated a differential activation of the IL-12p40 gene during diverse TLR stimulation. TLR9 agonist treatment resulted in constitutive IL-12p40 activation while TLR4 induced only transient IL-12p40 activation (146). The mechanism for how these TLR agonist differentially stimulate $p40_2$ production remains unknown. One potential hypothesis for these differences may be the different downstream signaling pathways employed by

TLR3 and TLR4. Both TLR3 and TLR4 utilize the adapter molecule TRIF to mediate downstream signaling (195). However, TLR4, but not TLR3, can mediate signals through an additional adapter MyD88 (195). Furthermore, it has been demonstrated that the outcome of TLR3 signaling and which proximal-TRIF pathways are utilized are cell type specific (196). Our data also demonstrate that stimulation of TLR4 and 7 lead to differential LNDC FasL expression despite both being capable of signaling through MyD88. One possibility for this is that TLR7 expression is restricted to pDC (17, 25, 27), which do not migrate directly into the lung draining LN, suggesting that TLR7 stimulation of rDC may be indirect through pDC. Together these studies suggest the possibility that TLR3 and TLR4 signaling may lead to a diverse cytokine array in rDC ultimately leading to differential $p40_2$ production.

During IAV infections the viral replication intermediate double stranded RNA is recognized by TLR3 leading to the generation of a proinflammatory response (76). However much of this double stranded IAV RNA is sequestered away from TLR3 by the viral polymerase complex and NS1 as well as the intracellular viral RNA receptor RIG I (76, 197, 198). The results presented in this chapter demonstrate that the double strand RNA mimic poly I:C induces higher levels of intracellular rDC $p40_2$ expression and subsequent LNDC FasL expression similar to lethal dose IAV infections. Interestingly, when mice are infected with a lethal dose of IAV, rDC become infected at an increased multiplicity of infection when compared to sublethal dose infection (VanOosten and Legge, manuscript submitted). The increase in infectious virus within rDC during lethal dose IAV infection may lead to saturation of NS1, viral polymerase, and RIG I leading to increased levels of free viral double stranded RNA within the cell suggesting the possibility of increased TLR3 signaling within rDC during lethal dose IAV infection. Further, there could also be an increase in extracellular IAV double stranded RNA generated from increased levels of necrosis from cells infected at high M.O.I. during

lethal dose IAV infection. This differential rDC infection level may represent a possible mechanism for the differential rDC $p40_2$ during lethal and sublethal dose IAV infections.

The data presented herein offer another example of DC mediated cross-talk between immune cells. Previous studies have demonstrated that migratory tissue derived DC cross-talk with LN resident DC through antigen transfer during a variety of infections, including IAV infections (10, 11). In addition to cross-talk with other DC subsets, DC also cross-talk with NK through IL-15 trans-presentation, allowing for the initiation of appropriate immune responses (199, 200). Furthermore IL-15 also plays a role in DC:T cell communication within the lungs. Results from McGill and Legge have demonstrated that subsequent to CD8 T cells initial antigen encounter with DC within the LN, activated CD8 T cells require a second hit from DC within the lungs through MHCI:antigen and trans presentation of IL-15 during IAV infections. Together these results highlight the importance of DC cross-talk through a variety of mediators and to multiple different cells of the immune system. Importantly, the results presented in chapter IV extend the idea of DC cross-talk by demonstrating that migration of rDC into the LN is required for $p40_2$ mediated expression of FasL on LNDC. However whether rDC:LNDC interaction through $p40_2$ requires cell contact similar to DC:NK and DC:T cell communication through IL-15 or if rDC cross-talk is mediated in a paracrine fashion remains unknown.

All together our studies demonstrate a critical role for rDC, and their production of $p40_2$, in modulating the phenotype of DC within the LN. Given the aberrant interaction of pDC with IAV-specific CD8 T cells during lethal dose IAV infections and the data presented herein demonstrating the rDC dependent regulation of FasL expression on pDC, together results suggest the possibility that rDC may control the overall magnitude of the CD8 T cell response generated during IAV infections both at the initial activation stage of adaptive responses but also via production of $p40_2$ and regulation of the magnitude of that response by $FasL^+$ pDC mediated apoptosis.

Figure 21. Depletion of respiratory phagocytic cells decreases rDC migration to the lung draining LN during lethal dose IAV infections. Wild-type mice were treated i.n. with or without liposomes containing clodronate, to deplete rDC, or PBS, as a control. 4 hours post depletion mice were treated i.n. with 25mM CFSE, and 2 hours post CFSE labeling infected with a $10LD_{50}$ dose of IAV. 18 hours p.i. lung draining LN were removed, pooled and the number of $CD11c^{+}MHCII^{+}CFSE^{+}$ rDC per LN was enumerated. Data are representative of 1 experiment.

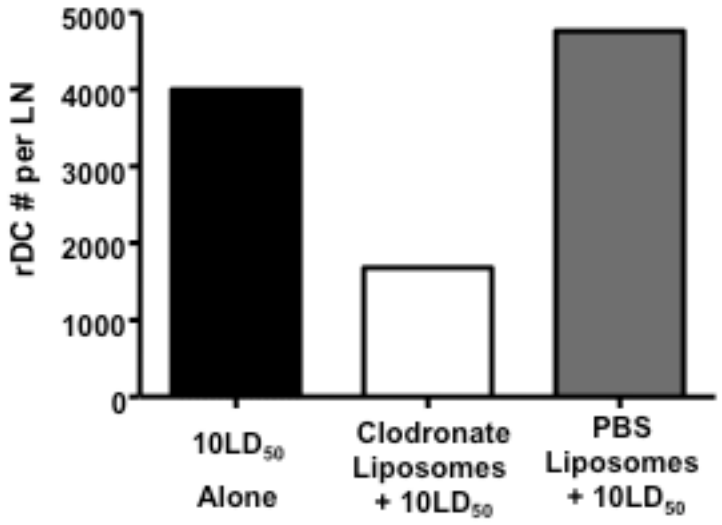

5000
4000
3000
2000
1000
0
rDC # per LN
10LD50 Alone
Clodronate Liposomes + 10LD50
PBS Liposomes + 10LD50

Figure 22. rDC migration into the lung draining LN controls LNDC $p40_2$ expression. rDC were depleted by treating mice i.n. with liposomes containing clodronate as in Figure 21. Six hours post-treatment mice were infected with a $10LD_{50}$ of IAV and the level of intracellular $p40_2$ in $CD11c^+CD103^-$, LN resident DC at 18 hours post infection was determined. LN were pooled from 3-5 mice per group. Data are representative of 2 independent experiments.

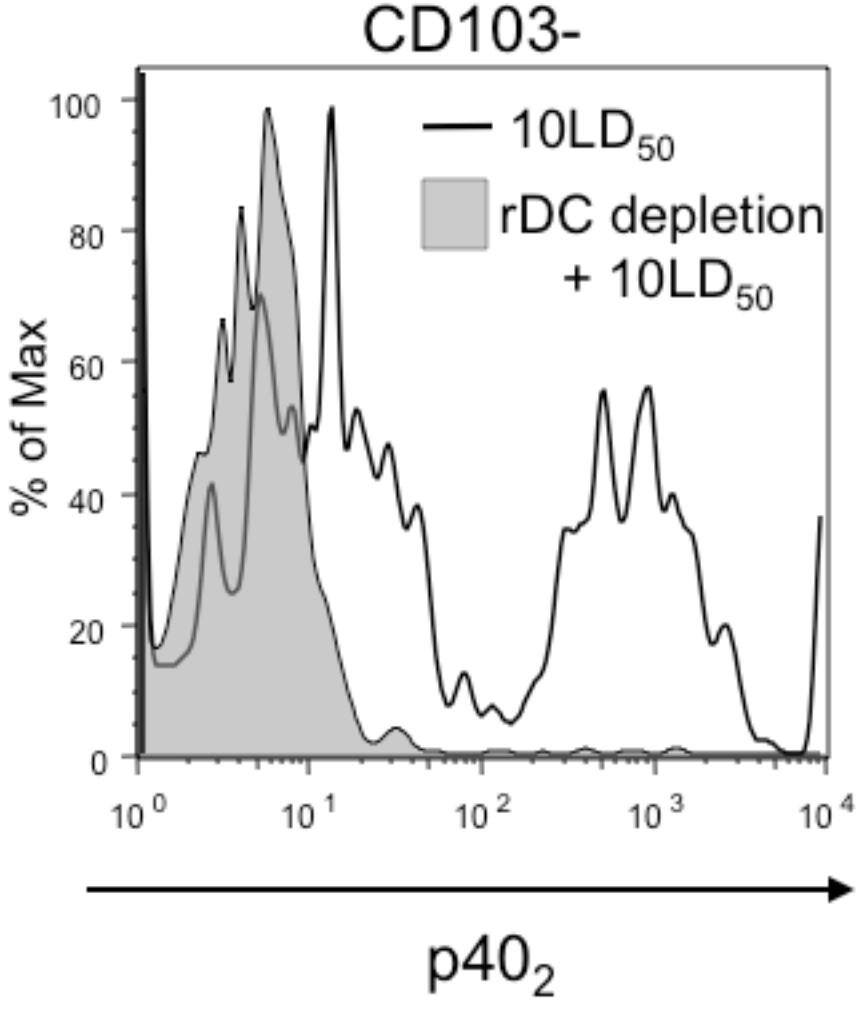

CD103-
10LD50
rDC depletion
+ 10LD50
% of Max
100
80
60
40
20
0
10 0
10 1
10 2
10 3
10 4
p40 2

Figure 23. Purified LNDC decrease FasL expression in isolation. Wild-type mice were infected with a $10LD_{50}$ dose of IAV and on day 3 p.i. $CD8\alpha^{+}$DC, $CD4^{+}$DC, DNDC and pDC were purified from lung draining LN. Purified DC were then either stained for FasL directly after purification or cultured in isolation for 6 hours and then the levels of FasL determined (similar results seen after 18 hours of isolation, not shown). pDC were stained with anti-FasL-biotin/texas red conjugated avadin (TR) all other subsets stained with anti-FasL-biotin/phycoerythrin conjugated avadin (PE). Data are representative of 3 independent experiments.

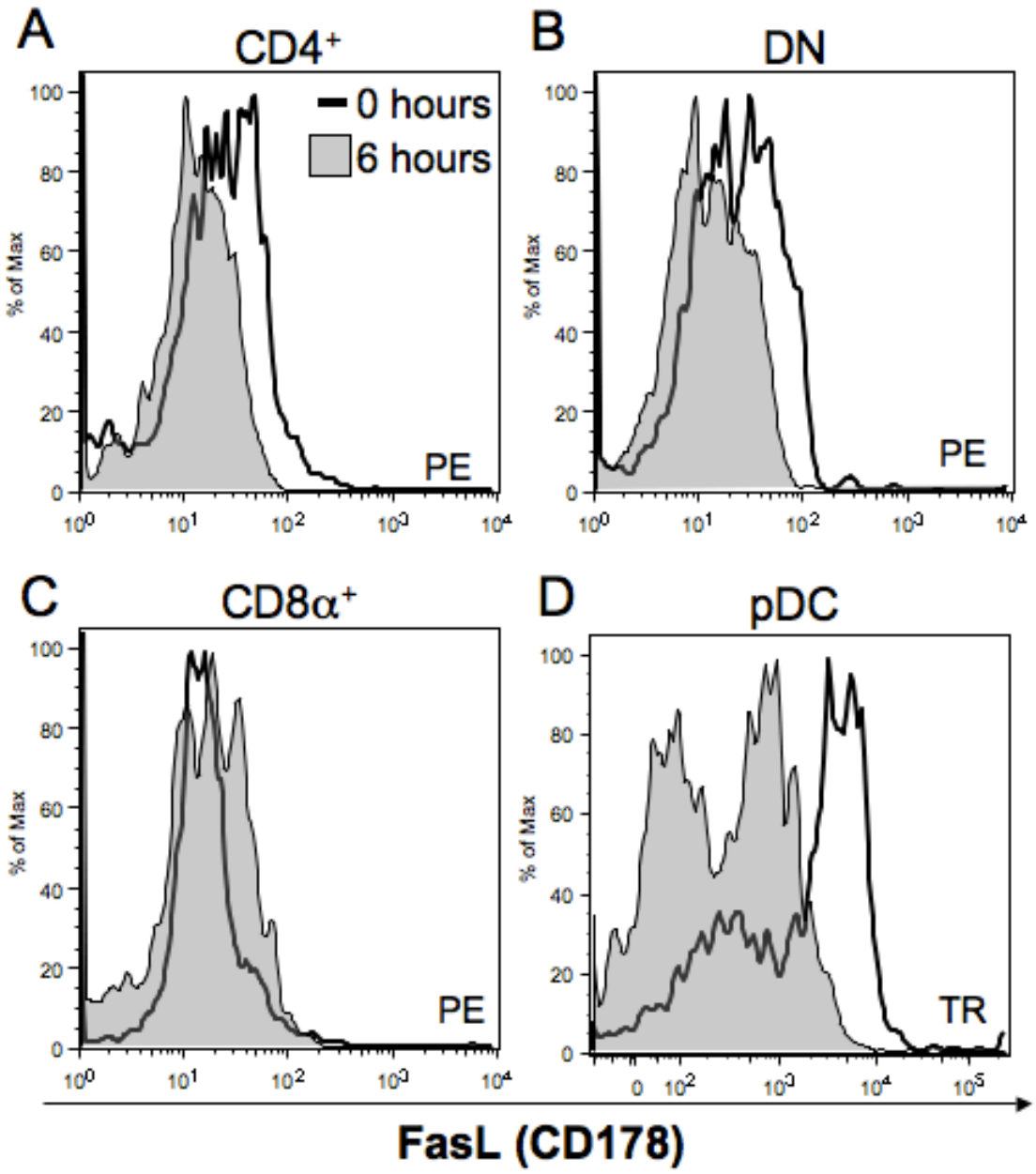

A
CD4+
0 hours
6 hours
PE
% of Max
B
DN
PE
C
CD8α+
PE
D
pDC
TR
FasL (CD178)

Figure 24. rDC migration into the lung draining LN controls LNDC FasL expression. rDC were depleted by treating mice i.n. with clodronate liposomes. Six hours post-treatment mice were infected with a $10LD_{50}$ of IAV. (A) $CD11c^{+}B220^{-}CD8^{+}$, $CD8\alpha^{+}$DC; (B) $CD11c^{+}B220^{-}CD8^{-}$, $CD8\alpha^{-}$DC; (C) $CD11c^{mod}B220^{+}$, pDC within the LN were analyzed for FasL expression. LN were pooled from 3-5 mice per group. Data are representative of 2 independent experiments.

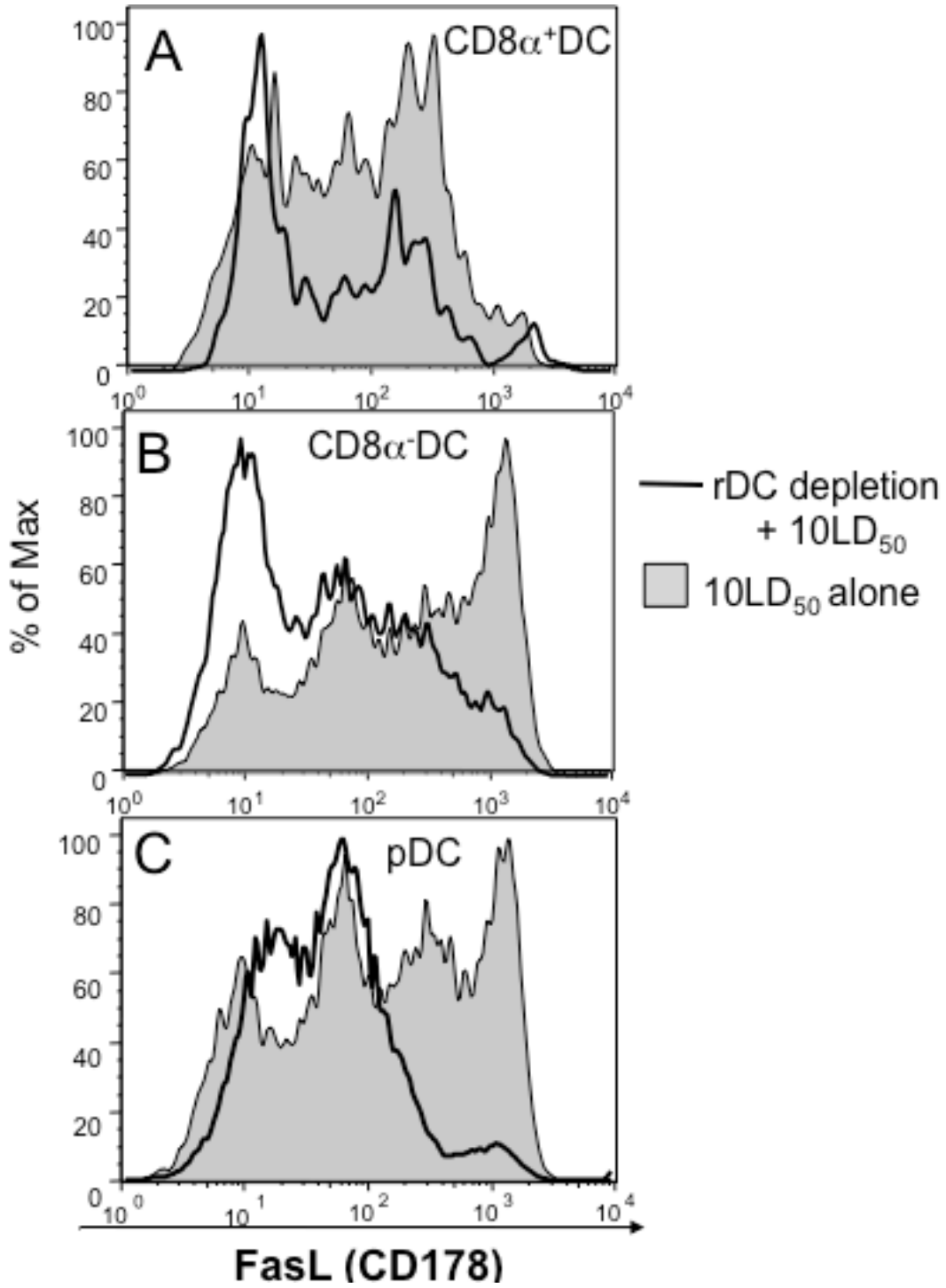

A
CD8α+DC
B
CD8α-DC
C
pDC
% of Max
FasL (CD178)
rDC depletion + 10LD50
10LD50 alone

Figure 25. rDC control FasL expression on LNDC during lethal dose IAV infections. rDC were purified from $10LD_{50}$ IAV infected wild-type IL-12p40$^{+/+}$ mice. Purified rDC were then incubated with intact LN from $10LD_{50}$ infected IL-12p40$^{-/-}$ mice and the level of FasL expression on CD11c^{+} LNDC was determined after 18 hour co-culture. As a positive control IL-12p40$^{-/-}$ LN were also incubated with $p40_2$. Data are representative of 4 independent experiments.

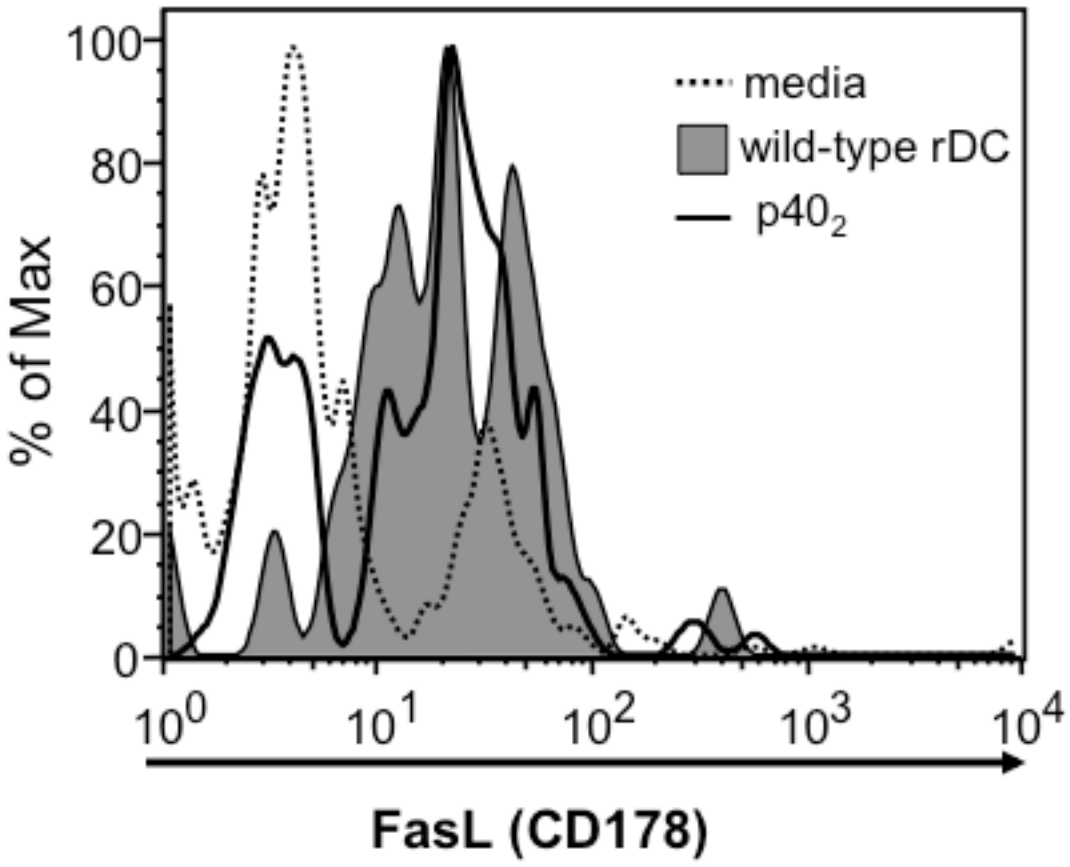
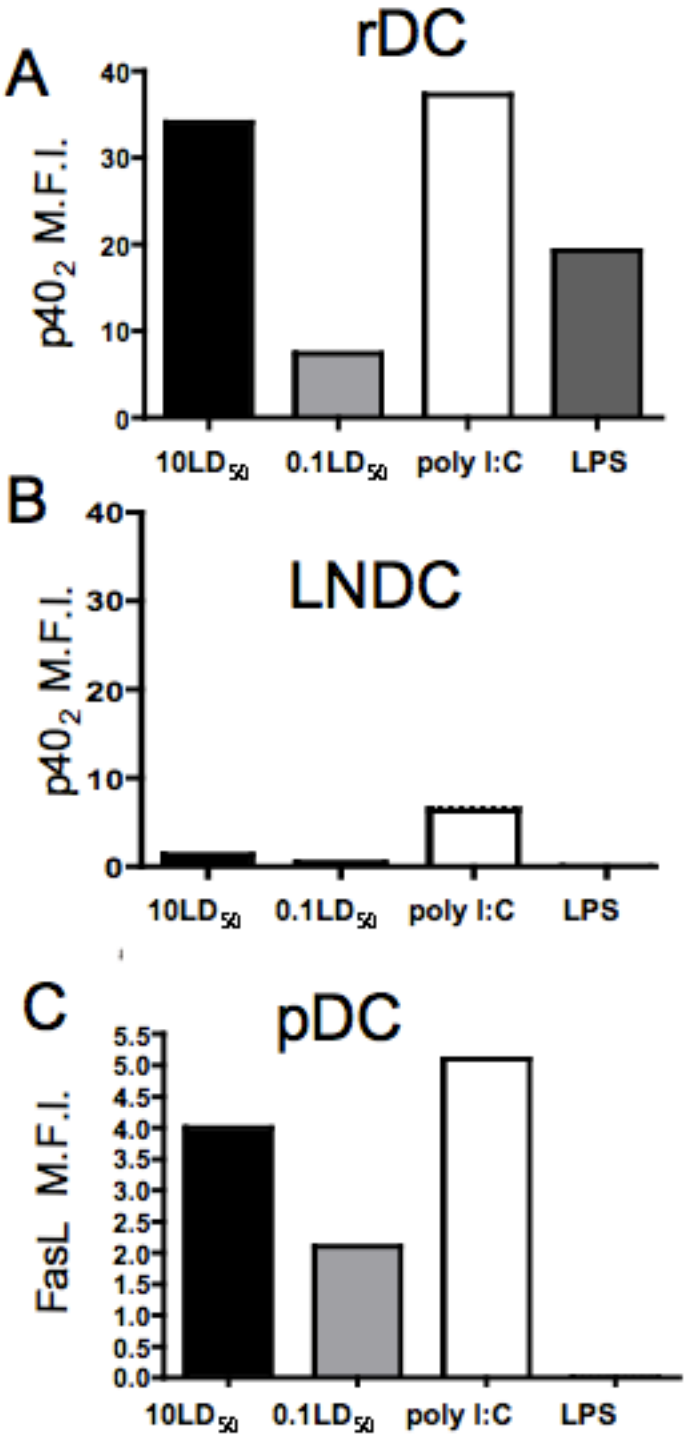

100
80
60
40
20
0
% of Max
media
wild-type rDC
p40$_2$
10^0
10^1
10^2
10^3
10^4
FasL (CD178)

Figure 26. Pulmonary TLR stimulation controls both rDC $p40_2$ production and pDC FasL expression. Mice were treated i.n. with LPS, poly I:C, or either a $10LD_{50}$ or $0.1LD_{50}$ of IAV and the level of intracellular $p40_2$ expression in (A) $CD11c^{+}CD103^{+}$, rDC and (B) $CD11c^{+}CD103^{-}$, LN resident DC was determined. (C) Mice were treated or infected as described above and on D3 post infection/treatment $CD11c^{mod}B220^{+}$, pDC FasL expression was determined. M.F.I. were subtracted from isotype controls. LN were pooled from 3-5 mice per group. Data are representative of 2-4 independent experiments.


A
rDC
p40$_2$ M.F.I.
40
30
20
10
0
10LD$_{50}$
0.1LD$_{50}$
poly I:C
LPS
B
LNDC
p40$_2$ M.F.I.
40
30
20
10
0
10LD$_{50}$
0.1LD$_{50}$
poly I:C
LPS
C
pDC
FasL M.F.I.
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10LD$_{50}$
0.1LD$_{50}$
poly I:C
LPS

CHAPTER V CONCLUSIONS

Synopsis

This study was undertaken to elucidate the interplay of specific LNDC subsets and rDC in the elimination of virus-specific CD8 T cells during lethal dose IAV infections. Previously it was determined that LNDC expressing regulated FasL during lethal dose IAV infections drive apoptosis of Fas^+ IAV-specific CD8 T cells through FasL:Fas (DC:CD8 T cell) interactions(38). In this dissertation I demonstrate that, despite FasL expression by all LN resident DC subsets during lethal dose IAV infections, only pDC are capable of directly eliminating IAV-specific CD8 T cells ex vivo. Furthermore pDC also eliminated IAV-specific CD8 T cells in vivo leading to enhanced mortality during lethal dose IAV infections. Interestingly, the discrepancy in other $FasL^+$ LNDC subsets inability to eliminate activated virus-specific CD8 T cells was determined to be dependent on viral antigen presentation. This outcome is consistent with other studies which have demonstrated that pDC isolated from the lung draining LN of IAV infected mice are incapable of presenting antigen to CD8 T cells, whereas all of the other LNDC subsets present IAV antigens (10, 11, 15, 97). However, MHC I on pDC experimentally loaded with IAV peptide inhibited pDC ability to eliminate IAV-specific CD8 T cells. Together our results suggest that during lethal dose IAV infections pDC are the cells within the lung draining LN that eliminate IAV-specific CD8 T cells through FasL:Fas interactions and that such killing occurs in the absence of CD8 T cells recognition of cognate viral antigen presentation.

Previous studies in our laboratory have also demonstrated that the differential FasL expression on LNDC during lethal versus sublethal dose IAV infections is regulated by the cytokine IL-12p40, specifically either the monomeric and/or homodimeric ($p40_2$) form (38). Herein I demonstrate that $p40_2$ and not p40 monomer, regulates FasL expression on bulk LNDC as well as on pDC. Furthermore the data presented in this

dissertation demonstrate that IL-12Rβ1, which binds to the IL-12p40 subunit, is critical for FasL expression on LNDC during lethal dose IAV infections. Surprising, both LN resident and respiratory migratory DC produced increased $p40_2$ during lethal dose IAV infections. Together these data suggest that either rDC and/or LNDC may be responsible for regulating LNDC FasL expression through their production of $p40_2$.

Lethal and sublethal dose IAV infections induce markedly different inflammatory environment within the lungs (173, 174). We therefore hypothesized that the differential pulmonary environment induced by lethal versus sublethal IAV infections may affect rDC activation and, upon migration into the draining LN, $p40_2$ production as well as corresponding LNDC FasL expression. Consistent with this idea when rDC migration was blocked by depleting phagocytic cells within the lungs, LNDC $p40_2$ expression and corresponding FasL expression was reduced suggesting that rDC control both LNDC $p40_2$ and FasL expression. Despite $p40_2$ expression directly ex vivo, LNDC failed to maintain FasL expression when cultured in isolation suggesting that another cell within the LN is required for LNDC FasL expression. Importantly purified rDC from lethal dose IAV-infected mice were sufficient to induce FasL expression on LNDC and pDC in mice deficient in IL-12p40. Together these data suggest that rDC are critical for the control of FasL expression on LNDC during lethal dose IAV infections.

Previous studies have demonstrated that differential IL-12p40 production is not unique to IAV infections as stimulation of DC with LPS leads to a rapid shut off of IL-12p40 production while CpG stimulation leads to enhanced IL-12p40 production (146). These data suggest that $p40_2$ may be differentially regulated by stimulation of DC with bacterial or virally derived TLR ligands. The data presented herein extend the previous in vitro data discussed above into an in vivo intranasal TLR stimulation model and further confirms that diverse TLR ligands can stimulate differential IL-12p40 (via $p40_2$) production in rDC in vivo. Furthermore our studies show that i.n. stimulation of TLR mediated regulation of rDC $p40_2$ production also translates into differential LNDC FasL

expression as TLR ligands that induce $p40_2$ production also induce downstream LNDC FasL expression. Together these data demonstrate that the regulation of rDC $p40_2$ production and subsequent LNDC FasL expression is not restricted to IAV infections and that CD8 T cell responses to pulmonary bacterial and viral infections may be regulated by differential pDC FasL expression.

Future directions

This dissertation highlights many novel aspects of both LNDC and rDC regulation of CD8 T cell numbers during IAV infections that necessitate further study. Chapter II details the elimination of IAV-specific CD8 T cells by pDC during lethal dose IAV infections. Our lethal dose infectious model system was chosen to mimic highly virulent IAV (i.e. 1918 and H5N1) that replicate to very high titers early following infection (173). Importantly during infection with these highly virulent IAV strains, severe lymphopenia develops likely from the increased corticosteroid production from the stress response (172-174). However, it will be important to determine in future studies if there is also an antigen-specific CD8 T cell lymphopenia that is mediated by similar pDC-FasL:CD8 T cell-Fas interactions as we have described in our system. If $FasL^+$ pDC are responsible for additional IAV-specific lymphopenia associated with highly pathogenic IAV infections these data would suggest that strategies aimed at inhibiting or blocking FasL expression or depletion of pDC may be capable of reversing some of the CD8 T cell lymphopenia and ultimately the outcome of infection.

Additionally, the data presented in chapter II demonstrate that pDC and $CD8\alpha^+$DC decrease FasL expression during sublethal compared to lethal dose IAV infections. This differential expression of FasL was found to be dependent on $p40_2$ expression within the LN. However the exact pathway through which $p40_2$ leads to FasL expression was not elucidated. One potential mechanism for $p40_2$-mediated induction of FasL expression is direct upregulation of the FasL gene. Future experiments aimed at

determining the activation of the FasL promoter during IAV infections will be critical to our understanding of IAV-associated LNDC and pDC FasL expression. Alternatively, another possibility for the differential LNDC FasL expression is the down regulation of proteins involved in posttranslational regulation FasL expression on the cell surface. Along these lines, IL-12 stimulation of ovarian cancers cells results in increased levels of FasL expression which was demonstrated to be dependent not on induction of the FasL gene but instead downregulation of matrix metalloproteases 3 and 7, which cleave FasL at the cell surface (201). Future experiments aimed at determining the levels of matrix metalloproteases, as well as other proteins capable of cleaving FasL from the cell surface such as ADAM10 (202), during lethal and sublethal dose IAV infections will be important in deciphering the mechanism of differential LNDC FasL expression.

Despite regulated FasL expression by both CD8α^+DC and pDC during IAV infections only pDC induce apoptosis of IAV-specific CD8 T cells directly ex vivo. One major difference between these cells during IAV infections is their overall accumulation within the LN and the putative effector to target ratio (i.e. DC:CD8 T cell). The ratio of pDC to IAV-specific CD8 T cells is approximately 9 times higher than CD8α^+DC to IAV-specific CD8 T cells suggesting that an increased effector to target ratio may play a critical role in the elimination of virus-specific CD8 T cells during IAV infections. Currently the location of this CD8 T cell apoptosis within the LN is unknown. pDC colocalize with CD8 T cells both within the T cell zones as well as at high endothelial venuoles (84, 164, 165), sites of T cell entry and egress suggesting that one or both of these sites may be involved. However CD8α^+DC only localize within the T cell zones of the LN. Furthermore, at either of these sites the local effector to target ratio (pDC:IAV-specific CD8 T cells) may in fact be higher than the overall effetor to target ratio for pDC allowing for increased chance of pDC:IAV-specific CD8 T cell interaction and therefore potentially increased FasL mediated apoptosis. Future studies aimed at determining the location of pDC induced IAV-specific CD8 T cell apoptosis as well as the local effector

to target ratio during lethal dose IAV infections will be important for our understanding of this detrimental interaction.

My results further demonstrate that the elimination of activated IAV-specific CD8 T cells by pDC only occurs in the absence of cognate viral antigen presentation (Figure 10). However, these experiments did not address how CD8 T cell cognate recognition of viral peptide:MHC I complexes protects them from FasL:Fas (DC:CD8 T cell) induced apoptosis. In this regard, studies have demonstrated that Fas ligation in the face of B cell antigen receptor stimulation does not lead to apoptosis (135). The pathway for this protection has not been fully elucidated. However, the current hypothesis is that antigen receptor stimulation leads to increased NFkB expression which subsequently protects the cells from apoptosis (134-137). In the future it will be important to determine the levels of NFκB in IAV-specific CD8 T cells incubated with pDC purified from lethal dose IAV infected mice in the presence or absence of IAV peptide. If NFκB expression correlates with protection the next step would be to determine which NFκB target(s) are responsible for protecting these cells from apoptosis. NFκB stimulates the transcription of a variety of molecules that can inhibit Fas mediated apoptosis. One potential target is cFLIP, which regulates Fas stimulated apoptosis by preventing the conversion of procaspase 8 into active caspase 8 (Figure 2B) (139). Other potential NFκB targets are the pro-survival molecules Bcl-2 and Bcl-X_L which both inhibit type II cell death by binding and blocking Apaf-1 and inhibiting apoptosis through caspase 9 (203). Finally, NFκB induces transcription of cIAP, which inhibits type I cell death by binding to and inhibiting the conversion of procaspase 3 and 7 into active caspase 3 and 7 (204). Any, or all of these NFκB induced genes could significantly alter the Fas mediated apoptosis pathway within activated CD8 T cells leading to protection from apoptotic Fas signaling.

Another intriguing hypothesis for antigen presentation mediated inhibition of LNDC FasL induced apoptosis of IAV-specific CD8 T cells is the location of Fas on the target cell surface. During antigen-specific DC:CD8 T cell interactions an immunological

synapse forms allowing for a stable long-term interaction (205). One protein critical for the formation and organization of the synapse in T cells, ezrin, also binds to the intracellular portion of Fas (206). Recent data have demonstrated that knockdown of ezrin in T cell lines enhanced FasL mediated apoptosis (207). Together, these data suggest the possibility that during an antigen-specific encounter with a viral antigen presenting DC, Fas may be sequestered into the synapse and away from FasL on the opposing cell, thereby preventing interaction between these molecules and the downstream induction of apoptosis. Currently, the location of FasL on the surface of DC during antigen presentation and therefore the ability to induce apoptosis, remains unknown. Therefore, in future studies it will be important to determine the distribution of FasL and Fas during DC and T cells interaction in the presence and absence of antigen presentation.

There are a variety of reasons for why $FasL^+$ pDC-mediated elimination of activated Fas^+ CD8 T cells in the absence of cognate antigen presentation would be beneficial to an animal. One possibility is that in the absence of infection, FasL expressing pDC may be capable of inducing apoptosis of aberrantly activated CD8 T cells. This could potentially help generate peripheral tolerance through elimination of self-reactive CD8 T cells and subsequently prevent autoimmune damage of pulmonary tissue following migration into the lungs. Therefore $FasL^+$ pDC may be gatekeepers allowing or denying access of CD8 T cells, activated within the LN, into the lungs similar to epithelial and endothelial cells inducing immune privilege through FasL within the eye (117). Given that $1LD_{50}$ IAV infections, like $10LD_{50}$ IAV infections, also have reduced IAV-specific CD8 T cell response compared to $0.1LD_{50}$ IAV infections (38) suggests the possibility that during these infection the potential elimination of IAV-specific CD8 T cells may in fact be beneficial for the host through the inhibition of a potentially over exuberant IAV-specific CD8 T cell responses and downstream CD8 T cell mediated immunopathology.

The data presented in this dissertation demonstrate that $p40_2$ controls FasL expression by LNDC during IAV infections. Importantly, chapter III also demonstrates that LNDC, that regulate FasL through $p40_2$, also express IL-12Rβ1. Given LNDC expression of IL-12Rβ1 and previous studies demonstrating that $p40_2$ signals through this receptor (156, 157) it was not surprising that mice deficient in IL-12Rβ1 have reduced LNDC FasL expression (Figure 16). However, how IL-12Rβ1 signals LNDC to upregulate FasL expression remains unknown. Interesting the cytoplasmic tail of IL-12Rβ1 is short and contains no phosphorylatable tyrosines (153). Despite the short cytoplasmic tail, TYK2 has been demonstrated to associate with IL-12Rβ1 (208) and may be capable of mediating $p40_2$ signaling. Importantly, results from Pahan and colleagues have demonstrated that $p40_2$ signals mediated through IL-12Rβ1 require both p38 MAPK and ERK as downstream signaling mediators (156). However the signaling intermediates in between the IL-12Rβ1/TYK2 and p38 MAPK/ERK remain unknown. One potential intermediate linking the IL-12Rβ1, TYK2 and the MAPK/ERK pathway is Rac1 which acts directly upstream of p38 MAPK/ERK (209, 210), as following TYK2 activation during type-1 IFN signaling VAV is phosphorylated and subsequently activates Rac1 which is subsequently able to activate p38 MAPK [INFR→tyk2→VAV→Rac1→p38 MAPK] (211). Future studies aimed at elucidating if Rac1 plays a role downstream of IL-12Rβ1 will be critical to our understanding of the $p40_2$:IL-12Rβ1 signaling pathway.

Another intriguing possibility for how IL-12Rβ1 mediates $p40_2$ signals despite a lack of phosphorylatable tyrosines is through pairing with a novel, as yet to be described signaling partner/receptor. One potential binding partner is IL-31Rα (also known as gp120 like protein) which is expressed on DC (212). The IL-31Rα has ~49% homology to IL-12Rβ1 (calculated by adding number of identical plus conserved amino acids and dividing by total) (Figure 27). The IL-31Rα contains phosphorylatable tyrosines and signals through STAT1, STAT3 and STAT5, which could potentially overcome the lack

of STAT signaling through IL-12Rβ1 alone. Importantly, activation of STAT1 has been demonstrated to activate the FasL gene (124), suggesting the possibility that IL-12Rβ1 and IL-31Rα may act in concert to mediate $p40_2$ signaling via STAT1 to drive upregulation of FasL expression through STAT1. During IL-12p70 signaling IL-12p40 binding to IL-12Rβ1 is thought to stabilize the interaction between IL-12p35 and IL-12Rβ2 (208). Interestingly, IL-12p70 is able to mediate weak signals through IL-12Rβ2 in the absence of IL-12Rβ1 (213). Given that loss of IL-12Rβ1 does not completely abrogate LNDC FasL, these data suggests that $p40_2$ may also be able to weakly signal through another receptor in the absence of IL-12Rβ1, possibly IL-31Rα, alone.

The data presented herein demonstrating $p40_2$ role in the regulation of LNDC FasL expression do not demonstrate whether or not $p40_2$ acts directly on LNDC to induce FasL expression, or if $p40_2$ acts indirectly through another cell or through another mediator to regulate FasL expression. To determine if $p40_2$ directly, or indirectly, induces FasL expression on LNDC, purified LNDC subsets from IL-12p40 and IL-12Rβ1 deficient mice could be incubated with either recombinant $p40_2$ or supernatant from cultured intact LN from lethal dose IAV infected wild-type mice and the levels of LNDC FasL determined after culture. If $p40_2$ upregulates FasL expression on purified LNDC subsets from IL-12p40 but not IL-12Rβ1 deficient DC these data would suggest that the $p40_2$-mediated control of FasL expression is direct. If, however, $p40_2$ cannot directly induce LNDC FasL expression and supernatants from lethal dose IAV infected LN (which contain $p40_2$ as well as all other cytokines induced during lethal dose IAV infection) induce FasL expression in DC from IL-12Rβ1$^{-/-}$ these data then would suggest that $p40_2$-mediated LNDC FasL expression is indirect and requires an additional cytokine.

Herein we further report a novel rDC-LNDC cross-talk mediated by differential production of $p40_2$ during lethal and sublethal dose IAV infections. Importantly, in chapter IV we found that rDC-LNDC cross-talk mediated by differential $p40_2$ expression

is not unique to IAV infections and can also be mediated by intranasal stimulation with a variety of TLR agonists. However, we did not directly determine if rDC-LNDC communication requires cell:cell contact or if this communication can instead can be mediated through paracrine mechanisms. One possibility is that $p40_2$ signaling to LNDC requires cell:cell contact to increase the local cytokine concentrations within the LN. In this regard, multiple investigators have found that cytokine signaling can require cell:cell contact for efficient cytokine signaling (214, 215). However, in this dissertation we found that incubation of intact LN with $p40_2$ was sufficient to induce LNDC FasL expression suggesting that paracrine cytokine signaling is sufficient to induce FasL expression. Consistent with this idea, a recent report has demonstrated that both IFNγ and IL-4 signaling within the LN does not require cell:cell conjugates and can instead mediate paracrine signals throughout the LN (216).

Chapter IV details the differential regulation of $p40_2$ expression and subsequent LNDC FasL expression following stimulation of rDC during lethal and sublethal dose IAV infections as well as during distinct intranasal TLR stimulation. However the mechanism for the differential regulation of $p40_2$ and subsequent LNDC FasL expression by IAV infections and TLR stimulation was not determined. One possibility for this difference is the selective induction IL-12p40 but not IL-12p40 binding partners, i.e. IL-23p19 and IL-12p35, which would potentially increase the chance for IL-12p40 to homodimerize. Interestingly preliminary data from our laboratory has demonstrated that prostaglandin E_2 is upregulated during IAV infections (Katewa and Legge, unpublished results). Importantly prostaglandin E_2 specifically induces IL-12p40 and not IL-12p35 (147) suggesting the possibility prostaglandin E_2 expression within the lungs during IAV infections may lead preferential IL-12p40 production from rDC increasing the likelihood for $p40_2$ formation. Another potential mechanism for differential $p40_2$ production during TLR3 and TLR4 stimulation is direct regulation of IL-12p40 homodimerization. In order for IL-12p40 to form a homodimer a chaperone protein, protein disulfide isomerase, is

required (148). Interestingly, studies have demonstrated that LPS signaling through TLR4 results in decreased levels of protein disulfide isomerase in macrophages (217). These data suggest that reduced protein disulfide isomerase, and therefore reduced ability to form $p40_2$, may be a potential mechanism for the decreased level of intracellular rDC $p40_2$ expression observed during intranasal LPS stimulation (Figure 26).

In addition to TLR stimulation during IAV infections, IAV also stimulate immune responses through another class of pattern recognition receptors within the cytoplasm, the Rig I like receptors (70, 77). During IAV infections, single stranded IAV RNA containing 5' triphosphates directly stimulates IL-6 production through RIG I in infected DC (70) and preliminary results from our laboratory have demonstrated that IL-6 is increased within the LN during lethal dose IAV infections (Legge and Braciale, unpublished results). Importantly IL-6 production is often associated with IL-12 production (218, 219) suggesting the possibility that RIG I signaling during IAV infection may directly lead to IL-12p40 production. If this is the case, these data would suggest that, given increased viral RNA within rDC during lethal dose IAV infection (VanOosten and Legge, manuscript submitted), triggering of RIG I within these may be a potential pathway for the increased intracellular $p40_2$ observed in rDC during lethal dose IAV infections.

Conclusions

Based on the data presented in chapters II-IV we propose the following model for the regulation of CD8 T cell responses during lethal dose IAV infections (Figure 28). During a lethal dose IAV infection, rDC migrate from the lungs to the regional LN translating the type of local inflammatory environment of the lungs into the LN through their production of cytokines, specifically $p40_2$. This $p40_2$ produced within the LN results in increased FasL expression on pDC. The consequence of FasL expression on pDC is the elimination of activated Fas^+ IAV-specific CD8 T cells. Importantly pDC, which are

incapable delivering a survival signal through presentation of IAV antigens via MHC I in vivo, instead only deliver an apoptotic signal through FasL. pDC elimination of IAV-specific CD8 T cells ultimately leads to an inability to control the infection resulting in death of the host. Conversely, during sublethal dose IAV infections rDC within the LN produce decreased $p40_2$. Consequently the reduction in $p40_2$ within the LN leads to decreased pDC FasL expression allowing activated Fas^+ IAV-specific CD8 T cells to escape pDC mediated FasL:Fas apoptosis. IAV-specific CD8 T cells then accumulate in the lungs in greater numbers allowing for clearance of the virus infection and survival of the host.

Taken together the results presented in this dissertation suggest that rDC mediated control of pDC FasL expression ultimately dictates the overall magnitude of the virus-specific CD8 T cell response generated during lethal dose IAV infections. These results highlight a novel interplay between rDC and pDC mediated by $p40_2$ production and suggest that rDC conditioned in different inflammatory environments can manipulate the ensuing adaptive immune response through $p40_2$ production. In the future manipulating the level of $p40_2$ or inhibiting the detrimental action of pDC through pDC depletion or by blockade of FasL, may represent a novel therapeutic approach to boost virus-specific CD8 T cell numbers in patients who are highly susceptible to IAV infections or during highly virulent IAV outbreaks.

Figure 27. Sequence alignment for IL-31Rα and IL-12Rβ1. Mouse IL-31R (top) and IL-12Rβ1 (bottom) were aligned using clustalw 2 software. (*) represents identical amino acids between the two sequences. (.) represents conserved aminio acid substitutions based on size and polarity. (:) represents semi-conserved amino acid substitutions. 115 out of 714 (16.1%) of amino acids are identical. 223 out of 714 are either conserved or semi-conserved (31.2).

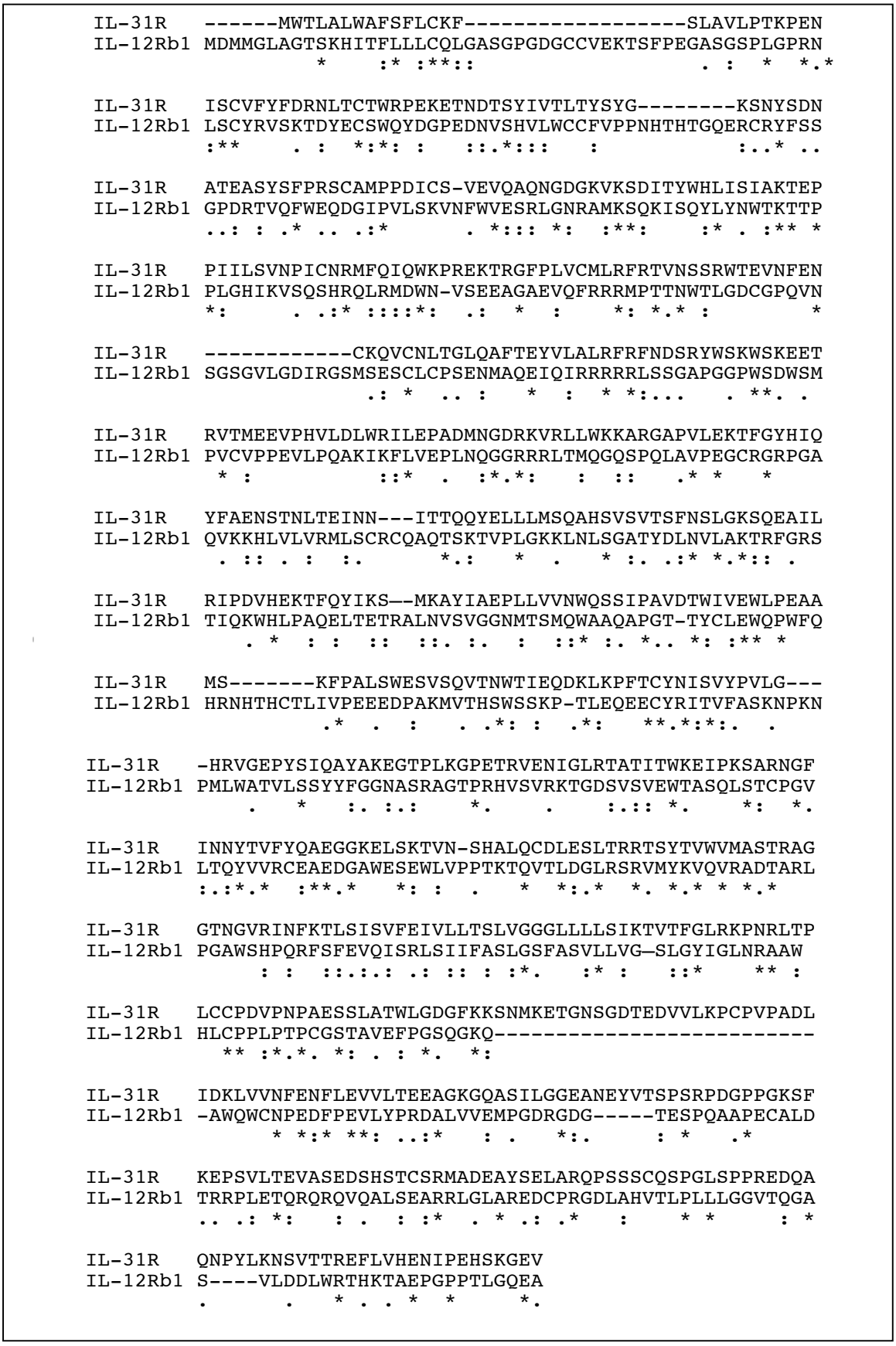
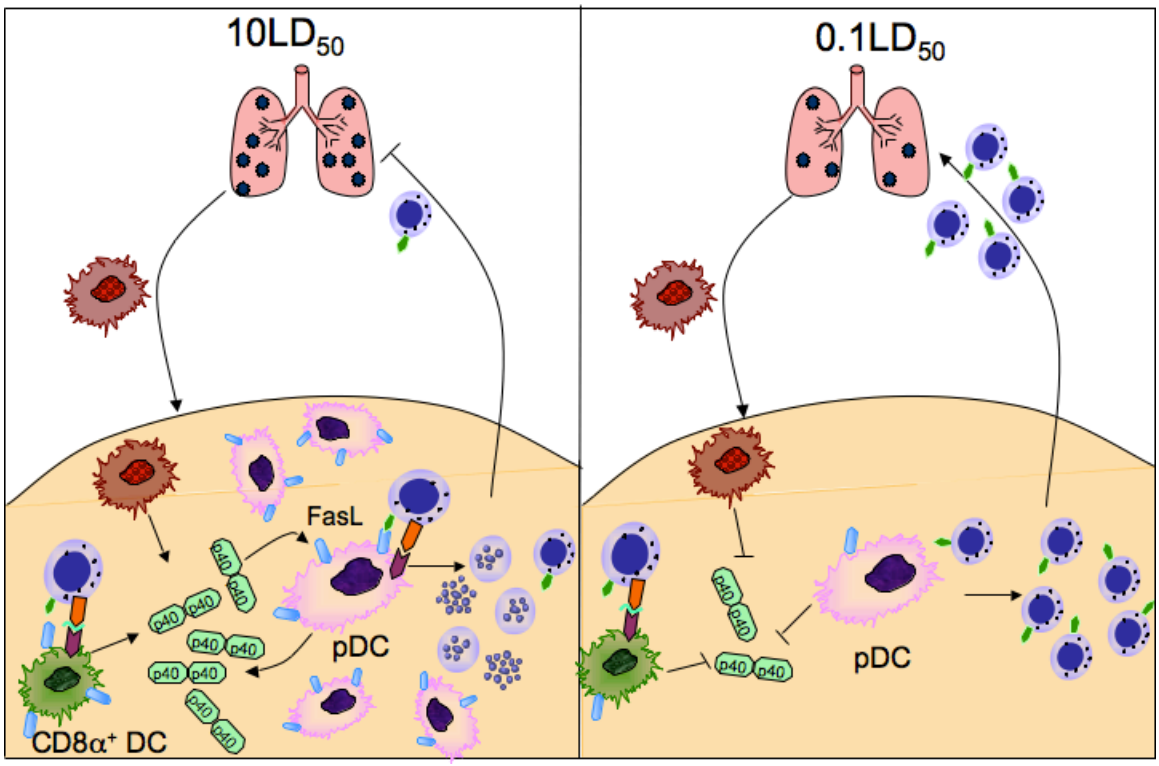

IL-31R ------MWTLALWAFSFLCKF-----------------SLAVLPTKPEN
IL-12Rb1 MDMMGLAGTSKHITFLLLCQLGASGPGDGCCVEKTSFPEGASGSPLGPRN
IL-31R ISCVFYFDRNLTCTWRPEKETNDTSYIVTLTYSYG--------KSNYSDN
IL-12Rb1 LSCYRVSKTDYECSWQYDGPEDNVSHVLWCCFVPPNHTHTGQERCRYFSS
IL-31R ATEASYSFPRSCAMPPDICS-VEVQAQNGDGKVKSDITYWHLISIAKTEP
IL-12Rb1 GPDRTVQFWEQDGIPVLSKVNFWVESRLGNRAMKSQKISQYLYNWTKTTP
IL-31R PIILSVNPICNRMFQIQWKPREKTRGFPLVCMLRFRTVNSSRWTEVNFEN
IL-12Rb1 PLGHIKVSQSHRQLRMDWN-VSEEAGAEVQFRRRMPTTNWTLGDCGPQVN
IL-31R ------------CKQVCNLTGLQAFTEYVLALRFRFNDSRYWSKWSKEET
IL-12Rb1 SGSGVLGDIRGSMSESCLCPSENMAQEIQIRRRRRLSSGAPGGPWSDWSM
IL-31R RVTMEEVPHVLDLWRILEPADMNGDRKVRLLWKKARGAPVLEKTFGYHIQ
IL-12Rb1 PVCVPPEVLPQAKIKFLVEPLNQGGRRRLTMQGQSPQLAVPEGCRGRPGA
IL-31R YFAENSTNLTEINN---ITTQQYELLLMSQAHSVSVTSFNSLGKSQEAIL
IL-12Rb1 QVKKHLVLVRMLSCRCQAQTSKTVPLGKKLNLSGATYDLNVLAKTRFGRS
IL-31R RIPDVHEKTFQYIKS--MKAYIAEPLLVVNWQSSIPAVDTWIVEWLPEAA
IL-12Rb1 TIQKWHLPAQELTETRALNVSVGGNMTSMQWAAQAPGT-TYCLEWQPWFQ
IL-31R MS-------KFPALSWESVSQVTNWTIEQDKLKPFTCYNISVYPVLG---
IL-12Rb1 HRNHTHCTLIVPEEEDPAKMVTHSWSSKP-TLEQEECYRITVFASKNPKN
IL-31R -HRVGEPYSIQAYAKEGTPLKGPETRVENIGLRTATITWKEIPKSARNGF
IL-12Rb1 PMLWATVLSSYYFGGNASRAGTPRHVSVRKTGDSVSVEWTASQLSTCPGV
IL-31R INNYTVFYQAEGGKELSKTVN-SHALQCDLESLTRRTSYTVWVMASTRAG
IL-12Rb1 LTQYVVRCEAEDGAWESEWLVPPTKTQVTLDGLRSRVMYKVQVRADTARL
IL-31R GTNGVRINFKTLSISVFEIVLLTSLVGGGLLLLSIKTVTFGLRKPNRLTP
IL-12Rb1 PGAWSHPQRFSFEVQISRLSIIFASLGSFASVLLVG—SLGYIGLNRAAW
IL-31R LCCPDVPNPAESSLATWLGDGFKKSNMKETGNSGDTEDVVLKPCPVPADL
IL-12Rb1 HLCPPLPTPCGSTAVEFPGSQGKQ--------------------------
IL-31R IDKLVVNFENFLEVVLTEEAGKGQASILGGEANEYVTSPSRPDGPPGKSF
IL-12Rb1 -AWQWCNPEDFPEVLYPRDALVVEMPGDRGDG-----TESPQAAPECALD
IL-31R KEPSVLTEVASEDSHSTCSRMADEAYSELARQPSSSCQSPGLSPPREDQA
IL-12Rb1 TRRPLETQRQRQVQALSEARRLGLAREDCPRGDLAHVTLPLLLGGVTQGA
IL-31R QNPYLKNSVTTREFLVHENIPEHSKGEV
IL-12Rb1 S----VLDDLWRTHKTAEPGPPTLGQEA

Figure 28. Model of rDC and pDC mediated control of the magnitude of IAV-specific CD8 T cells during lethal dose IAV infections. (left panel) During lethal dose IAV infections rDC migrate from the lungs into the draining LN, producing increased levels of $p40_2$ as well as controlling LNDC $p40_2$ production. $p40_2$ results in FasL expression on pDC, which accumulate at increased numbers within the LN and subsequently results in FasL:Fas mediated apoptosis of activated Fas^+ IAV-specific CD8 T cells. The loss of IAV-specific CD8 T cells within the LN results in a decreased CD8 T cell response in the lungs and death of the host. (right panel) During sublethal dose IAV infections rDC migrate from the lungs to the draining LN. However during sublethal dose IAV infections both rDC and LNDC produce decreased levels of $p40_2$. The decrease in $p40_2$ within the LN leads to decreased pDC FasL expression allowing activated Fas^+ IAV-specific CD8 T cells to escape pDC mediate FasL dependent apoptosis. This ultimately results in increased numbers of IAV-specific CD8 T cells that reach the lungs and resolution of the infection.


10LD50
0.1LD50
FasL
pDC
CD8α+ DC
p40
pDC

REFERENCES

1. Shortman, K., and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7:19-30.

2. Naik, S. H., P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, and K. Shortman. 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* 8:1217-1226.

3. Fogg, D. K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D. R. Littman, A. Cumano, and F. Geissmann. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311:83-87.

4. Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 164:2978-2986.

5. Vremec, D., and K. Shortman. 1997. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol* 159:565-573.

6. Bedoui, S., S. Prato, J. Mintern, T. Gebhardt, Y. Zhan, A. M. Lew, W. R. Heath, J. A. Villadangos, and E. Segura. 2009. Characterization of an immediate splenic precursor of CD8+ dendritic cells capable of inducing antiviral T cell responses. *J Immunol* 182:4200-4207.

7. Belz, G. T., C. M. Smith, D. Eichner, K. Shortman, G. Karupiah, F. R. Carbone, and W. R. Heath. 2004. Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses. *J Immunol* 172:1996-2000.

8. Pooley, J. L., W. R. Heath, and K. Shortman. 2001. Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. *J Immunol* 166:5327-5330.

9. den Haan, J. M., S. M. Lehar, and M. J. Bevan. 2000. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* 192:1685-1696.

10. Belz, G. T., S. Bedoui, F. Kupresanin, F. R. Carbone, and W. R. Heath. 2007. Minimal activation of memory CD8+ T cell by tissue-derived dendritic cells favors the stimulation of naive CD8+ T cells. *Nat Immunol* 8:1060-1066.

11. Belz, G. T., C. M. Smith, L. Kleinert, P. Reading, A. Brooks, K. Shortman, F. R. Carbone, and W. R. Heath. 2004. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. *Proc Natl Acad Sci U S A* 101:8670-8675.

12. Lee, H. K., M. Zamora, M. M. Linehan, N. Iijima, D. Gonzalez, A. Haberman, and A. Iwasaki. 2009. Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. *J Exp Med* 206:359-370.

13. Allan, R. S., J. Waithman, S. Bedoui, C. M. Jones, J. A. Villadangos, Y. Zhan, A. M. Lew, K. Shortman, W. R. Heath, and F. R. Carbone. 2006. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity* 25:153-162.

14. Carbone, F. R., G. T. Belz, and W. R. Heath. 2004. Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. *Trends Immunol* 25:655-658.

15. GeurtsvanKessel, C. H., M. A. Willart, L. S. van Rijt, F. Muskens, M. Kool, C. Baas, K. Thielemans, C. Bennett, B. E. Clausen, H. C. Hoogsteden, A. D. Osterhaus, G. F. Rimmelzwaan, and B. N. Lambrecht. 2008. Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J Exp Med* 205:1621-1634.

16. Belz, G. T., K. Shortman, M. J. Bevan, and W. R. Heath. 2005. CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol* 175:196-200.

17. Demedts, I. K., K. R. Bracke, T. Maes, G. F. Joos, and G. G. Brusselle. 2006. Different roles for human lung dendritic cell subsets in pulmonary immune defense mechanisms. *Am J Respir Cell Mol Biol* 35:387-393.

18. Toapanta, F. R., and T. M. Ross. 2009. Impaired immune responses in the lungs of aged mice following influenza infection. *Respir Res* 10:112.

19. Barchet, W., A. Krug, M. Cella, C. Newby, J. A. Fischer, A. Dzionek, A. Pekosz, and M. Colonna. 2005. Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways. *Eur J Immunol* 35:236-242.

20. Fitzgerald-Bocarsly, P. 1993. Human natural interferon-alpha producing cells. *Pharmacol Ther* 60:39-62.

21. Di Pucchio, T., B. Chatterjee, A. Smed-Sorensen, S. Clayton, A. Palazzo, M. Montes, Y. Xue, I. Mellman, J. Banchereau, and J. E. Connolly. 2008. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat Immunol* 9:551-557.

22. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.

23. Reis e Sousa, C. 2004. Activation of dendritic cells: translating innate into adaptive immunity. *Curr Opin Immunol* 16:21-25.

24. Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nat Rev Immunol* 6:476-483.

25. Chen, L., M. Arora, M. Yarlagadda, T. B. Oriss, N. Krishnamoorthy, A. Ray, and P. Ray. 2006. Distinct responses of lung and spleen dendritic cells to the TLR9 agonist CpG oligodeoxynucleotide. *J Immunol* 177:2373-2383.

26. Hammad, H., and B. N. Lambrecht. 2006. Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. *J Allergy Clin Immunol* 118:331-336.

27. Masten, B. J., G. K. Olson, C. A. Tarleton, C. Rund, M. Schuyler, R. Mehran, T. Archibeque, and M. F. Lipscomb. 2006. Characterization of myeloid and plasmacytoid dendritic cells in human lung. *J Immunol* 177:7784-7793.

28. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20:621-667.

29. Tough, D. F. 2004. Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation. *Leuk Lymphoma* 45:257-264.

30. Legge, K. L., and T. J. Braciale. 2003. Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity* 18:265-277.

31. Lopez, C. B., B. Moltedo, L. Alexopoulou, L. Bonifaz, R. A. Flavell, and T. M. Moran. 2004. TLR-independent induction of dendritic cell maturation and adaptive immunity by negative-strand RNA viruses. *J Immunol* 173:6882-6889.

32. Schlender, J., V. Hornung, S. Finke, M. Gunthner-Biller, S. Marozin, K. Brzozka, S. Moghim, S. Endres, G. Hartmann, and K. K. Conzelmann. 2005. Inhibition of toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. *J Virol* 79:5507-5515.

33. Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424:324-328.

34. Waithman, J., R. S. Allan, H. Kosaka, H. Azukizawa, K. Shortman, M. B. Lutz, W. R. Heath, F. R. Carbone, and G. T. Belz. 2007. Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. *J Immunol* 179:4535-4541.

35. Lambrecht, B. N., and H. Hammad. 2003. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat Rev Immunol* 3:994-1003.

36. Holt, P. G. 2005. Pulmonary dendritic cells in local immunity to inert and pathogenic antigens in the respiratory tract. *Proc Am Thorac Soc* 2:116-120.

37. Shibaki, A., and S. I. Katz. 2001. Activation through CD40 ligation induces functional Fas ligand expression by Langerhans cells. *Eur J Immunol* 31:3006-3015.

38. Legge, K. L., and T. J. Braciale. 2005. Lymph node dendritic cells control CD8+ T cell responses through regulated FasL expression. *Immunity* 23:649-659.

39. Suss, G., and K. Shortman. 1996. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J Exp Med* 183:1789-1796.

40. Chaperot, L., A. Blum, O. Manches, G. Lui, J. Angel, J. P. Molens, and J. Plumas. 2006. Virus or TLR agonists induce TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. *J Immunol* 176:248-255.

41. Fanger, N. A., C. R. Maliszewski, K. Schooley, and T. S. Griffith. 1999. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med* 190:1155-1164.

42. Taieb, J., N. Chaput, C. Menard, L. Apetoh, E. Ullrich, M. Bonmort, M. Pequignot, N. Casares, M. Terme, C. Flament, P. Opolon, Y. Lecluse, D. Metivier, E. Tomasello, E. Vivier, F. Ghiringhelli, F. Martin, D. Klatzmann, T. Poynard, T. Tursz, G. Raposo, H. Yagita, B. Ryffel, G. Kroemer, and L. Zitvogel. 2006. A novel dendritic cell subset involved in tumor immunosurveillance. *Nat Med* 12:214-219.

43. Mellor, A. L., and D. H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762-774.

44. Lamb, R. A., and P. W. Choppin. 1983. The gene structure and replication of influenza virus. *Annu Rev Biochem* 52:467-506.

45. Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 7:1306-1312.

46. Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 79:2814-2822.

47. Tumpey, T. M., and J. A. Belser. 2009. Resurrected pandemic influenza viruses. *Annu Rev Microbiol* 63:79-98.

48. Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev* 56:152-179.

49. Thomas, P. G., R. Keating, D. J. Hulse-Post, and P. C. Doherty. 2006. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 12:48-54.

50. Senanayake, S. N. 2009. A pandemic that's not bird flu? Pigs might fly. *Med J Aust* 191:38-40.

51. McGill, J., J. W. Heusel, and K. L. Legge. 2009. Innate immune control and regulation of influenza virus infections. *J Leukoc Biol* 86:803-812.

52. Grayson, M. H., and M. J. Holtzman. 2007. Emerging role of dendritic cells in respiratory viral infection. *J Mol Med* 85:1057-1068.

53. Hussell, T., and M. M. Cavanagh. 2009. The innate immune rheostat: influence on lung inflammatory disease and secondary bacterial pneumonia. *Biochem Soc Trans* 37:811-813.

54. Kuiken, T., and J. K. Taubenberger. 2008. Pathology of human influenza revisited. *Vaccine* 26 Suppl 4:D59-66.

55. Beadling, C., and M. K. Slifka. 2004. How do viral infections predispose patients to bacterial infections? *Curr Opin Infect Dis* 17:185-191.

56. Hers, J. F., J. Mudler, N. Masurel, K. L. vd, and D. A. Tyrrell. 1962. Studies on the pathogenesis of influenza virus pneumonia in mice. *J Pathol Bacteriol* 83:207-217.

57. Brown, D. M., E. Roman, and S. L. Swain. 2004. CD4 T cell responses to influenza infection. *Semin Immunol* 16:171-177.

58. Gerhard, W., K. Mozdzanowska, M. Furchner, G. Washko, and K. Maiese. 1997. Role of the B-cell response in recovery of mice from primary influenza virus infection. *Immunol Rev* 159:95-103.

59. Price, G. E., A. Gaszewska-Mastarlarz, and D. Moskophidis. 2000. The role of alpha/beta and gamma interferons in development of immunity to influenza A virus in mice. *J Virol* 74:3996-4003.

60. Sieczkarski, S. B., and G. R. Whittaker. 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. *J Virol* 76:10455-10464.

61. Lakadamyali, M., M. J. Rust, and X. Zhuang. 2004. Endocytosis of influenza viruses. *Microbes Infect* 6:929-936.

62. Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* 69:531-569.

63. Skehel, J. J., and D. C. Wiley. 1995. Influenza viruses and cell membranes. *Am J Respir Crit Care Med* 152:S13-15.

64. O'Neill, R. E., R. Jaskunas, G. Blobel, P. Palese, and J. Moroianu. 1995. Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. *J Biol Chem* 270:22701-22704.

65. Blaas, D., E. Patzelt, and E. Kuechler. 1982. Identification of the cap binding protein of influenza virus. *Nucleic Acids Res* 10:4803-4812.

66. Blaas, D., E. Patzelt, and E. Kuechler. 1982. Cap-recognizing protein of influenza virus. *Virology* 116:339-348.

67. Zheng, H., H. A. Lee, P. Palese, and A. Garcia-Sastre. 1999. Influenza A virus RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. *J Virol* 73:5240-5243.

68. Aragon, T., S. de la Luna, I. Novoa, L. Carrasco, J. Ortin, and A. Nieto. 2000. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. *Mol Cell Biol* 20:6259-6268.

69. Noda, T., H. Sagara, A. Yen, A. Takada, H. Kida, R. H. Cheng, and Y. Kawaoka. 2006. Architecture of ribonucleoprotein complexes in influenza A virus particles. *Nature* 439:490-492.

70. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314:997-1001.

71. Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre. 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* 74:7989-7996.

72. Kochs, G., A. Garcia-Sastre, and L. Martinez-Sobrido. 2007. Multiple anti-interferon actions of the influenza A virus NS1 protein. *J Virol* 81:7011-7021.

73. Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252:324-330.

74. Fernandez-Sesma, A., S. Marukian, B. J. Ebersole, D. Kaminski, M. S. Park, T. Yuen, S. C. Sealfon, A. Garcia-Sastre, and T. M. Moran. 2006. Influenza virus evades innate and adaptive immunity via the NS1 protein. *J Virol* 80:6295-6304.

75. Donelan, N. R., C. F. Basler, and A. Garcia-Sastre. 2003. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. *J Virol* 77:13257-13266.

76. Le Goffic, R., J. Pothlichet, D. Vitour, T. Fujita, E. Meurs, M. Chignard, and M. Si-Tahar. 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *J Immunol* 178:3368-3372.

77. Loo, Y. M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. Garcia-Sastre, M. G. Katze, and M. Gale, Jr. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *J Virol* 82:335-345.

78. Kanneganti, T. D., M. Body-Malapel, A. Amer, J. H. Park, J. Whitfield, L. Franchi, Z. F. Taraporewala, D. Miller, J. T. Patton, N. Inohara, and G. Nunez. 2006. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* 281:36560-36568.

79. Ichinohe, T., H. K. Lee, Y. Ogura, R. Flavell, and A. Iwasaki. 2009. Inflammasome recognition of influenza virus is essential for adaptive immune responses. *J Exp Med* 206:79-87.

80. Ichinohe, T., I. K. Pang, and A. Iwasaki. 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol* 11:404-410.

81. Bonjardim, C. A. 2005. Interferons (IFNs) are key cytokines in both innate and adaptive antiviral immune responses--and viruses counteract IFN action. *Microbes Infect* 7:569-578.

82. Lenschow, D. J., C. Lai, N. Frias-Staheli, N. V. Giannakopoulos, A. Lutz, T. Wolff, A. Osiak, B. Levine, R. E. Schmidt, A. Garcia-Sastre, D. A. Leib, A. Pekosz, K. P. Knobeloch, I. Horak, and H. W. t. Virgin. 2007. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci U S A* 104:1371-1376.

83. Koerner, I., G. Kochs, U. Kalinke, S. Weiss, and P. Staeheli. 2007. Protective role of beta interferon in host defense against influenza A virus. *J Virol* 81:2025-2030.

84. Barchet, W., A. Blasius, M. Cella, and M. Colonna. 2005. Plasmacytoid dendritic cells: in search of their niche in immune responses. *Immunol Res* 32:75-83.

85. Tumpey, T. M., A. Garcia-Sastre, J. K. Taubenberger, P. Palese, D. E. Swayne, M. J. Pantin-Jackwood, S. Schultz-Cherry, A. Solorzano, N. Van Rooijen, J. M. Katz, and C. F. Basler. 2005. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J Virol* 79:14933-14944.

86. Kumagai, Y., O. Takeuchi, H. Kato, H. Kumar, K. Matsui, E. Morii, K. Aozasa, T. Kawai, and S. Akira. 2007. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27:240-252.

87. Draghi, M., A. Pashine, B. Sanjanwala, K. Gendzekhadze, C. Cantoni, D. Cosman, A. Moretta, N. M. Valiante, and P. Parham. 2007. NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection. *J Immunol* 178:2688-2698.

88. Gazit, R., R. Gruda, M. Elboim, T. I. Arnon, G. Katz, H. Achdout, J. Hanna, U. Qimron, G. Landau, E. Greenbaum, Z. Zakay-Rones, A. Porgador, and O. Mandelboim. 2006. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nat Immunol* 7:517-523.

89. Bhardwaj, N., A. Bender, N. Gonzalez, L. K. Bui, M. C. Garrett, and R. M. Steinman. 1994. Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J Clin Invest* 94:797-807.

90. Hamilton-Easton, A., and M. Eichelberger. 1995. Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice. *J Virol* 69:6359-6366.

91. Nonacs, R., C. Humborg, J. P. Tam, and R. M. Steinman. 1992. Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J Exp Med* 176:519-529.

92. Mori, I., T. Komatsu, K. Takeuchi, K. Nakakuki, M. Sudo, and Y. Kimura. 1995. In vivo induction of apoptosis by influenza virus. *J Gen Virol* 76 (Pt 11):2869-2873.

93. Albert, M. L., S. F. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* 188:1359-1368.

94. Albert, M. L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392:86-89.

95. Sauter, B., M. L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 191:423-434.

96. Ballesteros-Tato, A., B. Leon, F. E. Lund, and T. D. Randall. Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. *Nat Immunol*.

97. Kim, T. S., and T. J. Braciale. 2009. Respiratory dendritic cell subsets differ in their capacity to support the induction of virus-specific cytotoxic CD8+ T cell responses. *PLoS ONE* 4:e4204.

98. Norbury, C. C., D. Malide, J. S. Gibbs, J. R. Bennink, and J. W. Yewdell. 2002. Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. *Nat Immunol* 3:265-271.

99. Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P. C. Doherty. 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. *J Exp Med* 174:875-880.

100. Topham, D. J., R. A. Tripp, and P. C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J Immunol* 159:5197-5200.

101. Graham, M. B., and T. J. Braciale. 1997. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J Exp Med* 186:2063-2068.

102. Brincks, E. L., A. Katewa, T. A. Kucaba, T. S. Griffith, and K. L. Legge. 2008. CD8 T cells utilize TRAIL to control influenza virus infection. *J Immunol* 181:4918-4925.

103. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature* 267:354-356.

104. Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink. 1977. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J Exp Med* 145:557-568.

105. Braciale, T. J. 1977. Immunologic recognition of influenza virus-infected cells. I. Generation of a virus-strain specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell Immunol* 33:423-436.

106. Gerhard, W. 2001. The role of the antibody response in influenza virus infection. *Curr Top Microbiol Immunol* 260:171-190.

107. Tripp, R. A., S. R. Sarawar, and P. C. Doherty. 1995. Characteristics of the influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2lAb gene. *J Immunol* 155:2955-2959.

108. Swain, S. L., R. W. Dutton, and D. L. Woodland. 2004. T cell responses to influenza virus infection: effector and memory cells. *Viral Immunol* 17:197-209.

109. Swain, S. L., J. N. Agrewala, D. M. Brown, D. M. Jelley-Gibbs, S. Golech, G. Huston, S. C. Jones, C. Kamperschroer, W. H. Lee, K. K. McKinstry, E. Roman, T. Strutt, and N. P. Weng. 2006. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol Rev* 211:8-22.

110. McGill, J., N. Van Rooijen, and K. L. Legge. IL-15 trans-presentation by pulmonary dendritic cells promotes effector CD8 T cell survival during influenza virus infection. *J Exp Med* 207:521-534.

111. McGill, J., N. Van Rooijen, and K. L. Legge. 2008. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *J Exp Med* 205:1635-1646.

112. Moyron-Quiroz, J., J. Rangel-Moreno, D. M. Carragher, and T. D. Randall. 2007. The function of local lymphoid tissues in pulmonary immune responses. *Adv Exp Med Biol* 590:55-68.

113. Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund, and T. D. Randall. 2004. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med* 10:927-934.

114. GeurtsvanKessel, C. H., M. A. Willart, I. M. Bergen, L. S. van Rijt, F. Muskens, D. Elewaut, A. D. Osterhaus, R. Hendriks, G. F. Rimmelzwaan, and B. N. Lambrecht. 2009. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. *J Exp Med* 206:2339-2349.

115. Xu, L., H. Yoon, M. Q. Zhao, J. Liu, C. V. Ramana, and R. I. Enelow. 2004. Cutting edge: pulmonary immunopathology mediated by antigen-specific expression of TNF-alpha by antiviral CD8+ T cells. *J Immunol* 173:721-725.

116. Takahashi, T., M. Tanaka, C. I. Brannan, N. A. Jenkins, N. G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76:969-976.

117. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270:1189-1192.

118. Griffith, T. S., J. M. Herndon, J. Lima, M. Kahn, and T. A. Ferguson. 1995. The immune response and the eye. TCR alpha-chain related molecules regulate the systemic immunity to antigen presented in the eye. *Int Immunol* 7:1617-1625.

119. Rathmell, J. C., and C. C. Goodnow. 1995. Autoimmunity. The Fas track. *Curr Biol* 5:1218-1221.

120. Rathmell, J. C., M. P. Cooke, W. Y. Ho, J. Grein, S. E. Townsend, M. M. Davis, and C. C. Goodnow. 1995. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 376:181-184.

121. Aguilar-Torrentera, F., and Y. Carlier. 2001. Immunological factors governing resistance and susceptibility of mice to Leishmania major infection. *Rev Latinoam Microbiol* 43:135-142.

122. Turner, J., C. D. D'Souza, J. E. Pearl, P. Marietta, M. Noel, A. A. Frank, R. Appelberg, I. M. Orme, and A. M. Cooper. 2001. CD8- and CD95/95L-dependent mechanisms of resistance in mice with chronic pulmonary tuberculosis. *Am J Respir Cell Mol Biol* 24:203-209.

123. Lee, H. O., and T. A. Ferguson. 2003. Biology of FasL. *Cytokine Growth Factor Rev* 14:325-335.

124. Xu, X., X. Y. Fu, J. Plate, and A. S. Chong. 1998. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 58:2832-2837.

125. Voss, M., M. Lettau, M. Paulsen, and O. Janssen. 2008. Posttranslational regulation of Fas ligand function. *Cell Commun Signal* 6:11.

126. Lettau, M., M. Paulsen, D. Kabelitz, and O. Janssen. 2008. Storage, expression and function of Fas ligand, the key death factor of immune cells. *Curr Med Chem* 15:1684-1696.

127. Brunner, T., C. Wasem, R. Torgler, I. Cima, S. Jakob, and N. Corazza. 2003. Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. *Semin Immunol* 15:167-176.

128. Andreola, G., L. Rivoltini, C. Castelli, V. Huber, P. Perego, P. Deho, P. Squarcina, P. Accornero, F. Lozupone, L. Lugini, A. Stringaro, A. Molinari, G. Arancia, M. Gentile, G. Parmiani, and S. Fais. 2002. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med* 195:1303-1316.

129. Monleon, I., M. J. Martinez-Lorenzo, L. Monteagudo, P. Lasierra, M. Taules, M. Iturralde, A. Pineiro, L. Larrad, M. A. Alava, J. Naval, and A. Anel. 2001. Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J Immunol* 167:6736-6744.

130. LA, O. R., L. Tai, L. Lee, E. A. Kruse, S. Grabow, W. D. Fairlie, N. M. Haynes, D. M. Tarlinton, J. G. Zhang, G. T. Belz, M. J. Smyth, P. Bouillet, L. Robb, and A. Strasser. 2009. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 461:659-663.

131. Peter, M. E., R. C. Budd, J. Desbarats, S. M. Hedrick, A. O. Hueber, M. K. Newell, L. B. Owen, R. M. Pope, J. Tschopp, H. Wajant, D. Wallach, R. H. Wiltrout, M. Zornig, and D. H. Lynch. 2007. The CD95 receptor: apoptosis revisited. *Cell* 129:447-450.

132. Siegel, R. M., F. K. Chan, H. J. Chun, and M. J. Lenardo. 2000. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 1:469-474.

133. Strasser, A., P. J. Jost, and S. Nagata. 2009. The many roles of FAS receptor signaling in the immune system. *Immunity* 30:180-192.

134. Dudley, E., F. Hornung, L. Zheng, D. Scherer, D. Ballard, and M. Lenardo. 1999. NF-kappaB regulates Fas/APO-1/CD95- and TCR- mediated apoptosis of T lymphocytes. *Eur J Immunol* 29:878-886.

135. Carey, G. B., D. Donjerkovic, C. M. Mueller, S. Liu, J. A. Hinshaw, L. Tonnetti, W. Davidson, and D. W. Scott. 2000. B-cell receptor and Fas-mediated signals for life and death. *Immunol Rev* 176:105-115.

136. Lu, B., L. Wang, D. Medan, D. Toledo, C. Huang, F. Chen, X. Shi, and Y. Rojanasakul. 2002. Regulation of Fas (CD95)-induced apoptosis by nuclear factor-kappaB and tumor necrosis factor-alpha in macrophages. *Am J Physiol Cell Physiol* 283:C831-838.

137. Schram, B. R., and T. L. Rothstein. 2003. NF-kappa B is required for surface Ig-induced Fas resistance in B cells. *J Immunol* 170:3118-3124.

138. Maksimow, M., T. S. Soderstrom, S. Jalkanen, J. E. Eriksson, and A. Hanninen. 2006. Fas costimulation of naive CD4 T cells is controlled by NF-kappaB signaling and caspase activity. *J Leukoc Biol* 79:369-377.

139. Budd, R. C., W. C. Yeh, and J. Tschopp. 2006. cFLIP regulation of lymphocyte activation and development. *Nat Rev Immunol* 6:196-204.

140. Newton, K., A. W. Harris, M. L. Bath, K. G. Smith, and A. Strasser. 1998. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J* 17:706-718.

141. Kang, T. B., T. Ben-Moshe, E. E. Varfolomeev, Y. Pewzner-Jung, N. Yogev, A. Jurewicz, A. Waisman, O. Brenner, R. Haffner, E. Gustafsson, P. Ramakrishnan, T. Lapidot, and D. Wallach. 2004. Caspase-8 serves both apoptotic and nonapoptotic roles. *J Immunol* 173:2976-2984.

142. Holscher, C. 2004. The power of combinatorial immunology: IL-12 and IL-12-related dimeric cytokines in infectious diseases. *Med Microbiol Immunol* 193:1-17.

143. Noben-Trauth, N., P. A. Schweitzer, K. R. Johnson, S. F. Wolf, B. B. Knowles, and L. D. Shultz. 1996. The interleukin-12 beta subunit (p40) maps to mouse chromosome 11. *Mamm Genome* 7:392.

144. Tone, Y., S. A. Thompson, J. M. Babik, K. F. Nolan, M. Tone, C. Raven, and H. Waldmann. 1996. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. *Eur J Immunol* 26:1222-1227.

145. Heinzel, F. P., A. M. Hujer, F. N. Ahmed, and R. M. Rerko. 1997. In vivo production and function of IL-12 p40 homodimers. *J Immunol* 158:4381-4388.

146. Bode, K. A., F. Schmitz, L. Vargas, K. Heeg, and A. H. Dalpke. 2009. Kinetic of RelA activation controls magnitude of TLR-mediated IL-12p40 induction. *J Immunol* 182:2176-2184.

147. Kalinski, P., P. L. Vieira, J. H. Schuitemaker, E. C. de Jong, and M. L. Kapsenberg. 2001. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* 97:3466-3469.

148. Martens, E., I. Alloza, C. J. Scott, A. Billiau, and K. Vandenbroeck. 2000. Protein disulfide isomerase-mediated cell-free assembly of recombinant interleukin-12 p40 homodimers. *Eur J Biochem* 267:6679-6683.

149. Germann, T., E. Rude, F. Mattner, and M. K. Gately. 1995. The IL-12 p40 homodimer as a specific antagonist of the IL-12 heterodimer. *Immunol Today* 16:500-501.

150. Gately, M. K., D. M. Carvajal, S. E. Connaughton, S. Gillessen, R. R. Warrier, K. D. Kolinsky, V. L. Wilkinson, C. M. Dwyer, G. F. Higgins, Jr., F. J. Podlaski, D. A. Faherty, P. C. Familletti, A. S. Stern, and D. H. Presky. 1996. Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. *Ann N Y Acad Sci* 795:1-12.

151. Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol* 25:200-206.

152. Ehlers, S., J. Lehmann, H. Mossmann, G. Alber, and C. Holscher. 2005. Interleukin-12p40 mediates transient protection against Mycobacterium avium infection in the absence of interleukin-12. *Immunobiology* 210:217-227.

153. Holscher, C., R. A. Atkinson, B. Arendse, N. Brown, E. Myburgh, G. Alber, and F. Brombacher. 2001. A protective and agonistic function of IL-12p40 in mycobacterial infection. *J Immunol* 167:6957-6966.

154. Russell, T. D., Q. Yan, G. Fan, A. P. Khalifah, D. K. Bishop, S. L. Brody, and M. J. Walter. 2003. IL-12 p40 homodimer-dependent macrophage chemotaxis and respiratory viral inflammation are mediated through IL-12 receptor beta 1. *J Immunol* 171:6866-6874.

155. Khader, S. A., S. Partida-Sanchez, G. Bell, D. M. Jelley-Gibbs, S. Swain, J. E. Pearl, N. Ghilardi, F. J. Desauvage, F. E. Lund, and A. M. Cooper. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp Med* 203:1805-1815.

156. Jana, M., S. Dasgupta, U. Pal, and K. Pahan. 2009. IL-12 p40 homodimer, the so-called biologically inactive molecule, induces nitric oxide synthase in microglia via IL-12R beta 1. *Glia* 57:1553-1565.

157. Jana, M., and K. Pahan. 2009. IL-12 p40 homodimer, but not IL-12 p70, induces the expression of IL-16 in microglia and macrophages. *Mol Immunol* 46:773-783.

158. Jana, M., and K. Pahan. 2009. Induction of lymphotoxin-alpha by interleukin-12 p40 homodimer, the so-called biologically inactive molecule, but not IL-12 p70. *Immunology* 127:312-325.

159. Brahmachari, S., and K. Pahan. 2009. Suppression of regulatory T cells by IL-12p40 homodimer via nitric oxide. *J Immunol* 183:2045-2058.

160. Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196:1645-1651.

161. Maldonado-Lopez, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* 189:587-592.

162. Maldonado-Lopez, R., T. De Smedt, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, C. R. Maliszewski, and M. Moser. 1999. Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo. *J Leukoc Biol* 66:242-246.

163. Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, and K. Shortman. 2001. The dendritic cell populations of mouse lymph nodes. *J Immunol* 167:741-748.

164. Yoneyama, H., K. Matsuno, Y. Zhang, T. Nishiwaki, M. Kitabatake, S. Ueha, S. Narumi, S. Morikawa, T. Ezaki, B. Lu, C. Gerard, S. Ishikawa, and K. Matsushima. 2004. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int Immunol* 16:915-928.

165. Krug, A., R. Uppaluri, F. Facchetti, B. G. Dorner, K. C. Sheehan, R. D. Schreiber, M. Cella, and M. Colonna. 2002. IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation. *J Immunol* 169:6079-6083.

166. Wolf, A. I., D. Buehler, S. E. Hensley, L. L. Cavanagh, E. J. Wherry, P. Kastner, S. Chan, and W. Weninger. 2009. Plasmacytoid dendritic cells are dispensable during primary influenza virus infection. *J Immunol* 182:871-879.

167. Fonteneau, J. F., M. Gilliet, M. Larsson, I. Dasilva, C. Munz, Y. J. Liu, and N. Bhardwaj. 2003. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. *Blood* 101:3520-3526.

168. Matsue, H., K. Matsue, M. Walters, K. Okumura, H. Yagita, and A. Takashima. 1999. Induction of antigen-specific immunosuppression by CD95L cDNA-transfected 'killer' dendritic cells. *Nat Med* 5:930-937.

169. Wolfe, T., C. Asseman, A. Hughes, H. Matsue, A. Takashima, and M. G. von Herrath. 2002. Reduction of antiviral CD8 lymphocytes in vivo with dendritic cells expressing Fas ligand-increased survival of viral (lymphocytic choriomeningitis virus) central nervous system infection. *J Immunol* 169:4867-4872.

170. Kim, S. H., N. Bianco, R. Menon, E. R. Lechman, W. J. Shufesky, A. E. Morelli, and P. D. Robbins. 2006. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther* 13:289-300.

171. de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. V. Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. V. Cam, Q. Ha do, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 12:1203-1207.

172. Tumpey, T. M., X. Lu, T. Morken, S. R. Zaki, and J. M. Katz. 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. *J Virol* 74:6105-6116.

173. Perrone, L. A., J. K. Plowden, A. Garcia-Sastre, J. M. Katz, and T. M. Tumpey. 2008. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS Pathog* 4:e1000115.

174. Baskin, C. R., H. Bielefeldt-Ohmann, T. M. Tumpey, P. J. Sabourin, J. P. Long, A. Garcia-Sastre, A. E. Tolnay, R. Albrecht, J. A. Pyles, P. H. Olson, L. D. Aicher, E. R. Rosenzweig, K. Murali-Krishna, E. A. Clark, M. S. Kotur, J. L. Fornek, S. Proll, R. E. Palermo, C. L. Sabourin, and M. G. Katze. 2009. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. *Proc Natl Acad Sci U S A* 106:3455-3460.

175. Chun, D. H., K. C. Jung, W. S. Park, I. S. Lee, W. J. Choi, C. J. Kim, S. H. Park, and Y. Bae. 2000. Costimulatory effect of Fas in mouse T lymphocytes. *Mol Cells* 10:642-646.

176. Maksimow, M., M. Santanen, S. Jalkanen, and A. Hanninen. 2003. Responding naive T cells differ in their sensitivity to Fas engagement: early death of many T cells is compensated by costimulation of surviving T cells. *Blood* 101:4022-4028.

177. Buonocore, S., N. O. Haddou, F. Moore, S. Florquin, F. Paulart, C. Heirman, K. Thielemans, M. Goldman, and V. Flamand. 2008. Neutrophil-dependent tumor rejection and priming of tumoricidal CD8+ T cell response induced by dendritic cells overexpressing CD95L. *J Leukoc Biol* 84:713-720.

178. Kusuhara, M., K. Matsue, D. Edelbaum, J. Loftus, A. Takashima, and H. Matsue. 2002. Killing of naive T cells by CD95L-transfected dendritic cells (DC): in vivo study using killer DC-DC hybrids and CD4(+) T cells from DO11.10 mice. *Eur J Immunol* 32:1035-1043.

179. Sanmiguel, J. C., F. Olaru, J. Li, E. Mohr, and L. E. Jensen. 2009. Interleukin-1 regulates keratinocyte expression of T cell targeting chemokines through interleukin-1 receptor associated kinase-1 (IRAK1) dependent and independent pathways. *Cell Signal* 21:685-694.

180. Wu, C., J. Ferrante, M. K. Gately, and J. Magram. 1997. Characterization of IL-12 receptor beta1 chain (IL-12Rbeta1)-deficient mice: IL-12Rbeta1 is an essential component of the functional mouse IL-12 receptor. *J Immunol* 159:1658-1665.

181. Presky, D. H., L. J. Minetti, S. Gillessen, U. Gubler, R. Chizzonite, A. S. Stern, and M. K. Gately. 1996. Evidence for multiple sites of interaction between IL-12 and its receptor. *Ann N Y Acad Sci* 795:390-393.

182. Jana, M., S. Dasgupta, R. N. Saha, X. Liu, and K. Pahan. 2003. Induction of tumor necrosis factor-alpha (TNF-alpha) by interleukin-12 p40 monomer and homodimer in microglia and macrophages. *J Neurochem* 86:519-528.

183. Mondal, S., A. Roy, and K. Pahan. 2009. Functional blocking monoclonal antibodies against IL-12p40 homodimer inhibit adoptive transfer of experimental allergic encephalomyelitis. *J Immunol* 182:5013-5023.

184. Pirhonen, J., S. Matikainen, and I. Julkunen. 2002. Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. *J Immunol* 169:5673-5678.

185. Novelli, F., and J. L. Casanova. 2004. The role of IL-12, IL-23 and IFN-gamma in immunity to viruses. *Cytokine Growth Factor Rev* 15:367-377.

186. van der Sluijs, K. F., L. J. van Elden, Y. Xiao, R. Arens, M. Nijhuis, R. Schuurman, S. Florquin, H. M. Jansen, R. Lutter, and T. van der Poll. 2006. IL-12 deficiency transiently improves viral clearance during the late phase of respiratory tract infection with influenza A virus in mice. *Antiviral Res* 70:75-84.

187. Kim, T. S., M. M. Hufford, J. Sun, Y. X. Fu, and T. J. Braciale. 2010. Antigen persistence and the control of local T cell memory by migrant respiratory dendritic cells after acute virus infection. *J Exp Med* 207:1161-1172.

188. Bosio, C. M., A. W. Goodyear, and S. W. Dow. 2005. Early interaction of Yersinia pestis with APCs in the lung. *J Immunol* 175:6750-6756.

189. Van Rooijen, N., and A. Sanders. 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174:83-93.

190. Cowdery, J. S., N. J. Boerth, L. A. Norian, P. S. Myung, and G. A. Koretzky. 1999. Differential regulation of the IL-12 p40 promoter and of p40 secretion by CpG DNA and lipopolysaccharide. *J Immunol* 162:6770-6775.

191. Choi, C., J. Y. Park, J. Lee, J. H. Lim, E. C. Shin, Y. S. Ahn, C. H. Kim, S. J. Kim, J. D. Kim, I. S. Choi, and I. H. Choi. 1999. Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma. *J Immunol* 162:1889-1895.

192. Ortiz-Arduan, A., T. M. Danoff, R. Kalluri, S. Gonzalez-Cuadrado, S. L. Karp, K. Elkon, J. Egido, and E. G. Neilson. 1996. Regulation of Fas and Fas ligand expression in cultured murine renal cells and in the kidney during endotoxemia. *Am J Physiol* 271:F1193-1201.

193. Yao, P. L., Y. C. Lin, P. Sawhney, and J. H. Richburg. 2007. Transcriptional regulation of FasL expression and participation of sTNF-alpha in response to sertoli cell injury. *J Biol Chem* 282:5420-5431.

194. Kitchen, S. G., N. R. Jones, S. LaForge, J. K. Whitmire, B. A. Vu, Z. Galic, D. G. Brooks, S. J. Brown, C. M. Kitchen, and J. A. Zack. 2004. CD4 on CD8(+) T cells directly enhances effector function and is a target for HIV infection. *Proc Natl Acad Sci U S A* 101:8727-8732.

195. Kawai, T., and S. Akira. 2006. TLR signaling. *Cell Death Differ* 13:816-825.

196. Gohda, J., T. Matsumura, and J. Inoue. 2004. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. *J Immunol* 173:2913-2917.

197. Libonati, M., A. Carsana, and A. Furia. 1980. Double-stranded RNA. *Mol Cell Biochem* 31:147-164.

198. Lu, Y., M. Wambach, M. G. Katze, and R. M. Krug. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. *Virology* 214:222-228.

199. 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.

200. 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.

201. Gorelik, E., R. P. Edwards, X. Feng, A. M. Marrangoni, J. R. Grandis, S. D. Drenning, L. Velikokhatnaya, J. A. Kwon, and A. E. Lokshin. 2004. IL-12 receptor-mediated upregulation of FasL in human ovarian carcinoma cells. *Int J Cancer* 112:620-627.

202. Schulte, M., K. Reiss, M. Lettau, T. Maretzky, A. Ludwig, D. Hartmann, B. de Strooper, O. Janssen, and P. Saftig. 2007. ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death. *Cell Death Differ* 14:1040-1049.

203. Zimmermann, K. C., C. Bonzon, and D. R. Green. 2001. The machinery of programmed cell death. *Pharmacol Ther* 92:57-70.

204. Verhagen, A. M., E. J. Coulson, and D. L. Vaux. 2001. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* 2:REVIEWS3009.

205. Stoll, S., J. Delon, T. M. Brotz, and R. N. Germain. 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science* 296:1873-1876.

206. Das, V., B. Nal, A. Roumier, V. Meas-Yedid, C. Zimmer, J. C. Olivo-Marin, P. Roux, P. Ferrier, A. Dautry-Varsat, and A. Alcover. 2002. Membrane-cytoskeleton interactions during the formation of the immunological synapse and subsequent T-cell activation. *Immunol Rev* 189:123-135.

207. Kuo, W. C., K. T. Yang, S. L. Hsieh, and M. Z. Lai. 2010. Ezrin is a negative regulator of death receptor-induced apoptosis. *Oncogene* 29:1374-1383.

208. Cooper, A. M., and S. A. Khader. 2007. IL-12p40: an inherently agonistic cytokine. *Trends Immunol* 28:33-38.

209. Clerk, A. 2001. Death by protein kinase C inhibitor: a stressful event. *J Mol Cell Cardiol* 33:1773-1776.

210. Mainiero, F., A. Soriani, R. Strippoli, J. Jacobelli, A. Gismondi, M. Piccoli, L. Frati, and A. Santoni. 2000. RAC1/P38 MAPK signaling pathway controls beta1 integrin-induced interleukin-8 production in human natural killer cells. *Immunity* 12:7-16.

211. Uddin, S., M. Sweet, O. R. Colamonici, J. J. Krolewski, and L. C. Platanias. 1997. The vav proto-oncogene product (p95vav) interacts with the Tyk-2 protein tyrosine kinase. *FEBS Lett* 403:31-34.

212. Diveu, C., E. Lelievre, D. Perret, A. H. Lak-Hal, J. Froger, C. Guillet, S. Chevalier, F. Rousseau, A. Wesa, L. Preisser, M. Chabbert, J. F. Gauchat, A. Galy, H. Gascan, and A. Morel. 2003. GPL, a novel cytokine receptor related to GP130 and leukemia inhibitory factor receptor. *J Biol Chem* 278:49850-49859.

213. Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C. Y. Wu, M. K. Gately, and U. Gubler. 1996. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci U S A* 93:14002-14007.

214. Maldonado, R. A., D. J. Irvine, R. Schreiber, and L. H. Glimcher. 2004. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature* 431:527-532.

215. Huse, M., B. F. Lillemeier, M. S. Kuhns, D. S. Chen, and M. M. Davis. 2006. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol* 7:247-255.

216. Perona-Wright, G., K. Mohrs, and M. Mohrs. 2010. Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. *Nat Immunol* 11:520-526.

217. Zhou, M., A. Jacob, N. Ho, M. Miksa, R. Wu, S. R. Maitra, and P. Wang. 2008. Downregulation of protein disulfide isomerase in sepsis and its role in tumor necrosis factor-alpha release. *Crit Care* 12:R100.

218. Alber, G., S. Al-Robaiy, M. Kleinschek, J. Knauer, P. Krumbholz, J. Richter, S. Schoeneberger, N. Schuetze, S. Schulz, K. Toepfer, R. Voigtlaender, J. Lehmann, and U. Mueller. 2006. Induction of immunity and inflammation by interleukin-12 family members. *Ernst Schering Res Found Workshop*:107-127.

219. Ozato, K., H. Tsujimura, and T. Tamura. 2002. Toll-like receptor signaling and regulation of cytokine gene expression in the immune system. *Biotechniques* Suppl:66-68, 70, 72 passim.