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Dendritic Cells Mediate the Dual Effects of IFN alpha/beta in HCV Immunity

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DENDRITIC CELLS MEDIATE THE DUAL EFFECTS OF IFNα/β IN HCV IMMUNITY

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Abstract

Hepatitis C virus (HCV) infects an estimated 170 million people and is responsible for considerable morbidity and mortality worldwide. Successful resolution of infection is achieved naturally in approximately 30% of infected individuals and correlates with a strong T cell response. Furthermore, treatment with interferon (IFN)α achieves sustained virologic response in approximately 50% of chronically infected individuals. Conventional dendritic cells (cDCs), as the most potent antigen presenting cells, and plasmacytoid DCs (pDCs), as the principal IFNα/β producing cells, serve as potential targets for both immune evasion and therapeutic strategies. In order to evaluate the effects of HCV on DC function, we compared phenotypic and functional characteristics of DCs from chronically infected patients to DCs from healthy donors. Our results suggest that despite non-specific decreases in total numbers, DC phenotypic and functional integrity is maintained. Consequently, we propose that other factors associated with HCV immunity may act on DCs to regulate pathogenesis and immunity. In light of the important roles of IFNα/β and CD4+ T cells in the clinical response to HCV, we focused on the interplay of these factors with DCs during CD8+ T cell priming. Using a human *in vitro* cross-presentation system, we found that IFNα/β acts on immature DCs (iDCs) to inhibit CD8+ T cell activation by cross-presentation. Further analysis revealed that STAT1-

dependent inhibition of CD40-induced IL-12 was responsible for this block. In contrast, a switch from STAT1 to STAT4 signaling enabled an immune enhancing effect of IFNα/β on mature DCs (mDCs) that potentiated CD8+ T cell activation. In order to evaluate this latter effect *in vivo*, we monitored the ability of pDC IFNα/β production to substitute for CD4+ T cells in CD8+ T cell priming. The results revealed that pDC activation overcomes the need for CD4 'help' in priming CD8+ T cell responses in an IFNα/β receptor-dependent manner. Importantly, IFNα/β produced by non-pDCs did not provide similar priming signals. These results provide support for HCV immunotherapy trials and offer a unique mechanism to account for the dual effects of IFNα/β in pathogenesis and immunity.

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LIST OF ABBREVIATIONS

AC	Apoptotic cell
ADAR	Adenosine deaminase
APC	Antigen presenting cell
BDCA	Blood dendritic cell antigen
CCR	Cys-Cys chemokine receptor
cDC	Conventional dendritic cell
CpG	Non-methylated bacterial CpG motifs
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
ELISPOT	Enzyme-linked immunospot
ER	Endoplasmic Reticulum
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
GAS	(Interferon) gamma-activated sequence
HAU	Hemagglutin units
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEV	High endothelial venules
HHD	Human HLA-A2.1 α1 α2 /Mouse D^b α3 monochain
HSV	Herpes Simplex Virus
H-Y	Minor histocompatibility antigens of the Y chromosome
ICCS	Intracellular cytokine staining

iDC	Immature dendritic cell
IFN	Interferon
IFNAR	Interferon α receptor
IL	Interleukin
ISRE	Interferon-stimulated response element
JAK	Janus kinase
LPS	Lipopolysaccharide
MCMV	Mouse Cytomegalovirus
mDC	Mature dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatability complex
MxA	Myxovirus resistance protein A
NS	Non-structural
OAS	Oligo-adenylate synthetase
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PGE	Prostaglandin E
PKR	Double-stranded RNA-activated protein kinase R
PTP	Protein tyrosine phosphatase
SAC	Staphylococcus aureus cowan I
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SVR	Sustained Virologic Responder
TCR	T cell receptor
TNF	Tumor necrosis factor

TLR	Toll receptor
T_{reg}	Regulatory T cell
WT	Wild type

Chapter 1: Defining a role for DCs in HCV pathogenesis

1.1 Introduction

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide and, as a leading cause of cirrhosis and liver disease, is associated with considerable morbidity and mortality. In order to develop vaccine strategies to reduce disease burden, a comprehensive understanding of the components of effective HCV immunity is needed. Two important discoveries have been critical in this regard. First, extensive characterization of the immune response to HCV infection revealed that strong cell-mediated immunity correlates with resolution of infection. Second, interferon-alpha (IFNα) is a highly effective treatment for chronic HCV infection. In light of these findings, I have focused on the role that two important dendritic cell (DC) subsets play in these phenomena—conventional DCs (cDCs) as potent stimulators of the adaptive immune response and plasmacytoid DCs (pDCs) as the main producers of IFNα. Moreover, I will discuss the mechanisms of IFNα/β production and signaling involved in its effects on adaptive immunity. A better understanding of the role of DCs in orchestrating effective HCV immunity will provide insight into the pathogenesis of and therapeutic strategies for HCV.

1.2. HCV Immunology

The immune system evolved to protect multicellular organisms from invading pathogens. Like any good security system, the immune system has surveillance and effector divisions to deal with these intruders; but, it is not just search and destroy. The immune systems must also strive to 'do no harm' to the host. Killing the pathogen at the expense of the host would not be evolutionarily advantageous. Similarly, invading pathogens have evolved in keeping with these rules; they maintain a vested interest in host survival to allow for replication and transmission. As such, they have evolved intricate mechanisms of immune evasion in order to maintain their survival. These mechanisms of evasion may either be direct antagonism of the immune response or immunologic jujitsu (using the host response for its own benefit). An understanding of these mechanisms offers insight into the important aspects for pathogen survival, key targets for therapeutic intervention, and fundamental mechanisms of the host immune response.

It is with these goals that I have considered the over-riding question of hepatitis C virus (HCV) immunology: Why do some people naturally clear HCV infection and some people progress to chronic infection? In order to address the question fully, I focus not only on the host's ability to respond, but also on the virus' ability to evade.

Pathogenesis and public health impact of HCV

Current estimates suggest that 3% of the world's population (approximately 170 million people) is infected with HCV[1]. Although HCV less commonly causes acute hepatitis (10-40% of infections), more than 70% of infected persons

progress to chronic infection[2] (Fig. 1.1). Chronic HCV is a major cause of chronic liver disease and hepatocellular carcinoma (HCC). According to the World Health Report of 2004, liver cirrhosis and HCC accounted for 1.4 million deaths, 20% of which are likely attributable to HCV infection[3]. In addition, HCV infection is linked to numerous co-mordibities (e.g. type II cyroglobulinemia) and is a leading cause of liver transplant in the USA and Europe.

The development of identification tools for hepatitis A and hepatitis B over 30 years ago allowed for the identification of non-A, non-B hepatitis transmissible by infected blood and blood products [4]. The causative agent was identified as HCV in 1989 by expression cloning of cDNAs from the serum of an infected patient[5]. Until effective screening measures where implemented in July 1992, infection was commonly passed through blood transfusions, hemodialysis, and organ transplantation [2]. Furthermore, the use of contaminated needles for vaccination campaigns from the 60's-70's are thought to have been responsible for high rates of exposures in some areas [6]. Worldwide public health initiatives to screen the blood supply for HCV and to provide clean medical supplies significantly reduced the incidence of HCV infection. According to the Center for Disease Control, the incidence of HCV infection dropped from 240,000/year in the 1980s to 30,000 in 2003[7]. Today, the highest incidence of HCV is among intravenous drug users. Shared risk factors with HIV make co-infection quite common. In fact, 50-90% of HIV infected injection drug users are co-infected and show more rapid progression to liver disease [8].

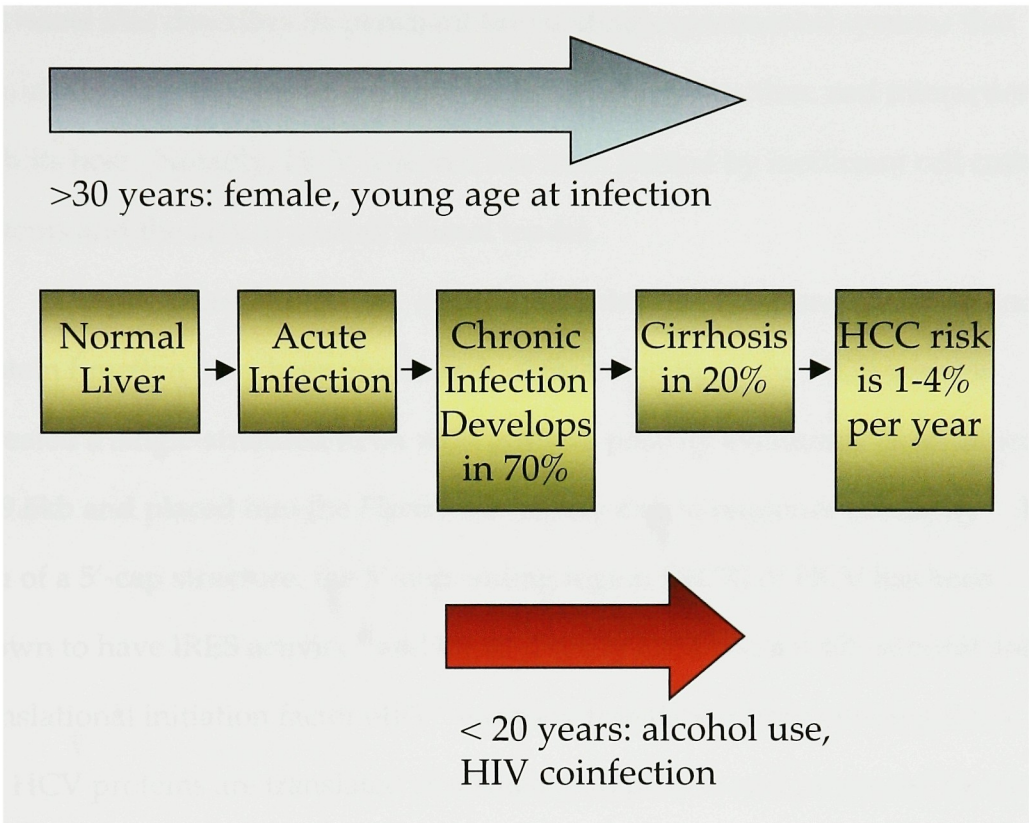

Figure 1.1 HCV Pathogenesis. Following exposure, acute HCV infection ensues. Although previous estimates underestimated the percentage of resolvers, chronic infection develops in the majority of infections and cirrhosis develops in 20% of those chronically infected. Progression to cirrhosis is usually slower in females and those infected at younger age. Progression to cirrhosis is generally accelerated in those with concomitant alcohol use or HIV co-infection. In patients with HCV cirrhosis, the risk of developing hepatocellular carcinoma (HCC) is 1-4% per year (adapted from Lauer and Walker, NEJM, 2001).

HCV—The stealth virus

HCV is often termed the stealth virus for its ability to establish chronic infection without efficient detection and clearance by the immune system. In addition, this name also describes its penchant for evading experimental systems that would allow for the characterization of its structure, function, and interaction with its host. Notably, HCV research has been limited by inefficient cell culture systems and the lack of a small animal model.

Despite these limitations, considerable details of genome structure and protein function have been elucidated. cDNA cloning of the HCV genome revealed a single-stranded RNA with positive polarity eventually determined to be 9.6kb and placed into the *Flaviviridae* family due to sequence similarity [5]. In lieu of a 5'-cap structure, the 5'-non coding region (NCR) of HCV has been shown to have IRES activity [9] and binds directly to ribosomal 40S subunit and translational initiation factor eIF3 [10] enabling translation from entering RNA. All ten HCV proteins are translated as a single polyprotein and processed by auto-catalytic cleavage or proteolytic cleavage (Fig. 1.2).

The amino-terminal section of the polyprotein contains the structural proteins (Core, E1, and E2) followed by a small integral membrane protein p7 which may function as an ion channel. Core protein is a highly conserved basic protein existing in 19- and 21-kd forms. Similar to other structural core proteins, HCV core protein promotes virion formation by binding viral RNA and associating with membranes at the cytoplasmic surface of the ER[11]. Envelope glycoproteins E1 and E2 likely play an important role in mediating viral entry. Biochemical data suggests that E2 binds to CD81, a tetraspanin expressed on

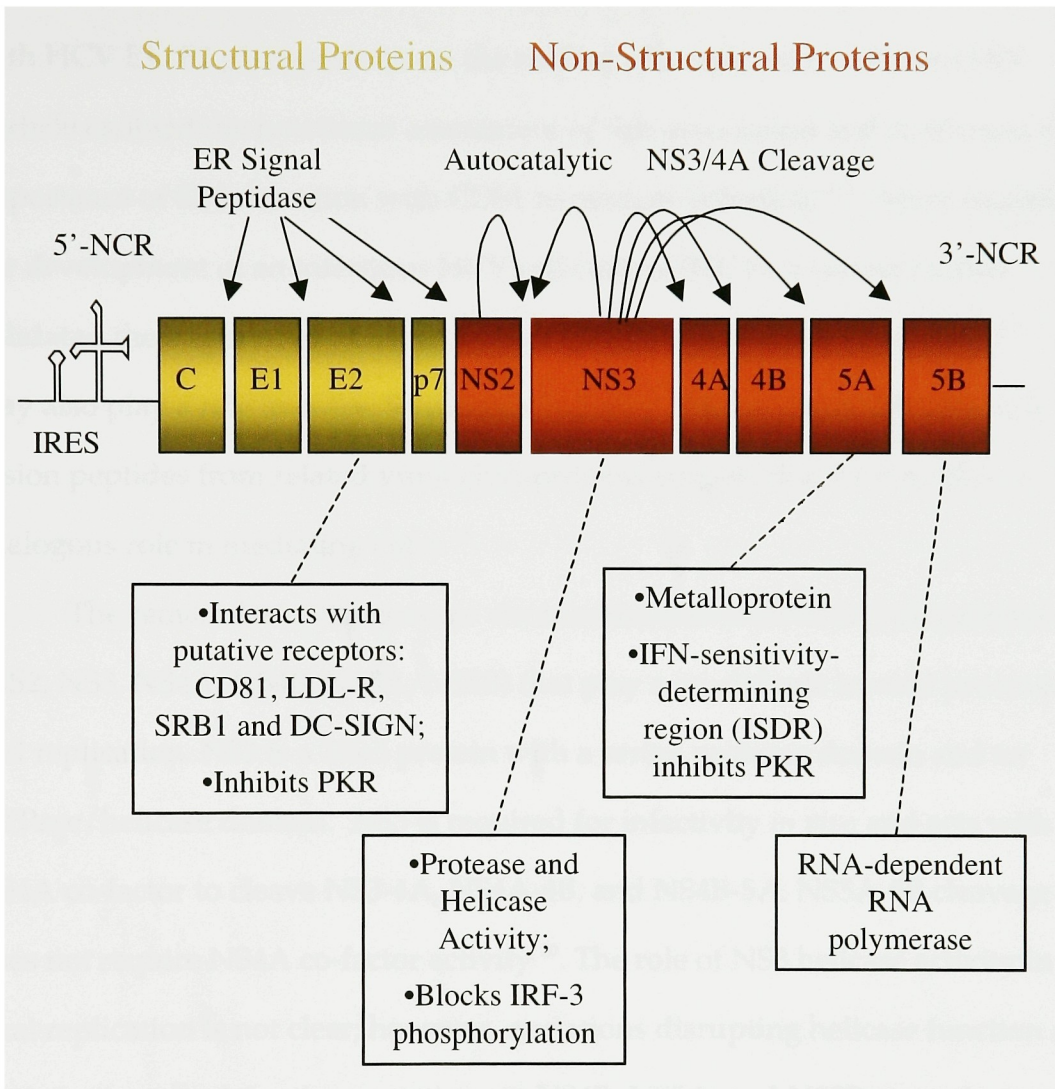

Figure 1.2. HCV structure and function. The HCV genome is represented schematically with all 10 proteins indicated. The 5′ NCR of HCV has an internal ribosomal entry site which drives translation of the HCV polyprotein. The polyprotein is cleaved as indicated in the figure. The putative functions of the proteins in the HCV life cycle are indicated.

various cell types including hepatocytes and B cells[12]. However, the lack of an infectious cell culture system prevented the functional evaluation of E2 and CD81 in veritable infection. The development of HCV pseudoparticles (HCVpp) with HCV E1/E2 glycoproteins on the surface of a replication deficient HIV particle enabled the functional assessment of this association and confirmed the importance of E2 interaction with CD81 to mediate infection[13-15]. More recently, the development of an infectious HCV cell culture (HCVcc) system further validated the importance of E2, as well as CD81, in mediating viral entry [16-18]. E1 may also play a role in entry. Sequence similarity of E1 residues 264-290 with fusion peptides from related virus glycoproteins suggest that E1 may play an analogous role in mediating entry [19].

The remainder of the genome encodes the non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) that play a crucial role in viral processing and replication. NS3 is a 70kd protein with a serine protease domain and an NTPase/helicase domain. NS3 is required for infectivity *in vivo* and acts with NS4A co-factor to cleave NS3-4A, NS4A-4B, and NS4B-5A. NS5A-5B cleavage does not require NS4A co-factor activity [20]. The role of NS3 helicase activity in viral replication is not clear; however, mutations disrupting helicase function are not infectious [21]. Adaptive mutations in NS4B, NS5A, and NS5B strongly enhance replication, suggesting that these proteins are important in the HCV replication machinery[22]. NS5A is a membrane associated zinc metalloprotein protein that exists in different phosphorylation states (56- and 58-kd). Zinc coordination is required for NS5A function in the HCV replication complex [23,24], but its exact function remains unclear. Finally, NS5B is the RNA-dependent RNA polymerase (RdRP) responsible for viral replication. Mutation of the

highly conserved GDD motif ablates *in vivo* infectivity[21]. The high error rate of the RdRP enables escape mutations and quasispecies to develop within the infected individual[25].

Understanding effective HCV immunity

Although the lack of a small animal model for HCV precludes extensive probing of host immunity, an important human model exists for the study of effective HCV immunity—natural resolvers. Recent estimates suggest that nearly 30% of those exposed to HCV clear the infection naturally and extensive work has been done to characterize the nature of the immune response to HCV in humans. Current results suggest that CD4+ and CD8+ T cells play an important role in controlling acute infection, but significant data also exist for the involvement of other cells. Moreover, chimpanzees also succumb to HCV infection. Studies with these animals have enabled extensive probing of the immune cells involved in protective immunity.

The role for B cells in HCV immunity is complex. The high prevalence of mixed cryoglobulinemia and lymphoma in chronic HCV has directed several groups to investigate the role for HCV in driving B cell abnormalities[26]. One study suggested a direct link between these abnormalities and HCV infection showing that 7 out of 7 patients with splenic lymphoma with villous lymphocytes in the setting of chronic HCV went into remission following treatment of their HCV with IFNα therapy [27]. E2 binding of CD81, a component of the B cell co-receptor complex, provided a possible mechanism for HCV antigen driving B cell abnormalities[12]; however, despite accumulation of naïve B

cells in the peripheral blood of HCV patients, antigenic stimulation does not seem to be responsible for B cell activation and differentiation [28].

Neutralizing antibodies play an important role in protective immunity to many viral infections;[29] however, the importance of antibodies in HCV immunity is not clear. HCV-specific antibodies usually appear 7-8 weeks after infection and there is evidence to suggest that they may neutralize infection both *in vitro* [30] and *in vivo* [31]. The putative target of some of these neutralizing antibodies is the N-terminal region of E2 which contains a hypervariable region (HVR1) [30]. Sequence changes in the HVR-1 of E2 that occurred simulataneously with antibody seroconversion suggest that antibody drives viral mutations. However, early studies in chimpanzees and humans suggest that antibody seroconversion does not protect against re-infection[32,33]. Furthermore, resolution may occur in the absence of antibody seroconversion[34,35]. Specific qualitative properties of B cell function and antibody specificity may be important parameters for conferring protection *in vivo*. A more extensive evaluation of neutralizing antibodies with the recently developed HCVcc system may offer new insight into their importance.

Natural Killer (NK) cells may play an important role in mediating effective innate immunity in HCV infection. When activated by virally infected cells, NK cells release IFNγ and cytolytic granules. *In vitro* studies suggest that E2 engagement of CD81 on NK cells may modulate cytokine production and NK cell effector function [36]. A recent study aimed at identifying genetic susceptibility to chronic HCV infection showed that patients with a specific NK inhibitory receptor (KIR2DL3) and HLA haplotype (HLA-C) were more likely to clear infection [37]. The inability of HLA-C to interact with KIR2DL3 may lower the

threshold for NK cell activation. One caveat of this study is that the correlation only held for patients infected from "low dose" exposures.

Considerable effort has been made to understand the role of HCV-specific T cells in viral clearance. Specifically, CD8+ T cells are crucial in host defense against viral infection. CD8+ 'killer' T cells or cytotoxic T lymphocytes (CTLs) recognize infected cells in an antigen specific manner by T cell receptor engagement of foreign peptides bound to MHC class I (MHCI) molecules on the cell surface of the infected cell. Thus engaged, the cytotoxic effect can be achieved by release of apoptosis inducing granzymes or pore forming peforins as well as a membrane-bound apoptosis-inducing ligand called Fas. In addition, CTLs can also produce IFNγ which can inhibit viral replication, induce MHCI upregulation, and activate macrophage effector functions[38]. Consequently, both the cytolytic and noncytolytic functions of CTLs are important in controlling viral infection[39].

Early work showed that CD8+ T cell immune responses are important prognostic indicators in acute infections[40] (Fig. 1.3). Similarly, protective immunity in chimpanzees correlates with HCV-specific cellular immunity, not antibody response [34]. Numerous studies have offered correlative support for the role of T cell immunity in HCV clearance[41]. Direct evaluation of the role for CD8+ T cells by *in vivo* depletion resulted in prolonged viremia offering proof that CD8+ T cells play a crucial role in mediating resolution[42]. However, CD8+ T cell responses are also seen in chronic infection and do not always correlate with clinical outcome[43]. As such, differences in CTL effector function in the tissue may

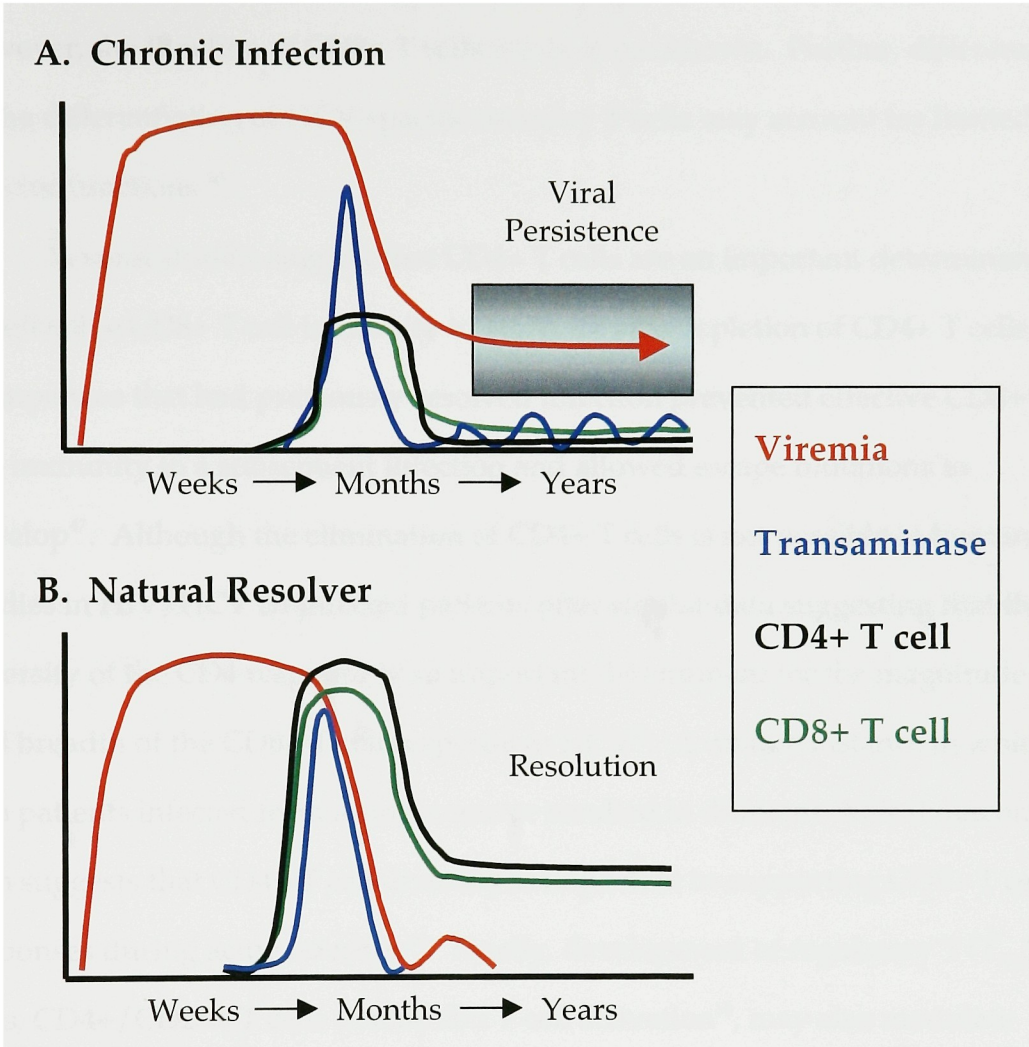

Figure 1.3. T cell immunity plays a key role in the outcome of HCV infection. Liver injury is observed weeks to months following initial viremia as evidenced by the rise in transaminase levels. **A.** Although T cell responses are variable during progression to chronic infection, both the CD8+ and CD4+ T cell responses are generally blunted. **B.** In contrast, robust CD8+ and CD4+ T cell responses correlate with viral resolution and normal transminase (adapted from Bowen and Walker, 2005 Nature).

account for their variable efficacy in viral clearance. Several reports have suggested impaired cytokine secretion and cytotoxic function *in vitro* [44,45]; however, the function of CD8+ T cells *in situ* is not known. Further, differences in the differentiation of HCV specific memory T cells may account for limited effector functions [46].

Several studies suggest that CD4+ T cells are an important determinant for effective CD8+ T cell immunity to HCV. *In vivo* depletion of CD4+ T cells in a chimpanzee that had previously resolved infection prevented effective CD8+ T cell immunity in a subsequent infection and allowed escape mutations to develop[47]. Although the elimination of CD4+ T cells is not possible in humans, studies in HIV/HCV co-infected patients offer similar data suggesting that the diversity of the CD4 response is an important determinant for the magnitude and breadth of the CD8+ T cell response to HCV[48]. A unique instance in which two patients infected from a single source resulted in different clinical outcomes also suggests that CD4+ T cell diversity is important in supporting CD8+ T cell responses during acute infection[49]. Finally, development of regulatory T (T_{reg}) cells, CD4+/CD25+ T cells that inhibit T cell activation[50], may also modulate HCV specific T cell function[51], but the antigen specificity of T_{reg} cell activity is still unclear.

The strong evidence suggesting the importance of cell-mediated immunity in an effective HCV immune response has raised many questions concerning the components involved in generating this response. In order to explore these questions, I have focused on two aspects of HCV immunity: the role of dendritic cells (DCs) and the effect of IFNα/β. First, we will consider the DC as a pivotal player in the priming of a T cell response.

1.3. Dendritic Cells

The 'sentinels' of the immune system

The initiation of an immune response is a complex process. The invading antigens and the lymphocytes that respond to their assault have long been appreciated as crucial components of the host/pathogen interaction. However, the spatial separation between the antigen in the site of infection or tumor and the lymphocytes in the lymph nodes creates the need for a messenger system—the antigen presenting cell (APC). Although not appreciated as such originally, these APCs were first visualized in the skin by Paul Langerhans in 1868. DCs are considered the most potent APCs and exist throughout the skin, mucousal surfaces, and peripheral tissue[52]. They can efficiently stimulate both B and T lymphocytes. Importantly, DCs are required for priming naïve T lymphocytes [52]. The existence of a DC 'sentinel' system offers the immune system the ability to receive information of potential dangers in a centralized lymph area where it can subsequently identify and prime antigen specific effectors.

Maturation alters DC phenotype, function, and location

Two distinct differentiation states of DCs have been described that facilitate their ability to act as 'sentinels' of the immune system (Fig. 1.4). In tissue, DCs are present in what has been termed the 'immature' state. Immature DCs (iDCs) are characterized by their primary role—antigen capture[53]. iDCs utilize four main pathways to capture antigen: phagocytosis (via receptors including $\alpha_v\beta_5$, phosphatidylserine receptor, and CD36), micropinocytosis, macropinocytosis, and receptor mediated endocytosis (via receptors including MMR, DEC-205,

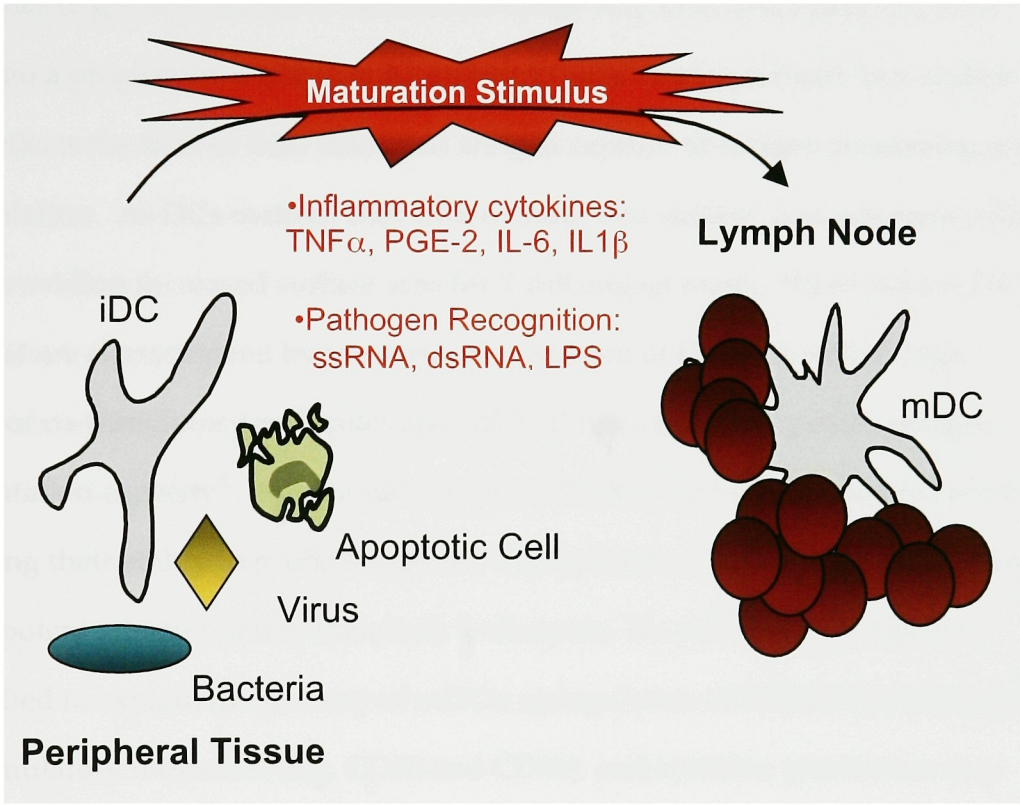

Figure 1.4. DCs are the 'sentinels' of the immune system. DCs serve as the messenger between the site of infection and the priming of the adaptive immune response in the lymph node. Immature DCs (iDCs) in the periphery are uniquely suited to capture antigen. In response to maturation stimuli, DCs migrate through the afferent lymph to the lymph node where they can present antigen to T cells in secondary lymphoid organs. Mature DCs (mDCs) are aptly suited for priming T cells with high levels of surface MHC and co-stimulatory molecule expression.

FcRγ and FcRε). iDCs are characterized immunohistochemically by their lack of macrophage markers such as CD14, absence of the mature DC marker CD83 and low surface expression of co-stimulatory molecules and MHC II (consistent with their primary role being antigen capture). Upon exposure to inflammatory cytokines (e.g.TNFα, IL-1β) or microbial stimuli (e.g. LPS, SAC, dsRNA), DCs undergo a programmed phenotypic and functional change termed 'maturation' that reflects the shift in their role from antigen capture to antigen processing and presentation. As DCs mature, they differentiate into stellate, non-adherent cells thus providing increased surface area for T cell engagement. These mature DCs (mDCs) are characterized by the surface expression of CD83 as well as high levels of co-stimulatory molecules and MHC II reflecting their potent antigen presentation capacity[52]. Functional activity of mDCs can be evaluated *in vitro* by assaying their ability to prime allogeneic T lymphocytes[54]. DCs are 100-1000 fold more potent allostimulators than bulk leukocytes. No one molecule has been identified to explain the potency of mDCs; upregulation of MHCII (10-100-fold), co-stimulatory molecules (e.g. CD80 and CD86), and cytokine production (e.g. IL-12) [55,56] all may play a role.

Coincident with these maturation changes, DCs upregulate CCR7 to facilitate migration from the tissue to the lymph node[57]. Physiologically, this migration puts mDCs in proximity of both naïve and central memory T cells. As such, the location and unique phenotypic profile puts the mDC in the right place with all the tools for initiating a T cell response, be it activation or tolerance. Accordingly, I focus in depth in chapter 2 on the maturation profiles of DCs during chronic HCV and the role they may play in HCV immunity.

DCs specialize in antigen capture and presentation

The central feature of the DC 'sentinel' system is the endowment with a unique ability to capture, process, and present antigen for an effective immune response. The ability of iDCs to capture antigen is not unique—in fact, macrophages phagocytose more efficiently than DCs. However, while macrophages phagocytose and subsequently degrade antigen, DCs sequester antigen in MHCII-rich compartments (MIICs). These MIICs convert from late-endosomal to non-lysosomal compartments upon maturation, enabling MHCII-peptide complex discharge to the surface [58]. A recent report suggests that low levels of lysosomal proteases allow DCs to sequester and process antigen, thus facilitating antigen presentation [59].

DCs also have specialized machinery for MHCI presentation. In contrast to MHCII presentation, MHCI presentation is classically restricted to endogenously expressed antigens processed by the proteasome and loaded onto MHCI in the ER. This classical model however fails to account for the generation of CD8+ T cell responses to tissue-restricted antigen (e.g. tumor antigen or antigen from viruses that do not infect APCs). Early work by Michael Bevan challenged the classical model for MHCI antigen presentation by showing that exogenous antigen can be used to generate epitopes for CD8+ T cell priming[60]. This mechanism was termed cross-priming due to the crossing of the canonical boundaries of MHCI antigen presentation. This pathway plays an important role in generating CD8+ T cell responses to tissue-restricted antigen [61-63]. Notably, DCs have a unique capacity to cross-present antigen on MHCI in order to prime a CTL response[64]. In addition to apoptotic cells, antigen from viral particles as

well as immune complexes may be cross-presented to engage antigen-specific CD8+ T cells[65].

The mechanism by which HCV is presented to the immune system by APCs is not clear. In the following chapters, I review and evaluate the ability of HCV to directly infect APCs. Furthermore, in chapter 4, I detail the development of an *in vivo* system to evaluate cross-presentation of full length HCV.

Immunologic ouctomes of antigen presentation

Following antigen processing and presentation, DCs engage and activate T cells. Initial models suggested that T cell activation requires two signals: 'signal 1' (MHC-peptide complex) to engage antigen specific T cells and 'signal 2' (co-stimulation) to enable T cell activation. Co-stimulatory molecules CD80 and CD86 are uniquely expressed on APCs and provide 'signal 2' via CD28 engagement on T cells. However, more recent data argues that the generation of effective CTLs requires 'signal 3' or 'help,' provided either by "danger" signals[66] or in an antigen specific fashion by CD4+ T helper cells[67] (Fig.1.5). Although immune response to several infectious agents were traditionally characterized as helper-independent, recent studies suggest that CD4+ T cell help is an absolute requirement for the generation of CD8+ T cell memory [68,69]. *In vivo* models of cross-priming (via i.v. injection of irradiated OVA-loaded β2 microglobulin-/- spleen cells) require CD4+ T cell 'help' to generate antigen-specific CTLs[70]. The requirement for CD4+ T cell 'help' for cross-priming CTLs suggests two potential scenarios: (1) A three-

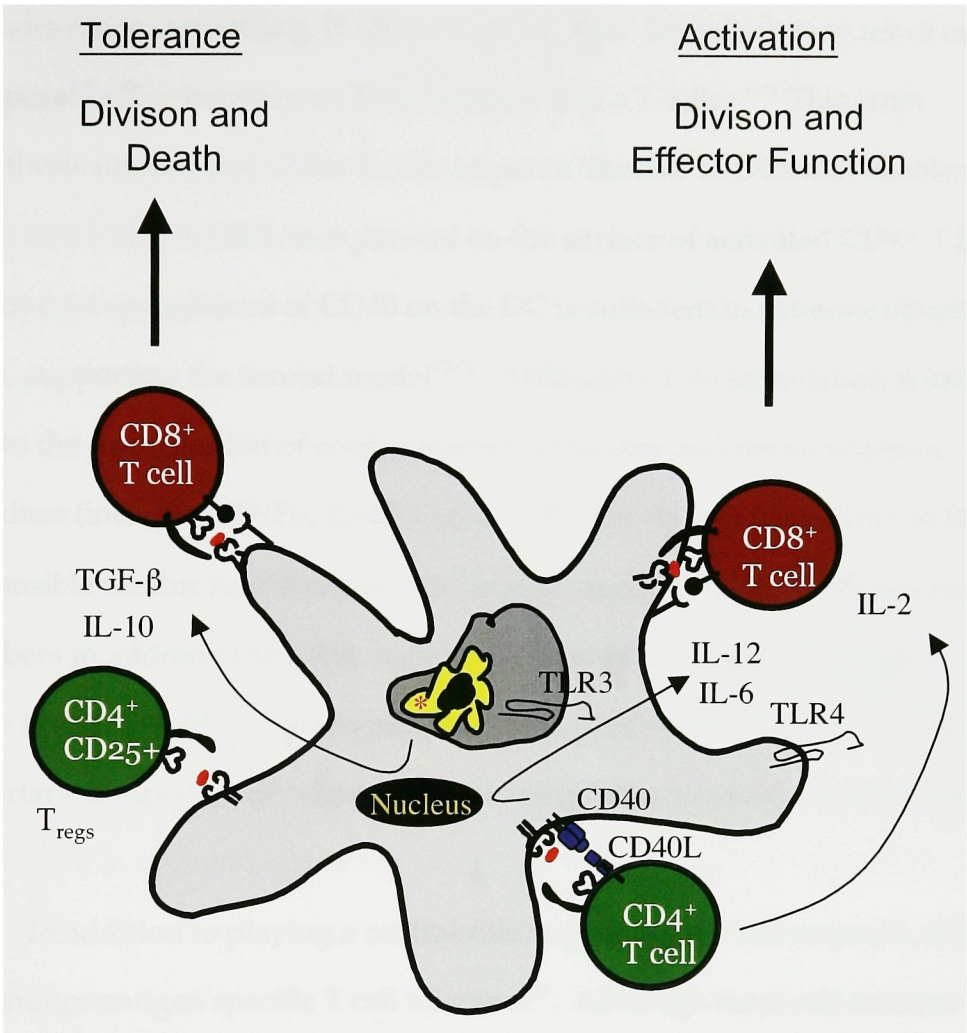

Figure 1.5. DCs mediate activation and tolerance of CD8+ T cells. Antigen presentation by mDCs can result in distinct immunologic outcomes: activation or tolerance. In the absence of CD4 help or other danger signals, cross-presentation of antigen to CD8+ T cells leads to tolerance via division and death. Production of TGF–β by DCs following phagocytosis of apoptotic cells or IL-10 production by T_{regs} may skew towards tolerance. In contrast, in the presence of 'help' provide by CD40L ligation from CD4 T cells, LPS engagement of TLR4, or dsRNA engagement of TLR3, DCs are licensed to prime CD8+ T cells. IL-12 and IL-6 from the DC as well as IL-2 produce by CD4 helper cell are important cytokines for priming the CD8+ T cell response.

cell interaction (DC, CD4+ and CD8+ T cell) in the lymph node suggesting a role for short-range signals (e.g. IL-2) or (2) a 'licensed' DC which may serve as a "temporal bridge between a CD4+ T-helper and a T-killer."[71] This latter hypothesis implies that CD4+ T cells impart a 'license' to DCs that enables them to activate CTLs. CD40L is expressed on the surface of activated CD4+ T helper cells and its engagement of CD40 on the DC is sufficient to generate effective CTLs, supporting the second model[72-74]. Although CD40 stimulation is known to lead to the upregulation of co-stimulatory molecules and the secretion of cytokines (including TNFα, IL-1β, and IL-12)[75], the signaling pathway in DCs responsible for this functional switch remains unclear. Other TNF superfamily members in addition to CD40L may also activate DCs to produce 'signal 3.'[62] There is strong evidence to suggest that IL-12 produced by the DC may be an important component of 'signal 3' in allowing full activation of naïve CD8 T cells[76].

In addition to playing a central role in priming a T cell response, DCs can also induce antigen specific T cell tolerance[77]. Although most self-reactive T cells are deleted during development in the thymus in a process called central tolerance, peripheral tolerance is thought to be responsible for inactivating T cells reactive to developmentally expressed and tissue-restricted antigens. The induction of T cell anergy, deletion, and cytokine skewing are all mechanisms of functional tolerance. All three mechanisms require engagement of T cell with MHC-peptide complex. Due to the suggestion that naïve T cells do not circulate[78], peripheral CD8+ T cell tolerance to tissue restricted antigens depends on a cross-presenting APC to deliver antigen to the lymph node [79]. *In vitro* studies have identified the DC as the crucial APC required for CD8+ T cell

tolerance to cross-presented antigen [62]; this finding is supported by the recent *in vivo* work of others[80].

Some reports suggest that the maturation state of the DC controls the immunologic outcome of the T cell response[81]. In other words, in the steady state iDCs capture antigens and silence T cell responses either by deleting them or expanding T_{reg} cells. However, this poses two problems regarding T cell tolerance by DCs *in vivo*. First, as mentioned above, since naïve T cells do not circulate, peripheral CD8+ T cell tolerance likely occurs in the T cell rich regions of the lymph node or secondary lymphoid tissue. However, DC chemotaxis into the T cell areas of the draining lymph organs, enabled by the upregulation of CCR7, requires a maturation stimulus [57]. Second, DC / T cell engagement is required for active induction of antigen specific T cell tolerance. However, iDCs do not acidify their endosomal system and as a result the antigen captured is maintained in an unprocessed holding compartment until a maturation signal is received[82]. Therefore, it is difficult to envision how antigen captured in the periphery can be efficiently presented to T cells for the induction of antigen specific tolerance by iDCs.

Instead, it has been demonstrated that mDCs can mediate both activation and tolerance depending on the presence of 'signal 3' stimuli[62]. Using an *in vitro* cross-presentation system, both activation (defined by proliferation and CTL activity) and tolerance (defined by T cell division and death) required phenotypically (i.e. CD83+, CD25+, $CD86^{high}$, HLA DR^{high}) and functionally (i.e. potent inducers of allogeneic mixed leukocytes reaction) mature DCs [62]. The crucial switch between DCs inducing T cell activation or tolerance is not maturation, but rather 'signal 3' provided by CD40 engagement on the DC. IL-12

production in response to CD40 engagement may act as a 'signal 3' in skewing cross-tolerance to cross-priming[76,83].

The regulation of T cell activation *versus* T cell tolerance in HCV infection is not well understood. The lack of a small animal model creates a significant obstacle for extensive characterization of the immune response. In lieu of this model, I evaluate, in chapters 3 and 4, the role that CD4+ T cells and virally induced IFNα/β play in modulating these signals to control the immunological outcome.

DC interaction with viruses

Infection of DCs can alter function. Some viruses, such as influenza, promote maturation [84]. In the case of influenza, maturation may protect from viral cytopathicity as well as enable DC migration for T cell engagement. This maturation response, however, is not common to all viruses and may even depend on the multiplicity of infection. Infection of DCs with herpes simplex virus (HSV) and measles virus (MV) leads to submaximal DC maturation and impaired T cell activation [85-87]. In contrast, two flaviviruses, bovine viral diarrheal virus (BVDV) and classic swine fever virus (CSFV) do not induce maturation or disrupt the ability to stimulate T cells [88,89]. Similar results are seen with a related flavivirus, yellow fever 17D [90](see Appendix 3).

There is precedent for DC-specific interactions with flaviviruses—specifically dengue virus (DV). DV selectively infects a DC population in the skin called langerhan cells (LCs) and causes infected LCs to migrate out of the tissue [91]. DC specific ICAM-3 grabbing non-integrin (DC-SIGN) is sufficient for DV infection [92] and accounts for the selectivity of DV for LCs and iDCs [93].

Interestingly, HCV E2 interacts with DC-SIGN [94,95]. One report suggests that VSV E1/E2 pseudotype particles enter DCs via DC-SIGN [96], but the same results are not seen with HCVpp[13-15]. In addition to infection, binding of HCV to DCs may also play a role in pathogenesis. For example, interaction of HIV with DC-SIGN for binding but not for entry has suggested a mechanism whereby HIV migrates with DCs to the lymph node to gain access to CCR5 positive CD4+ T cells [97]. DC-SIGN interaction is not, however, a general feature of flavivirus host interaction. It is not sufficient for yellow fever infection or necessary for West Nile virus (WNV) infection [92]. A recent report shows $\alpha_v\beta_3$ integrin as the receptor for WNV[98].

1.4. Interferon (IFN)-α/β

In addition to the data from natural resolvers, another patient population provides important data for understanding the components of a successful response—sustained virologic responders (SVRs). SVRs are defined as patients that have cleared HCV following IFNα therapy and remain HCV negative 6 months after discontinuation of therapy. The second major aim of my thesis is to understand the role that IFNα may play in modulating the immune response at the DC:T cell interface, during pathogenesis and therapy.

IFNα: The standard of care

Currently, the most effective treatment for HCV is pegylated IFNα. It slows progression of liver disease and eradicates virus in approximately 50% of cases[99]. IFNα was originally defined as an antiviral agent, but has been used therapeutically for the treatment of viral and non-viral diseases. The original IFNα monotherapy for chronic HCV with three injections per week was approved in 1990 and the latest regimen, combination of ribavirin and pegylated IFNα was approved in 2002 [100]. Response rates to IFNα therapy stratify by genotype with SVR rates of 42-46% for genotypes 1 and 4; 76-80% for genotypes 2 and 3 [101,102]. Analysis of sequence determinants correlating with response to IFNα therapy revealed an interferon sensitivity-determining region (ISDR) within genotype 1b seen in some cohorts[103,104] but not others[105].

IFNs were discovered by Alick Isaacs and Jean Lindemann in 1957 as an undefined substance with antiviral activity[106]. They showed that supernatant from influenza-infected chicken embryos cleared of virus was able to inhibit

influenza infection when added to a fresh set of chicken embryos. Work within the last several decades has defined this antiviral substance more carefully as type I IFNs in contrast to type II IFNs (IFNγ). There are seven different classes of type I IFNs (α, β, δ, ε, κ, ω, and τ). IFNα and IFNβ are best characterized of the type I IFNs. While most cells can produce both, depending on the stimulus, the primary producers of IFNα and IFNβ are leukocytes and fibroblasts, respectively[107]. At least 14 IFNα subtypes exist, but only one IFNβ. For the most part, the antiviral and antiproliferative effects of the multiple IFNα subtypes are functionally redundant; however, large differences exist in their ability to activate NK cells[108]. Nonetheless, all use a single IFNα/β receptor (IFNAR).

The molecular mechanisms associated with IFNα/β's defining antiviral effect are numerous; however, four proteins play an important role—2,5-oligoadenylate synthetase (2',5'-OAS), protein kinase R (PKR), myxovirus resistance protein A (MxA), and RNA-specific adenosine deaminases (ADAR). 2',5'-OAS induces an antiviral state by activating RNase L, a cellular endoribonuclease, which degrades viral RNA [109]. PKR is a double-stranded RNA-dependent protein kinase which inhibits peptide chain initiation and viral translation by phosphorylation of eIF-2α [110]. MxA is an IFN-induced GTPase originally characterized by its ability to block influenza A infection[111]. Although the mechanism of its antiviral activity is not completely understood, MxA antagonizes viral polymerase activity[112] and blocks redistribution of viral capsid proteins[113]. Finally, the activation of ADAR inactivates viral RNA by RNA editing[114].

The signaling mechanisms associated with the induction of this antiviral response have been extensively characterized (Fig. 1.6). The IFNAR is a heteromdimeric receptor consisting of subunits IFNAR1 and IFNAR2. IFNAR2 exists in three different forms: IFNAR-2a (short), IFNAR-2b (soluble), and IFNAR-2c (long) [115]. IFNAR1 and IFNAR2c are constitutively associated with intracellular Janus kinases (JAKs), Jak1 and Tyk2, respectively[116]. IFNα/β engagement causes IFNAR dimerization and/or oligomerization. This rearrangement enables Jak1 and Tyk2 activation by trans-phosphorylation. Subsequent phosphorylation of receptor cytoplasmic tails enables STAT2 recruitment. In contrast to activation of STAT1 by IFNγ, IFNα/β signaling requires STAT2 for STAT1 phosphorylation [117]. Tyrosine phosphorylation enables STAT1/STAT2 heterodimerization and interaction with p48 (IRF-9) forming interferon-stimulated gene factor-3 (ISGF-3). ISGF-3 translocates to the nucleus, binds to interferon stimulated response elements (ISRE), and activates transcription [118]. IFNα/β also activates STAT3 and STAT4. Similar to STAT2, STAT3 can form heterodimers with STAT1 to activate ISRE. In contrast, STAT4 homodimerizes and binds to (IFN)-gamma-activated sequence (GAS) elements[119].

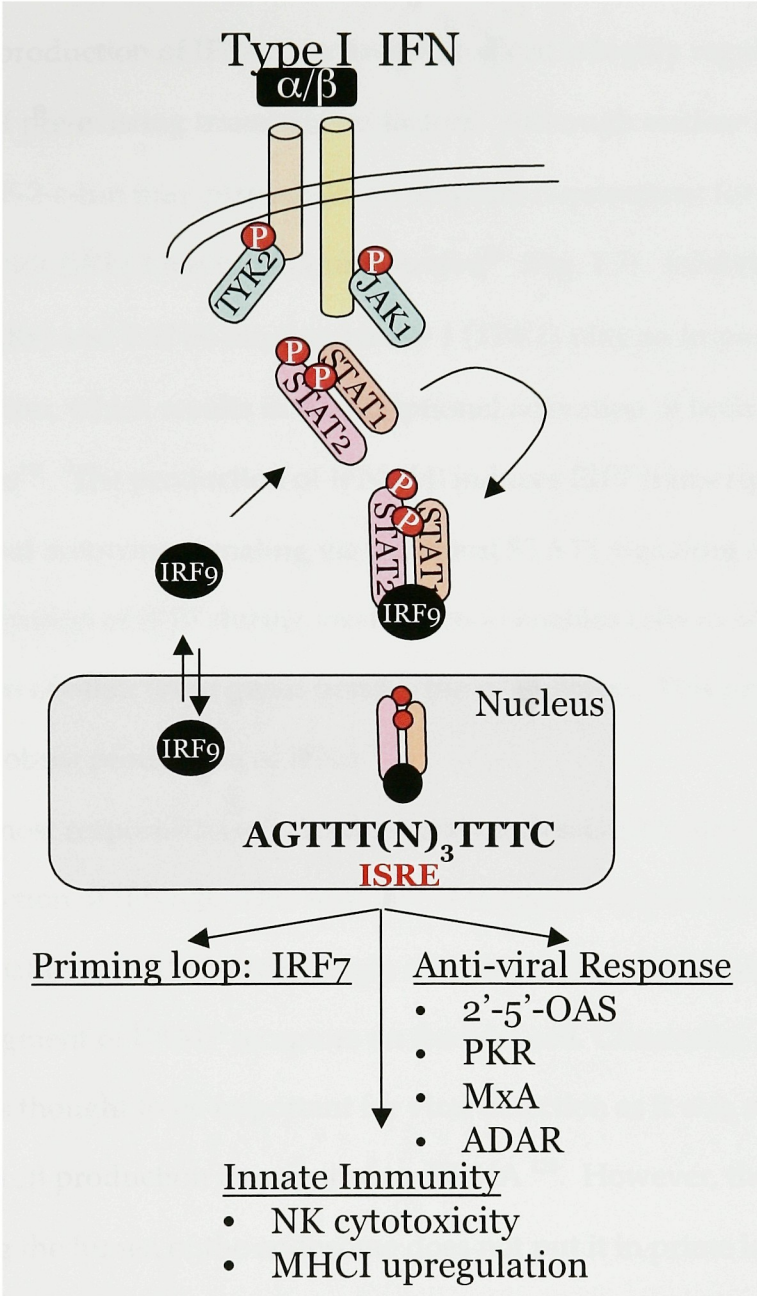

Figure 1.6. IFNα/β signaling. Engagement of the IFNα/β receptor activates Jak1 and Tyk2 which in turn phosporylate STAT1 and STAT2. Heterodimerization and association with IRF9 to form ISGF3 results in translocation to the nucleus and activation of gene transcription from interferon stimulated response elements (ISRE). ISRE transcription drives the IFN priming loop as well as other anti-viral and innate immunity effector functions.

IFNα/β production triggered by virus detection

The production of IFNα/β by an infected cell is highly regulated by the activation of pre-existing transcription factors. Although nuclear factor-κB (NF-κB) and ATF-2-c-Jun may play a role, an absolute requirement for interferon response factor (IRF) 3 has been demonstrated[120] (Fig. 1.7). Inhibitor of NF-κB kinase-ε (IKKε) and TANK-binding kinase 1 (TBK1) play an important role in IRF3 activation, which results in transcriptional activation of both IFNβ and IFNα4 genes[121]. The production of IFNα4/β induces IRF7 transcription by both paracrine and autocrine signaling via canonical STAT1 signaling disccused above. Activation of IRF7 during viral infection enables cells to activate transcription of other IFNα genes besides the α4 subtype. This priming loop is crucial for robust production of IFNα [122].

The host response has evolved several mechanisms to link viral detection with production of IFNα/β. This response is triggered by pathogen-associated molecular patterns (PAMPs) (e.g. single stranded (ss) or double stranded (ds) RNA) engagment of PAMP receptors on the host cell. Classically, toll receptor (TLR) 3 was thought to be important for viral detection as it was required to trigger IFNα/β production in response to dsRNA [123]. However, the location of TLR3 facing the lumen of the endosome does not put it in prime location for detecting ssRNA viruses. Another toll receptor, TLR9 was originally defined to detect non- methylated CpG motifs specific for bacterial DNA[124]. Although TLR9 agonists are a strong trigger for IFNα/β, it was not originally clear how they were involved in a viral response. Subsequently, it was found that in addition to

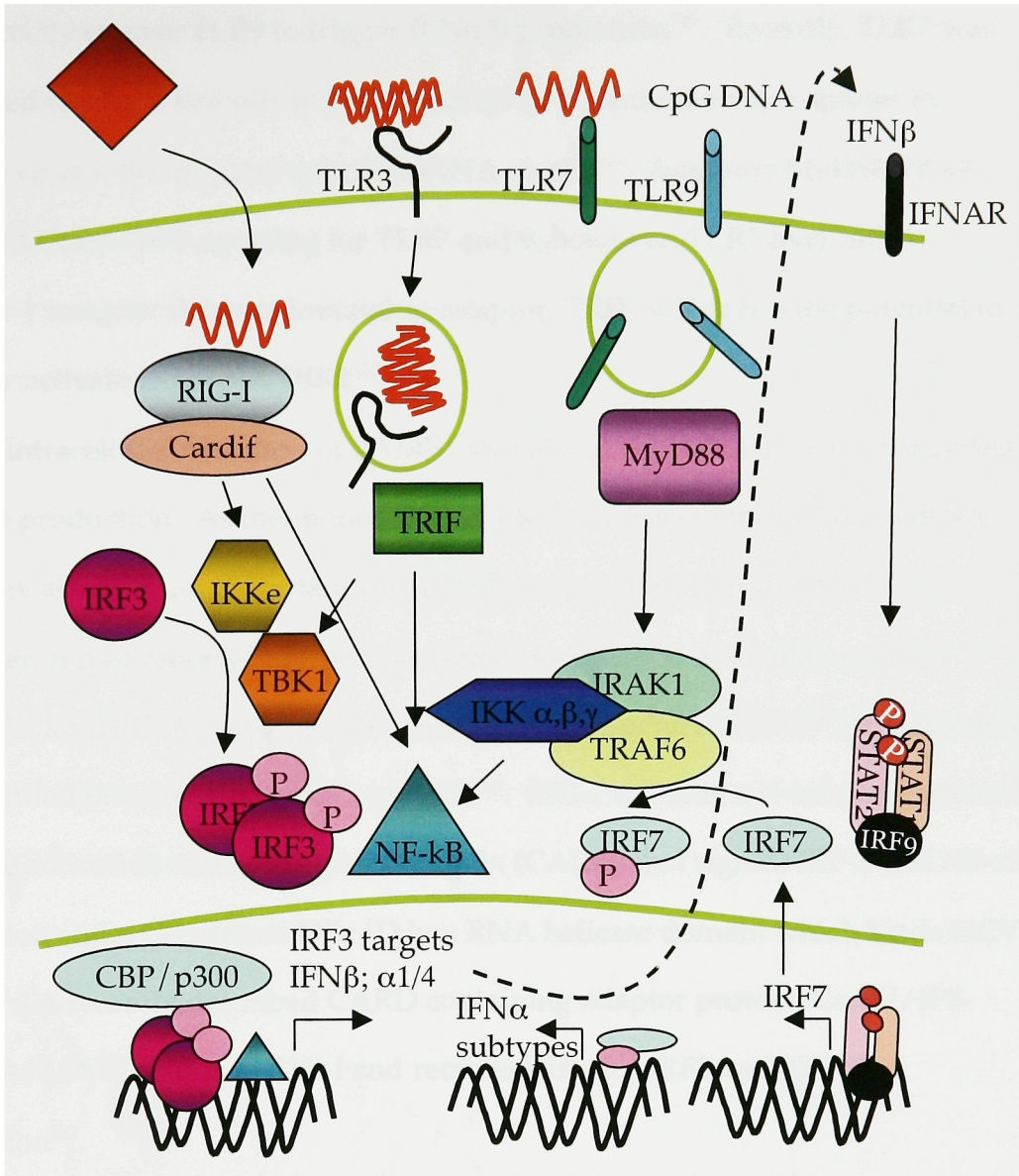

Figure 1.7. PAMP receptors detect viral infection and trigger IFNα/β response via the IFNα/β priming loop. Three key PAMP receptors, TLR3, TLR7, and TLR9, detect dsRNA, ssRNA, and CpG DNA, respectively, in endosomal compartments. Intracellular ssRNA triggers RIG-I and activation of IKKs as well as NF-κB via Cardif. TLR3 activates NF-κB and TBK1 via TRIF. Phosphorylation of IRF3 by IKKε and TBK1 allows for dimerization and interaction with CBP/p300 to trigger IFNβ transcription. IFNβ signaling triggers IRF7 expression. TLR7 and TLR9 signal via MyD88 to activate NF-κB and phosphorylate IRF7. The molecules involved in MyD88 dependent IRF3 activation are not known.

bacterial CpG motifs, viral DNA from herpesviruses (eg. HSV-1 and MCMV) can also directly engage TLR9 to trigger IFNα/β production[125]. Recently, TLR7 was proposed to play a key role in triggering IFNα/β production in response to ssRNA virus influenza and specific ssRNA motifs[126]. Adaptors MyD88, IRAK, and TRAF6 mediate signaling for TLR7 and 9; however, TLR3 may utilize Toll/IL-1 receptor domain-containing adaptor, TRIF, which has the potential to directly activate IKKε and TBK1[127].

Intracellular detection of PAMPs also plays a significant role in triggering IFNα/β production. As mentioned above, PKR can detect intercellular dsRNA and may also play an important role in triggering IFNα/β production [128]; however, recent reports have demonstrated the function of another crucial intracellular dsRNA sensor, retinoic-acid-inducible gene I (RIG-I)[129] or melanoma differentiation-associated gene 5 (mda-5)[130]. RIG-I contains a N-terminal tandem caspase activation and recruitment domain (CARD) that signals IRF-3 and NF-κB activation and a C-terminal DEx/D box RNA helicase domain which binds HCV RNA[131]. A recently described CARD containing adaptor protein, Cardif/IPS-1/VISA/MAVS, binds to RIG-I and recruits IKKs for IRF3 and NF-κB activation[132].

IFNα/β during HCV infection

In light of the potent antiviral effects of IFNα/β on HCV replication, it is understandable why HCV has evolved numerous mechanisms to disrupt IFNα/β signaling. Both and NS5A [133] and E2 [134] have been shown to inhibit PKR via direct

interaction. More recently, the NS3-4A protease has been shown to play a key role in disrupting IRF-3 phosphorylation[135] by cleaving Cardif[136] and TRIF[137].

Despite the multiple strategies to inhibit IFNα/β signaling, intrahepatic IFNα/β production during HCV infection is a unique characteristic of HCV infection. In contrast to hepatitis B virus (HBV) infection, HCV reaches high viral titers within 1 week of infection [138] and induces a robust IFNα/β response detected by IFN stimulated gene (ISG) expression [139]. Notably, induction of ISGs following infection does not correlate with viral titer and outcome suggesting that other factors such as genetics or concomitant risk factors play an important role. Alternatively, other features of IFNα/β's complex role in immunoregulation may impact the outcome of HCV infection[140].

The main producers of IFNα/β during HCV infection have not been identified. Clearly, hepatocytes are the main target of infection[141] and posses both RIG-I and TLR3 signaling pathways for triggering IFNα/β production[142]. However, although induction of ISGs are seen in liver tissue, IFNα/β transcripts are seen in PBMCs suggesting that a cellular component of PBMCs may be responsible for ISG induction in liver tissue during acute infection[143].

Plasmacytoid DCs: The Interferon Producing Cells

Although many cells are capable of producing IFNα/β in response to appropriate stimuli, the natural interferon producing cell (NIPC) has long been recognized as the most potent producer of IFNα/β among PBMCs[144]. These cells remained poorly characterized until the observation was made that NIPCs were a DC subset called plasmacytoid DCs (pDCs)[145]. In humans, pDCs were first

described as a CD11c negative DC subset [146,147] and proposed to be the poorly characterized plasmacytoid T cells long described by pathologists in lymphoid tissue [148]. These cells were originally characterized by their location in the T cell-rich extra-follicular regions and high expression levels of IL-3Rα (CD123) [149]. Subsequent studies have shown that IL-3 acts as a survival factor, but the immunological significance of pDC location is not clear.

Originally, pDCs were identifiable only by lineage negative and non-specific markers CD4, CD123, and HLA-DR. The generation of antibodies specific for pDCs allowed the direct enumeration, purification, and functional assessment of pDCs in circulating blood [150]. One of these antibodies recognizes a type II C-type lectin called blood DC antigen (BDCA)-2 [151]. Importantly, BDCA-2 is downregulated upon activation and antibodies to BDCA-2 inhibit IFNα/β production. Recently, antibodies against specific mouse pDC markers were developed (120G8 and PDCA-1) [152]. Hopefully, these reagents will give us a clear sense of pDC and pDC-derived IFNα/β in coordinating adaptive immunity.

As the most potent producers of IFNα/β, these cells play an important role in the response to viral infection. Notably, they are capable of producing robust amounts of IFNα/β after exposure to DNA and RNA viruses as well as bacteria. Further work showed that human pDCs express TLR7 and TLR9 [153], making them poised to respond to infectious pathogens. Interestingly, pDCs constitutively express IRF7 [154] allowing faster IFNα/β production, independent of feedback. Following activation by TLR or CD40L engagement, pDCs move to lymph nodes and produce IFNα/β in the T cell-rich areas [155,156]. A recent report describing the IRF7-/- mouse links a defect in pDC activity to a decrease in CpG

adjuvant effect on transgenic CD8+ T cells, but the immunologic significance of the pDC response in coordinating T cell activation is not well defined[157].

The role for pDCs in HCV pathogenesis is an area of intense research. As the main producers of IFNα/β, pDCs serve as a likely target for immune evasion strategies. As such, in chapter 2, I evaluate pDC function during chronic HCV infection. The question of pDC function is critical not only for understanding pathogenesis but also for evaluating the rationale of TLR7 and TLR9 agonist therapies for chronic HCV. A better understanding of the role for pDCs and IFNα/β in regulating HCV-specific T cell immunity may offer insight into pathogenic and therapeutic strategies for HCV.

1.5. The questions

In this thesis, I have endeavored to explore the role of DCs in HCV pathogenesis and the factors that act on DCs to regulate the immunologic outcome during HCV infection. I began these studies by setting up a clinical study at the Rockefeller University Hospital, CRI-0505, to explore the function of DCs in chronic HCV. The results of this study, discussed in chapter 2, pushed us to redefine the mechanisms by which HCV may affect DC function and immunologic outcome. In particular, I focused on the interplay between IFNα/β and CD4 help acting on DCs to regulate CD8+ T cell priming. Our surprising results, discussed in chapter 3, led us to explore the regulation of IFNα/β signaling by DC maturation and the implications of spatial location in dictating effects of cytokines on immunological outcome. In light of these results, I set up *in vivo* models to evaluate the ability of pDC IFNα/β production in the lymph node as a source of helper signals for CD8+ T cell priming, discussed in chapter 4. The results presented here offer significant support for HCV immunotherapy trials and provide a unique mechanism for IFNα/β in pathogenesis and immunity. It is my hope that these results will inspire support for new therapeutic strategies for HCV.

Chapter 2: Redefining the role for DCs in HCV pathogenesis: Normal DC function in patients chronically infected with HCV

2.1 Summary

HCV-specific T cell responses and IFNα play important roles in HCV immunity. Since conventional DCs (cDCs) are required for priming T cells *in vivo* and pDCs (pDCs) are the principal cell type responsible for endogenous IFNα/β production[158], an understanding DC function during HCV infection may offer insight into the crucial components of immunity. Initial reports showed impaired maturation and allostimulation of monocyte derived DCs [159-161] and pDCs[162] from chronic HCV patients suggesting a model of "DC dysfunction" to account for impaired immunity in chronic HCV. In order to evaluate the HCV specificity of these reports, we did a more careful analysis of absolute numbers, phenotypic markers, and functional activity of these DCs. In contrast to previous reports, we find no overt phenotypic or functional changes in monocyte derived DCs or circulating cDCs from chronically infected patients. Although our findings agree with previous reports of lower percentages of circulating pDCs in chronic HCV infection, single cell analysis suggests no overt defect in pDC IFNα production. This data challenges the old model of "DC dysfunction" in accounting for impaired HCV immunity. Furthermore, our analysis supports alternate models for weak T cell immunity in chronic HCV and offers data in support of cDC and pDC immunotherapy for chronic HCV.

2.2 Introduction

Role for DC subsets in HCV infection

As mentioned above the CD8+ T cell response has been identified to correlate with response to HCV infection. In light of the crucial role for cDCs in priming these CD8+ T cells, attention in the HCV field has been given to understanding the interaction of HCV with DCs (Fig. 2.1). The key questions in understanding this interaction center around infection of DCs as well as modulation of function. The prior lack of an infectious viral system and the technical difficulty in establishing reliable negative strand detection assays has precluded sound conclusions regarding DC infection[141,163]. In contrast, many tools exist to study the function of DCs both *in vitro* and *ex vivo*—but the data remain controversial as to the effect of chronic HCV infection on DC function.

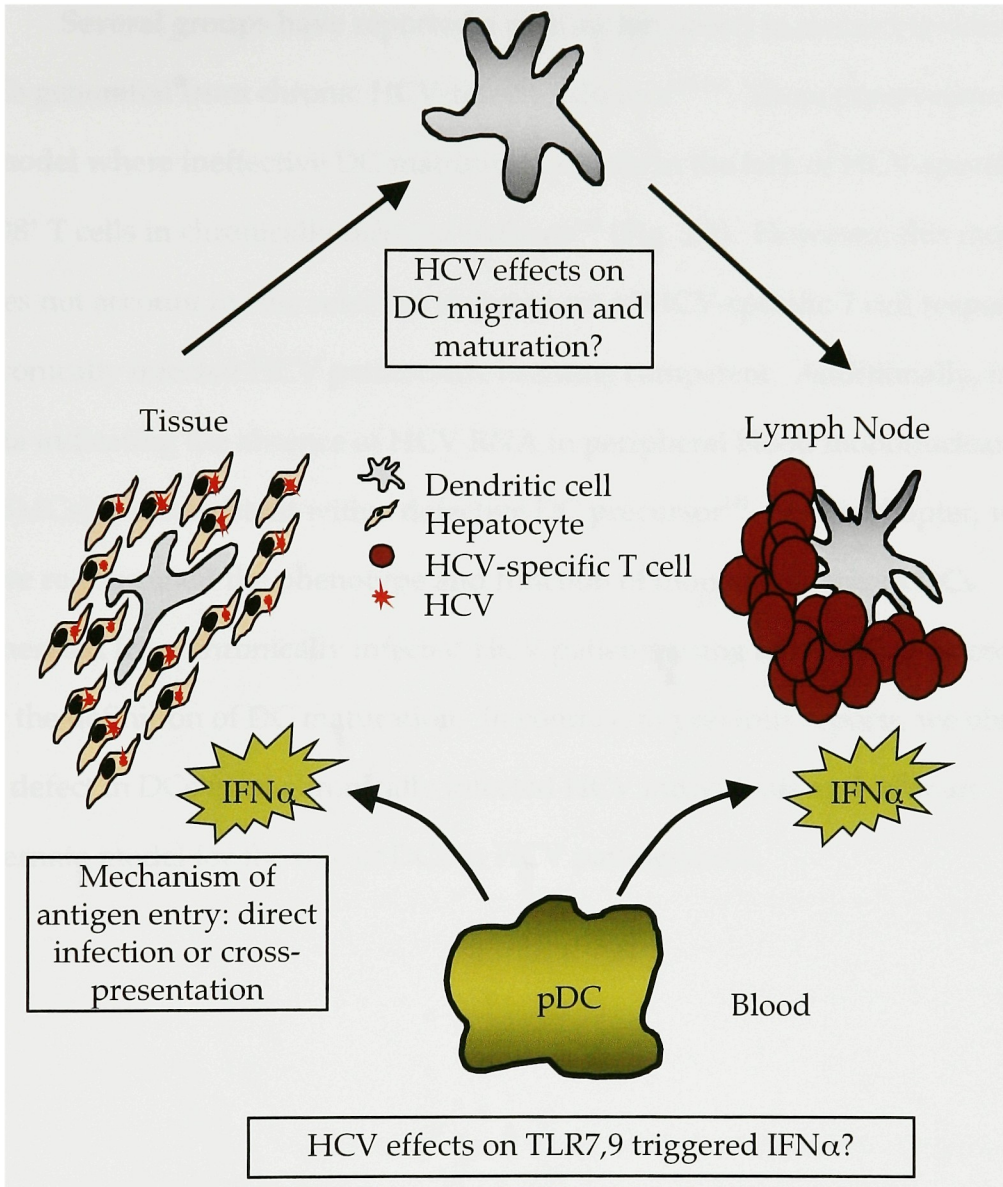

Figure 2.1. DCs play a pivotal role in HCV immunity. cDCs play a pivotal role in presenting antigen to the adaptive immune system. Antigen capture by direct infection or phagocytosis allow DCs to traffic antigen to the lymph node for T cell priming. pDCs are the main producers of IFNα/β and may influence infection in the tissue or immunity in the lymph node.

The old model: DC dysfunction in chronic HCV

Several groups have reported a maturation defect in monocyte-derived DCs generated from chronic HCV-infected donors[159-161]. These observations led to a model where ineffective DC maturation results in the lack of HCV-specific $CD8^+$ T cells in chronically infected patients[160] (Fig. 2.2). However, this model does not account for the selective impairment of HCV-specific T cell responses as chronically infected HCV patients are immune competent. Additionally, *in vivo* data indicating the absence of HCV RNA in peripheral blood mononuclear cells (PBMCs) is inconsistent with a defective DC precursor[141]. In this chapter, we have re-examined the phenotype and function of monocyte-derived DCs generated from chronically infected HCV patients using more stringent criteria for the definition of DC maturation. In contrast to previous reports, we observe no defect in DCs from chronically infected HCV individuals and offer an alternate model for the role of DCs in HCV pathogenesis.

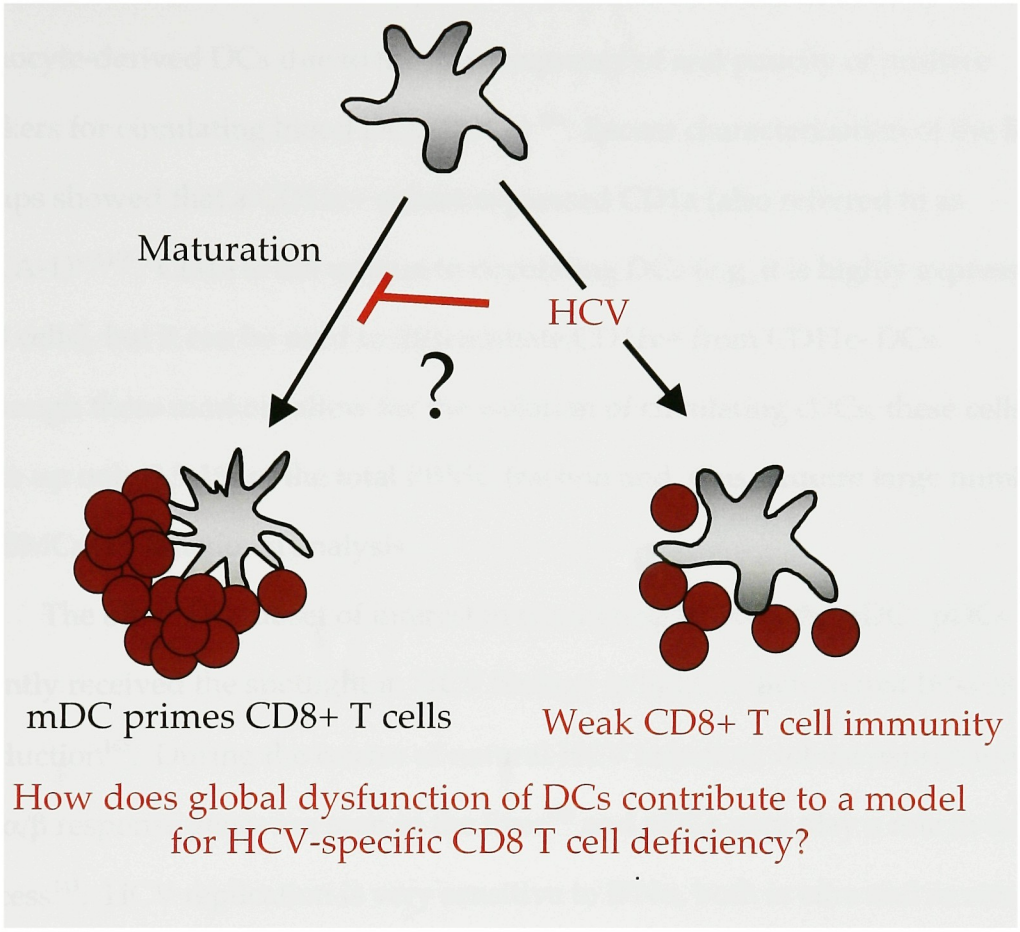

Figure 2.2. The "old model" for DCs in HCV pathogenesis. Previous reports have suggested that HCV inhibits DC maturation and consequently efficient T cell priming. However, this model does not account for the selective impairment of HCV-specific T cell responses as chronically infected HCV patients are immune competent.

Circulating cDCs and pDCs in human blood

A significant caveat of all the studies mentioned above is that they focus on *in vitro* derived DCs. In fact, the majority of human DC studies are based on monocyte-derived DCs due to the low frequency of and paucity of positive markers for circulating blood DCs (BDCs) [146]. Recent characterization of the BDC groups showed that a CD11c+ subset expressed CD1a (also referred to as BDCA-1)[164,165]. CD1a is not unique to circulating DCs (e.g. it is highly expressed on B cells), but it can be used to differentiate CD11c+ from CD11c- DCs. Although these markers allow for the isolation of circulating cDCs, these cells make up only 0.5-1% of the total PBMC fraction and, thus, require large numbers of PBMCs for functional analysis.

The other DC subset of interest in circulating blood is the pDC. pDCs recently received the spotlight in HCV biology in light of their robust IFNα/β production[145]. During the course of natural HCV infection, robust expression of IFNα/β response genes are seen in the liver[139] and pDCs may play a role in this process[143]. HCV replication is very sensitive to IFNα, both *in vitro* and *in vivo*, but while HCV has numerous mechanisms to avoid the downstream effects of IFNα[166], direct modulation of pDC function by HCV has not been shown. Furthermore, the immunoregulatory role of IFNα/β during HCV infection is not well understood.

pDCs may be distinguished based on surface expression of CD123, BDCA-2 or BDCA-4[150]. Although they exist at very low frequency (0.2 to 0.5% of PBMCs), cytometric analysis permits simultaneous gating of cell populations of interest and determination of functional activity (e.g. production of IFNα)[167]. In

this chapter, we provide a careful analysis of both cell populations in chronic HCV infection and conclude that there are no phenotypic or functional defects in the cDCs and no decrease in the amount of IFNα produced on a per pDC basis.

2.3 Study Design

CRI-0505: Chronically Infected HCV Patients (see Appendix 3)

Patient material was obtained as per Weill Medical College of Cornell University and The Rockefeller University Hospital Institutional Review Board approved protocols and all patients gave written informed consent. HCV infection was confirmed based on serology and/or plasma HCV titers. Chronic HCV was defined as detectable HCV viral load and sustained liver injury for greater than 6 months (as monitored by liver function tests and/or liver biopsy). IFNα / ribavirin therapy had been discontinued at least 6 months prior to sample collection. In addition, patients had not received any immune modulators or suppressors within 30 days prior to study entry. All patients tested negative for HIV, syphilis, and HBV within 30 days prior to study entry and were free of uncontrolled medical illness (including decompensated cirrhosis and any rheumatologic or immunologic disease). Cohort 1 (n = 16) was used to analyze monocoyte derived DCs (Table 2.1) and cohort 2 (n = 17) was used to evaluate circulating DC populations (Table 2.2). Samples were also collected from 17 non-HCV healthy donors. Sustained virologic responders (SVR, n=6) were HCV RNA negative six months after completion of pegylated-IFNα/ribavirin therapy. PBMCs were also obtained from patients with non-HCV liver disease (n=7) including hepatitis B, granulomatous hepatitis, and cirrhosis.

2.4 Materials and Methods

Isolation and preparation of cells

PBMCs, DCs, and T cells were prepared as previously described[168]. Briefly, PBMCs were isolated from whole blood by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ). T cell enriched and T cell depleted fractions were prepared by adherence to plastic in 1% single donor plasma. iDCs were prepared from the T cell depleted fraction by culturing cells in the presence of 1000 U/mL GM-CSF (Berlex, Seattle, WA) and 500-1000 U/mL IL-4 (R&D Systems, Minneapolis, MN) for 6 days[169,170]. To generate mDCs, cultures were stimulated on day 6 with 50 ng/mL TNFα (Alexis Biochemicals) and 0.1μM PGE-2 (Sigma) for 36-48 hrs[171]. cDCs were isolated using a BDCA-1 Isolation Kit (Miltenyi Biotec, Auburn, CA). Mature cDCs cultures were generated after purification with 50ng/mL TNFα (AlexisBiochemicals, San Diego, CA) and 0.1μM PGE-2 (Sigma, St. Louis, MO) for 36-48 hrs[171].

Allogeneic Mixed Leukocyte Reaction (Allo-MLR)

2×10^5 purified T cells/well were plated with cDCs at indicated ratios in 5% pooled human serum (Labquip LTD). Cultures were incubated for 4-5 days at 37°C and pulsed with 1μCi ^{3}H-thymidine during the last 14-16 hrs of culture. ^{3}H-thymidine incorporation was measured using a liquid scintillation counter (Packard Topcount). Allostimulatory index was generated by dividing the observed proliferation by the average maximal proliferation achieved when using mature cDCs at a ratio of 30 T cells : 1 DC. In all cases, maximal proliferation was > 100,000 counts per minute / 10^6 T cells used.

Detection of influenza-specific T cells by ELISPOT

mDCs were infected with influenza A/PR/8 (Spafas, Conn.) for 1hr at 37°C, as described[168]. DCs and T cells were plated in 96-well Millipore plates coated with 5mg/mL of anti-IFNγ mAb (Mabtech, Mab-1-D1K). Cultures were incubated for 24-36 hrs at 37°C, washed with mild detergent, and incubated with 1μg/mL biotin-conjugated anti-IFNγ mAb (Mabtech, MAB 7BG-1). Spots were visualized using Vectastain Elite Kit (Vector Laboratories). Evaluation was performed in a blinded fashion by an independent service (Zellnet Consulting, Inc., NY) using an automated ELISPOT reader (Carl Zeiss, Inc., NY). Spots represent IFNγ production by single cells and are reported as spot forming cells (SFCs)/10^6 cells.

Cell enumeration

Flow cytometric analysis was employed to enumerate different hematopoetic lineages in PBMCs using a Blood Dendritic Cell Enumeration Kit (Miltenyi Biotec, Auburn, CA). Briefly, scatter properties of the cells as well as CD19 and CD14 staining enabled enumeration of B cells and monocytes, respectively. Simultaneous labeling with anti-CD1c (BDCA-1) and anti-BDCA-2 monoclonal antibodies (mAb) allowed selection of CD19 negative cells and enumeration of the BDCA-1^+ cDCs and the BDCA-2^+ pDC populations. Absolute numbers of the respective cell populations were normalized to the total number of PBMCs collected to obtain the relative percentage of each cell type.

IFNα production by Intracellular Cytokine Staining (ICCS) and ELISA

PBMCs were resuspended in DMEM 10% FBS at 5 x 10^6/mL and stimulated with either media alone; 50 hemagglutinin units (HAU)/ml Influenza A/PR8/1976; or 5μg/mL CpG (2216: 5'-GGGGGACGATCGTCGGGGGG-3'). Cultures were incubated for 5 hrs, harvested and fixed using Cytoperm/Cytofix kit (BD Pharmingen) and stained with anti-IFNα (MMHA-11, PBL Laboratories, Piscataway, NJ) followed by goat anti-mouse PE (Jackson Immunochemical, West Grove, PA). BDCA-2 FITC and BDCA-4 APC antibodies were used to label pDCs in the PBMC fraction, post-stimulation. To measure the quantity of total IFNα produced, supernatants from parallel cultures were collected after 24 or 48 hrs and assayed using a human IFNα ELISA (PBL laboratories, Piscataway, NJ). Importantly, all PBMC samples used for IFNα assays were freshly prepared and used within 12 hrs as IFNα production was significantly affected by freeze-thaw (>10-fold decrease in IFNα production in parallel samples). IFNα production was normalized per pDC based on the percentage of pDCs in the PBMC fraction.

Statistics

Two tailed non-parametric comparisons (Mann-Whitney test) were used to calculate *p* values for differences in cDC and pDC enumeration, as well as total IFNα production by PBMCs, as this method allows for data with a non-Gaussian distribution. *p* values for normalized data in Fig. 2D was evaluated with a two-tailed parametric comparison (unpaired t test, Welch corrected).

J6/JFH-1 chimeric infections

High titer J6/JFH-1 chimeric HCV was obtained from Brett Lindenbach (Rice lab, RU). Briefly, this virus was created from the JFH-1 HCV cDNA (which can replicate *in vitro* without adaptive mutations[18]) recombined with core-NS2 region from a related genotype 2a strain, J6[16]. iDCs and a hepatoma cell line (Huh7.5) were exposed to J6 / JFH chimeric HCV or mock virus supernatant for one hour at 37°C at 10^6/mL. DCs were washed and resuspended in 1% human serum with maturation cocktail TNFα / PGE-2. Huh7.5 cells were washed and fresh media was added. Infection was assayed by FACS analysis of NS5A expression.

NS5A expression by FACS analysis

Indicated cells were resuspended at 10^6/mL, washed in PBS, and fixed for 10 minutes in 100μl cytoperm/cytofix (BD Pharmingen) per 10^5 cells at 4°C. Cells were washed twice in 1x perm wash (BD Pharmingen) and stained with mouse monoclonal anti-NS5A (9E10, generated by Tim Tellinghuisen, Rice lab, RU) at 1:50 for 15 minutes at 4°C and goat anti-mouse IgGs PE (Jackson Immunoresearch) at 1:400 for 15 minutes.

2.5 Results

Functional monocyte-derived DCs can be generated from patients with chronic HCV.

PBMCs were isolated from chronic HCV patients (cohort 1, Table 2.1) and used for the generation of iDCs. Surface expression of CD14, CD25, CD40, CD54, CD83, CD86 and HLA-DR measured by FACS analsysis showed similarity to normal iDCs (Fig. 2.3A). Following exposure to TNFα and PGE-2, patient DCs

Table 2.1. Characteristics of study participants for monocyte-derived DCs (Cohort 1)

Patient ID	Age	Sex	Geno type	ALT/ AST	Viral Load (10^5)*	Histology	Date of Dx or Prior Tx
3 (Chronic†)	52	M	1a	41/42	189.0	Fibrosis: Stage 2	Dx in 2/99; Peg-Ribavirin/IFN
33 (Chronic)	52	F	1a	NA	4.6	Fibrosis: Grade 2, Stage 2	Unknown
51 (Chronic)	47	M	1a	76/48	6.9	Fibrosis: Grade 2, Stage 2	Dx in '96; Tx naive
104 (Chronic)	53	M	1b	45/41	14.8	Fibrosis: Grade 2, Stage 2	Dx in '65; Peg-Ribavirin x 3
126 (Chronic)	48	M	2a	59/31	NA	No biopsy taken	Tx Naive
351 (Chronic)	61	F	1b	80/96	5.2	Fibrosis: Grade 2, Stage 3	Tx Naive
402 (Chronic)	20	M	N/A	41/31	1.3	No biopsy taken	Dx '01; Tx naive
689 (Chronic)	69	M	1a	88/55	47.1	Fibrosis: Grade 2, Stage 2-3	Peg-Ribavirin/IFN
794 (Chronic)	49	F	N/A	35/70	0.5	Cirrhosis: Grade 3, Stage 4	Dx in '78; Tx naive
804 (Chronic)	22	M	1b	188/ 113	11.6	Advanced Fibrosis, Grade 2, Stage 4	Dx in 3/02; Tx naive
812 (Chronic)	65	F	1	51/42	16.2	Fibrosis: Stage 1	Dx in '60s; Peg-Ribavirin/IFN
815 (Chronic)	68	M	N/A	247/ 112	4.5	No biopsy taken	Dx in 9/01; Tx naive
936 (Chronic)	42	F	1a	19/14	9.6	Fibrosis	Dx in '95; Tx naive
237 (SVR§)	52	M	1a	14/17	BD**	Cirrhosis: Stage 4, Grade 4	Dx '00; Peg-Ribavirin/IFN
813 (SVR)	42	F	4a	5/9	BD	No biopsy taken	Dx in '97; Peg-Ribavirin/IFN
976(SVR)	41	M	N/A	131/25	BD	No biopsy taken	Dx '79; Peg-Ribavirin/IFN

† Chronic patient defined as individual with HCV viral load and sustained liver injury for greater than 6 months as monitored by ALT/AST and/or liver biopsy.
§ Sustained Virologic Responder (SVR) defined as individual with evidence of HCV infection but maintained undetectable viral load more than 6 months after termination of therapy.
* Viral load defined by quantitative PCR at time of blood draw (Roche Amplicor™). Data not available (NA) for PT #126; prior data confirmed status as chronically infected.
** BD stands for below detection.

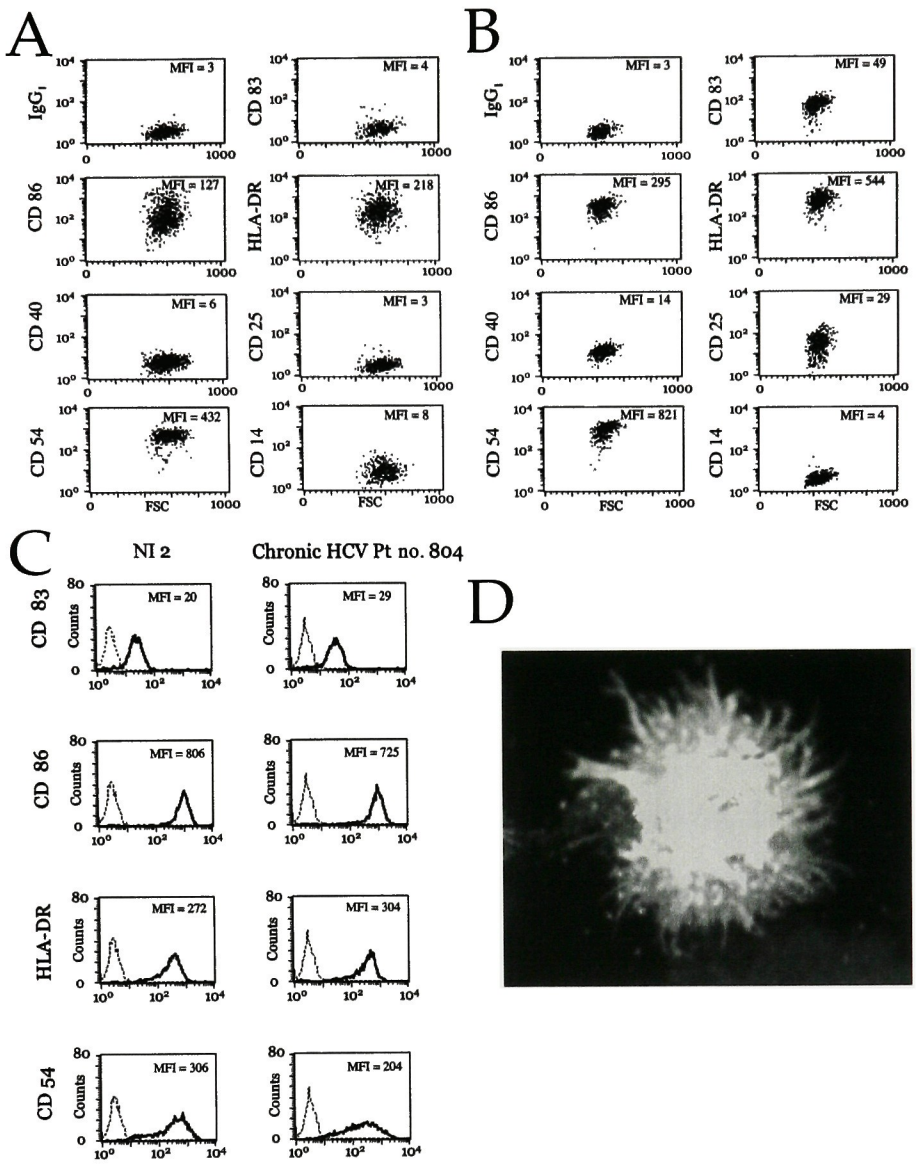

Figure 2.3. Monocyte-derived DCs from chronic HCV patients undergo normal TNF-α dependant maturation. A, B. Representative examples of iDCs (A) and mDCs (B) from a chronic HCV patient #3 are shown. Geometric mean fluorescence intensity (MFI) is indicated. **C.** Representative examples of mDCs from a normal donor and chronic HCV patient #804 are shown. Surface expression of the indicated marker (dark lines) is overlaid with the isotype control (dotted line). All antibodies were obtained from BD Biosciences. **D.** mDCs from chronic HCV patient #936 were adhered to Alcian blue treated coverslips, fixed with 3.4% PFA and stained with anti-MHC II (clone 93C9), followed by secondary anti-mouse IgG Alexa 488. Cells were visualized using a Zeiss Axioplan 200 microscope.

expressed surface markers characteristic of mDCs (Fig. 2.3B). Direct comparison of mDCs from chronic HCV patients and normal donors showed similar expression of CD83, CD86, HLA-DR, and CD54 (Fig. 2.3C). Furthermore, patient DCs displayed a stellate morphology characteristic of mDCs (Fig. 2.3D). Maturation was TNFα-dependent, as PGE-2 alone did not induce phenotypic maturation.

We next evaluated patient DCs for their ability to prime allogeneic T lymphocytes as a measure of their functional capacity. Notably, proliferation was robust (>10^5 CPM at 90:1 T:DC ratio, Fig. 2.4A) and specific, as demonstrated by the lack of proliferation in syngeneic T cells (data not shown). Comparison of T cell priming capacity of phenotypically normal iDCs and mDCs from chronic HCV and normal donors provides functional data to support the normal TNFα-dependent maturation in both patient populations (Fig. 2.4A). Normalized allostimulatory capacity of DCs from healthy *versus* chronic HCV-infected donors is shown (Fig. 2.4B). We conclude that monocyte-derived DCs generated from patients chronically infected with HCV are phenotypically and functionally normal.

While it is difficult to demonstrate DC function *in vivo*, one measure employed is the evaluation of circulating memory T cells. We show that influenza-reactive T cells derived from patients can be effectively re-stimulated using syngeneic mDCs, as measured in an IFNγ ELISPOT (Fig. 2.4C). Precursor frequency of influenza-reactive T cells in HCV patients (n=5), measured by spot forming cells (SFCs) / 10^6 T cells, fell within the range of normal individuals

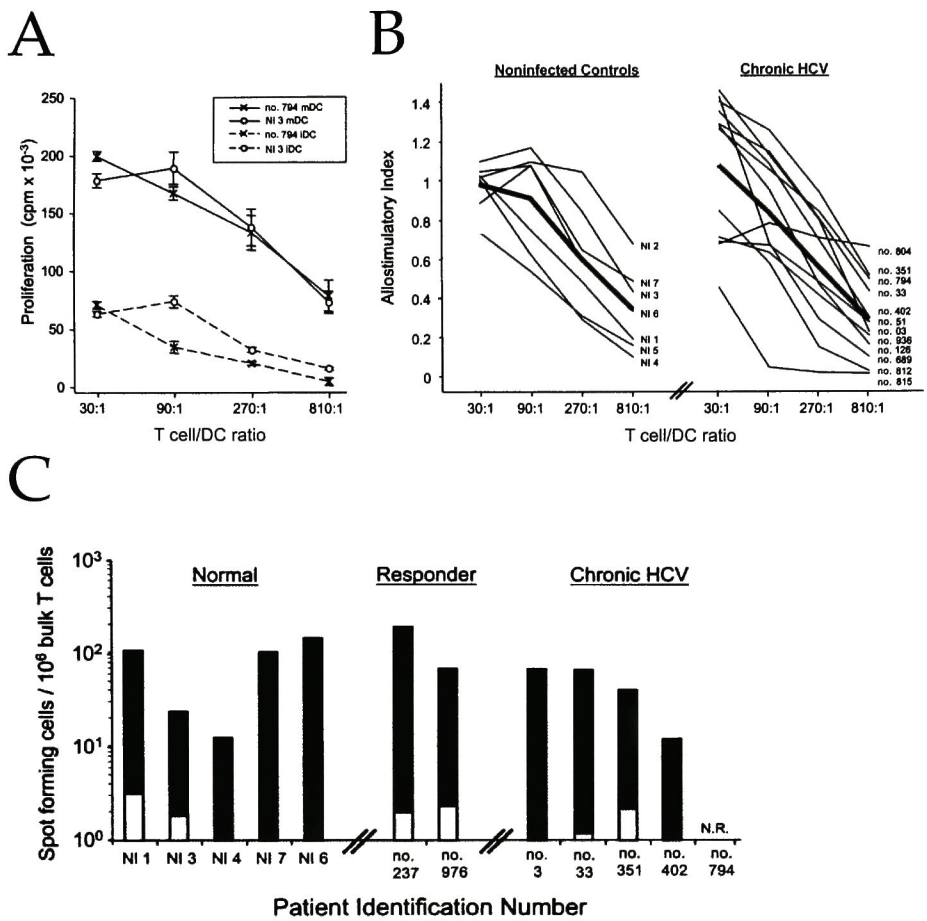

Figure 2.4. Mature DCs derived from chronic HCV patients prime allogeneic T cells and stimulate antigen specific syngeneic memory T cells. A. Allo-MLR T cell priming by iDCs and mDCs from normal (Nl) donor 3 and chronic HCV patient #794 are shown. Error bars indicate +/- SD. **B.** Allostimulatory responses of mDCs from all tested normal donors and chronic HCV patients were normalized to values of the normal donor at 30:1 to generate an allostimulatory index and plotted as thin lines with patient identification numbers noted. Thick black line represents mean allostimulatory capacity. **C.** IFN-γ production by influenza-specific T cells stimulated with either uninfected (white portion of bar) or infected mDCs (black bar) from all patients tested. Data from normal, responder and chronic HCV patients are shown as spot forming cells (SFCs) per 10^6 T cells, plotted on a logarithmic scale. NR indicates <50 SFC per 10^6 T cells.

(pCTL range in humans is 0.02-0.15% based on seven control patients studied here and >350 ELISPOT assays performed in our laboratory).

Lower numbers of circulating DCs in chronic HCV patients

Despite the *in vivo* implications of this work, the caveat remains that these are data for *in vitro* derived cells. In order to explore the small populations of circulating DCs *in vivo* during chronic HCV infection (cohort 2, Table 2.2), we employed flow cytometric analysis. Using the scatter properties of the cells (size and granularity), as well as staining with anti-CD19 and anti-CD14 mAbs, it was possible to enumerate B cells (Fig. 2.5A, R2) and monocytes (Fig. 2.5A, R3), respectively. Cells were simultaneously labeled with anti-CD1c (BDCA-1) and anti-BDCA-2 mAbs, thus allowing selection of CD19- cells (Fig. 2.5A, R4) and enumeration of the BDCA-1^+ cDCs (Fig. 2.5A, R5) and the BDCA-2^+ pDC (Fig. 2.5A, R6) populations. Absolute numbers of the respective cell populations were normalized to the total number of PBMCs collected to obtain the relative percent of each cell type (Fig. 2.5B). Despite greater heterogeneity in the percentage of B cells in HCV patients, seen here, as well as previous studies[28], we observed no statistical differences in the median percentage of B cells or monocytes. We did, however, reveal a small but marginally significant difference in the percentage of cDCs in chronic HCV compared to normals (0.62 vs. 0.83, p=0.05, respectively). In addition, the data indicate a reduction in the percentage of pDCs in chronic HCV patients (0.11 vs. 0.34, p=0.004) as compared to normal individuals. While potentially important to disease progression, this observation is not specific for chronic HCV infection. We find lower numbers of DCs in sustained virologic

Table 2.2. Characteristics of HCV patients in DC subset evaluation (Cohort 2)

Patient	Age, years	Sex	Genotype	ALT/AST score	HCV load, IU/mL ($\times 10^6$)[a]	Histological finding[b]	Treatment
1181[c]	55	M	NA	60/89	62.5	NA	Naive
347[c,d]	51	F	1a	207/145	431	Grade 3, stage 2	Peg-IFN/RBV
1142 [c,d]	46	F	1b	40/43	128	Grade 2, stage 3–4	Naive
1060[c]	38	F	1	129/77	42.2	Stages 1–2	Naive
107	33	M	1a	74/28	2.5	Grade 3, stage 3	Peg-IFN/RBV (treated in 2003)
1128	57	F	1b	51/NA	45	Stage 2	Naive
1127[d]	54	F	2a/c	138/93	14.6	Grade 1, stage 1	Naive
959	29	M	2b	235/87	385	Grade 3, stage 3	Naive
967	64	M	1a	48/31	NA	Grade 3, stage 3	Naive
1133	47	M	1a	48/42	182	NA	NA
840	43	M	1b	66/40	222.2	Grade 1, stage 3	Naive
501	58	F	2b	29/30	>100	Grade 3, stage 1	Naive
522	60	F	1b	82/60	27 7	Grade 2, stage 2	Naive
573[d]	52	M	1a	24/33	NA	Grade 2, stage 2	Peg-IFN/RBV (treated in 2003)
1166[d]	46	M	1a/b	63/51	872	NA	Naive
1161[d]	70	F	1a	204/113	264	Grade 2, stage 1	Naive
400[d]	53	M	1a	104/106	13	Grade 3, stage 4	Naive

NOTE, A patient with chronic HCV infection was defined as an individual with a detectable HCV load and sustained liver injury for >6 months, as monitored by liver function tests (alanine aminotransferase [ALT]/aspartate aminotransferase [AST]) and/or liver biopsy. IFN, interferon; NA, not available; PEG, pegylated; RBV, ribavirin.

[a] HCV load defined by quantitative polymerase chain reaction at the time of collection of blood samples (Roche Amplicor). Data were not available for patients 967 and 573; prior data confirmed that they had chronic HCV infection.

[b] Histological criteria defined as in [16].

[c] Indicates leukapheresis sample.

[d] Indicates fresh sample used for analysis of IFN-α production; no marking indicates a frozen sample.

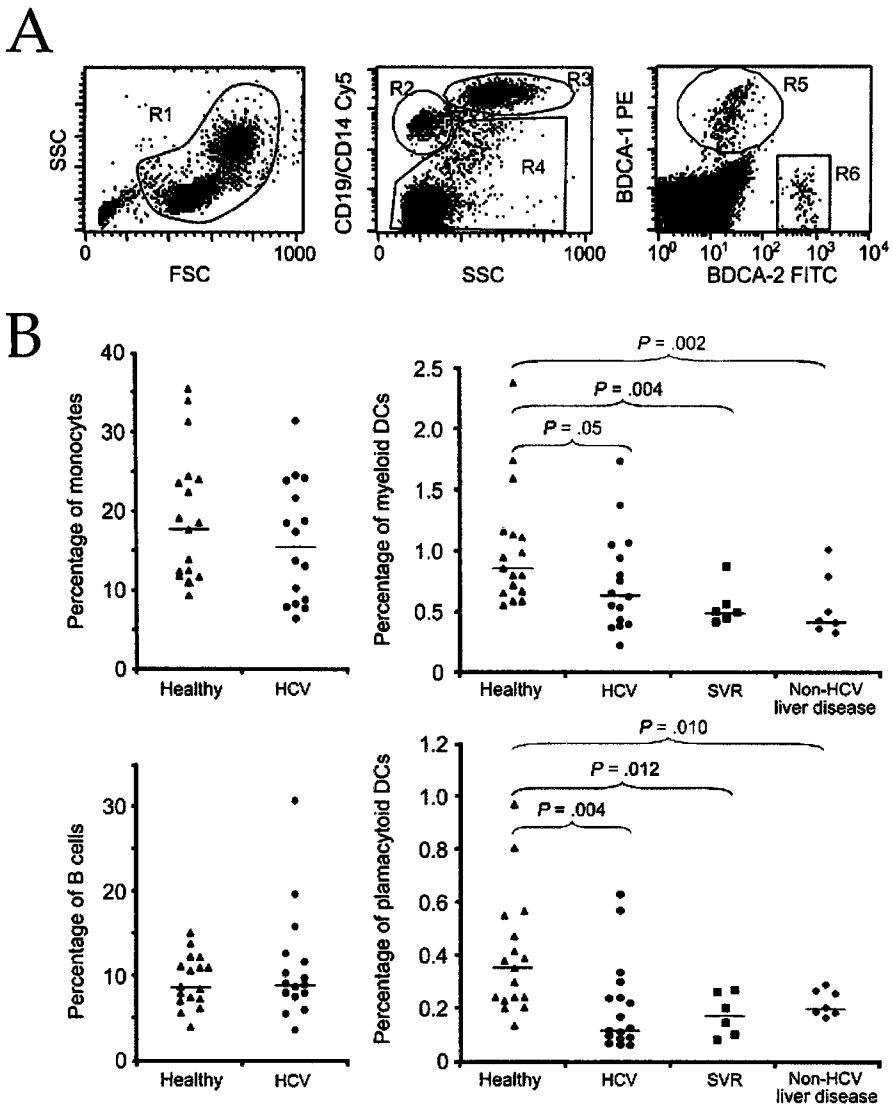

Figure 2.5. DC Enumeration in chronic HCV. A. Cytometric-based enumeration strategy. PBMCs are gated in R1 based on Forward Scatter (FSC)/Side Scatter (SSC). B cells and monocytes are identified by CD19 and CD14 Cy5 labeling, respectively, and are differentiated based on SSC profile. B cells are gated by R2 and monocytes are gated by R3. CD19- and CD14 low cells are gated by R4 and used to enumerate BDCA-1 PE DCs (R5) and BDCA-2 FITC (R6). **B.** The percentage of monocytes and B cells are graphed for normal (filled triangles) or chronic HCV (filled circles) donors. The percentage of DCs (BDCA-1$^+$) and plasmacytoid DCs (BDCA-2$^+$) in normal, chronic HCV (HCV), sustained virologic responders (SVR, filled squares), and non-HCV liver disease (filled diamonds) are also displayed graphically. A Mann-Whitney test was used to generate p values for comparison of normal donors (n=17) and individuals with chronic HCV (n=17).

responders (n=6) as well as patients with non-HCV liver disease (n=7). Low numbers of pDC have also been observed in patients with HIV / AIDS [172].

Normal phenotype and function of circulating cDCs and pDCs

In order to isolate cDCs and evaluate their functional characteristics, large numbers of PBMCs were collected by leukapheresis from four chronically infected patients. Surface expression of phenotypic markers was measured by flow cytometric analysis and indicated a phenotype suggestive of immature DCs—$CD14^-$, $CD25^-$, $CD40^-$, $CD83^{lo}$, $CD86^{int}$ and $HLA\text{-}DR^{int}$ (Fig. 2.6A, gray line). Following exposure to TNFα, upregulation of maturation markers CD25, CD40, CD83, CD86, and HLA-DR was observed (Fig. 2.6A, thick black line). This upregulation corresponded with a functional change in allostimulatory potential characteristic of a mDC (Fig. 2.6B). Of note, the phenotype and priming capacity of cDCs derived from patients with chronic HCV was equivalent to that observed in normal individuals (Fig. 2.6B).

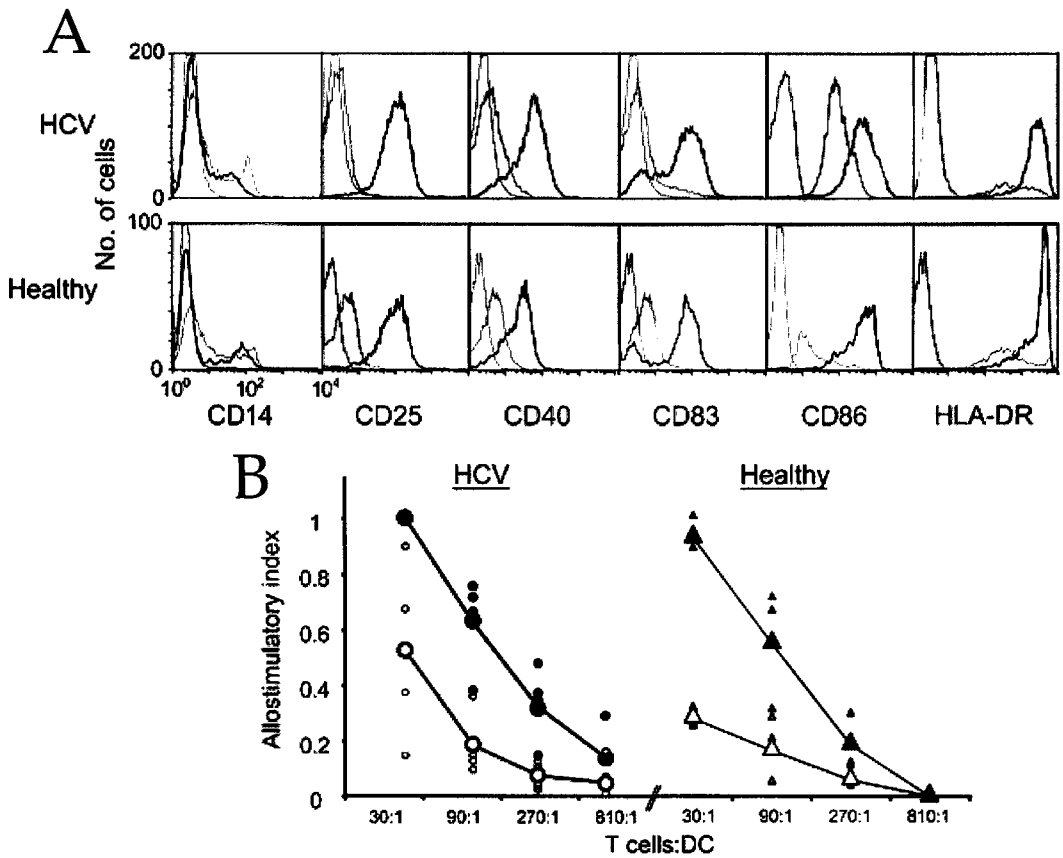

Figure 2.6. Normal phenotype and function of myeloid DCs in chronic HCV patients. A. FACS phenotype of myeloid DCs (BDCA-1$^+$) is shown for representative chronic HCV patient #1181. Analysis was performed following CD19 depletion and BDCA-1 positive selection (90% purity, data not shown). Thick black line, myeloid DCs + TNFα; thick gray line, myeloid DCs; thin black line, isotype control. **B.** Allostimulatory responses for myeloid DCs purified from 4 chronic HCV patients and 3 normal individuals were evaluated. BDCA-1$^+$ purified cells alone or cells exposed to TNFα, were placed into co-culture with allogeneic T cells, and cultured for 5 days. Proliferation was monitored by thymidine incorporation and data was normalized to the average maximum stimulation by mature myeloid DC as described in the study design section. Open circle, myeloid DCs or filled circle, myeloid DCs + TNFα (HCV patients); open triangle, myeloid DCs or filled triangle, myeloid DCs + TNFα (Normal controls). Average proliferation responses across the patient populations are indicated by solid lines.

We next addressed the function of the pDCs in chronic HCV patients. In humans, pDCs produce robust amounts of IFNα after engagement of TLR7, a receptor for ssRNA, or TLR9, a receptor for CpG motifs[153]. We stimulated the PBMCs with live influenza virus or CpG-2216 and measured IFNα production by ELISA (Fig. 2.7A). Notably, IFNα production was lower in patients with chronic HCV as compared to normal individuals when stimulated with 50 HAU of influenza (3.9 ng/ml vs. 12.3 ng/ml, p=0.07, respectively) or CpG-2216 (4.9ng/ml vs. 26.1 ng/ml, p=0.04, respectively). However, when normalized to the respective percentage of pDCs in the PBMC sample, the difference in IFNα secretion was no longer statistically significant between chronic HCV patients and normals for influenza (1.3 pg/ml vs. 1.9 pg/ml, respectively) or CpG (2.0 pg/ml vs. 2.9 pg/ml, respectively) (Fig. 2.7B). These findings suggest that while lower in number, the pDCs are unimpaired in their production of IFNα in response to TLR ligands.

In order to verify IFNα production by pDCs and to evaluate the percentage of IFNα+ pDCs, we assayed stimulated PBMCs for intracellular IFNα by flow cytometric analysis after 5 hr of stimulation with influenza or CpG-2216 (Fig. 2.7C). Only the pDCs produced measurable IFNα under these conditions and by gating on this population, the percentage of IFNα producing pDCs could be assessed (Fig. 2.7D). The data indicate that despite the decreased percentage of pDCs in the circulating blood, the pDCs respond equally well to influenza virus and CpG motifs on a per cell basis (Fig. 2.7B, D).

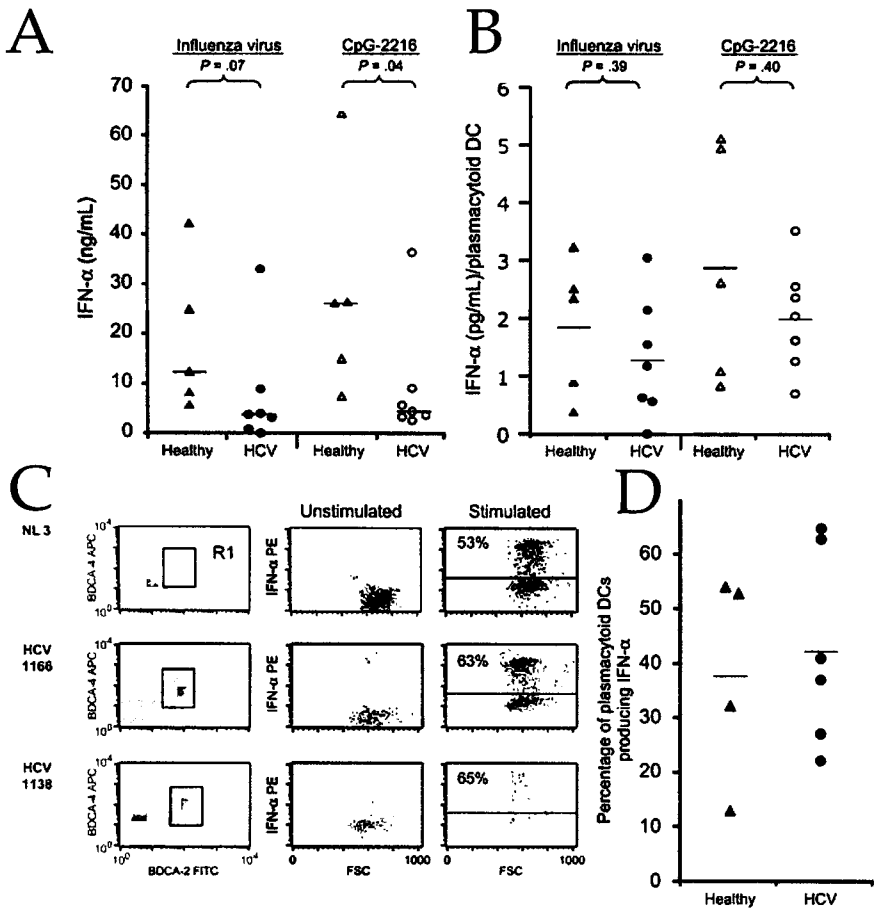

Figure 2.7. pDCs from chronic HCV patients produce IFNα in response to TLR stimulation. A. IFNα production in the supernatant of PBMCs stimulated with 50 HAU of influenza or 5μg/ml CpG-2216 was evaluated by ELISA. Median values are indicated by black lines and *p*-values were determined using a Mann-Whitney test. **B.** Data from the ELISA was normalized based on the respective percentage of pDCs present in the PBMC fraction. *p*-values were determined using an unpaired t test, Welch corrected. **C.** pDCs were identified in the PBMC population using lineage-specific markers BDCA-4 APC and BDCA-2 FITC (Gate R1). Intracellular IFNα staining in unstimulated or stimulated (50 HAU influenza) conditions was measured by cytometric analysis and the percentage of IFNα$^+$ pDCs is represented in a dot-plot. Patient #1166 represents a chronic HCV donor with normal pDC (BDCA-2$^+$) numbers (0.62%). Patient #1138 represents a chronic HCV donor with low pDC (BDCA-2$^+$) numbers (0.05%). **D.** The percentage of pDCs responding to influenza stimulation and producing IFNα in 6 chronic HCV and 4 normal donors is graphically represented. Black lines indicate average percentage of IFNα$^+$ pDCs across the respective patient population tested.

No robust infection of DCs in vivo or in vitro

Finally, we evaluated productive infection in these DC subsets as detected by non-structural protein expression. Although RNA and structural proteins may stick to circulating DCs via binding receptors such as DC-SIGN, non-structural protein expression indicates viral replication. Purified cDCs and pDCs were permeabilized and stained with anti-NS5A. In all cases, neither cDCs nor pDCs showed expression of NS5A as monitored by FACS analysis (Fig. 2.8A).

Recently, infectious chimeric HCV J6/JFH was reported to infect and replicate in hepatoma cell lines *in vitro*[16]. In order to test if this virus could infect DCs, monocyte derived DCs were exposed to virus and analyzed after 3 days. Despite robust infection of Huh7.5 hepatoma cells, no infection of monocyte derived DCs was seen 3 days post-infection, as monitored by intracellular NS5A staining (Figure 2.8B).

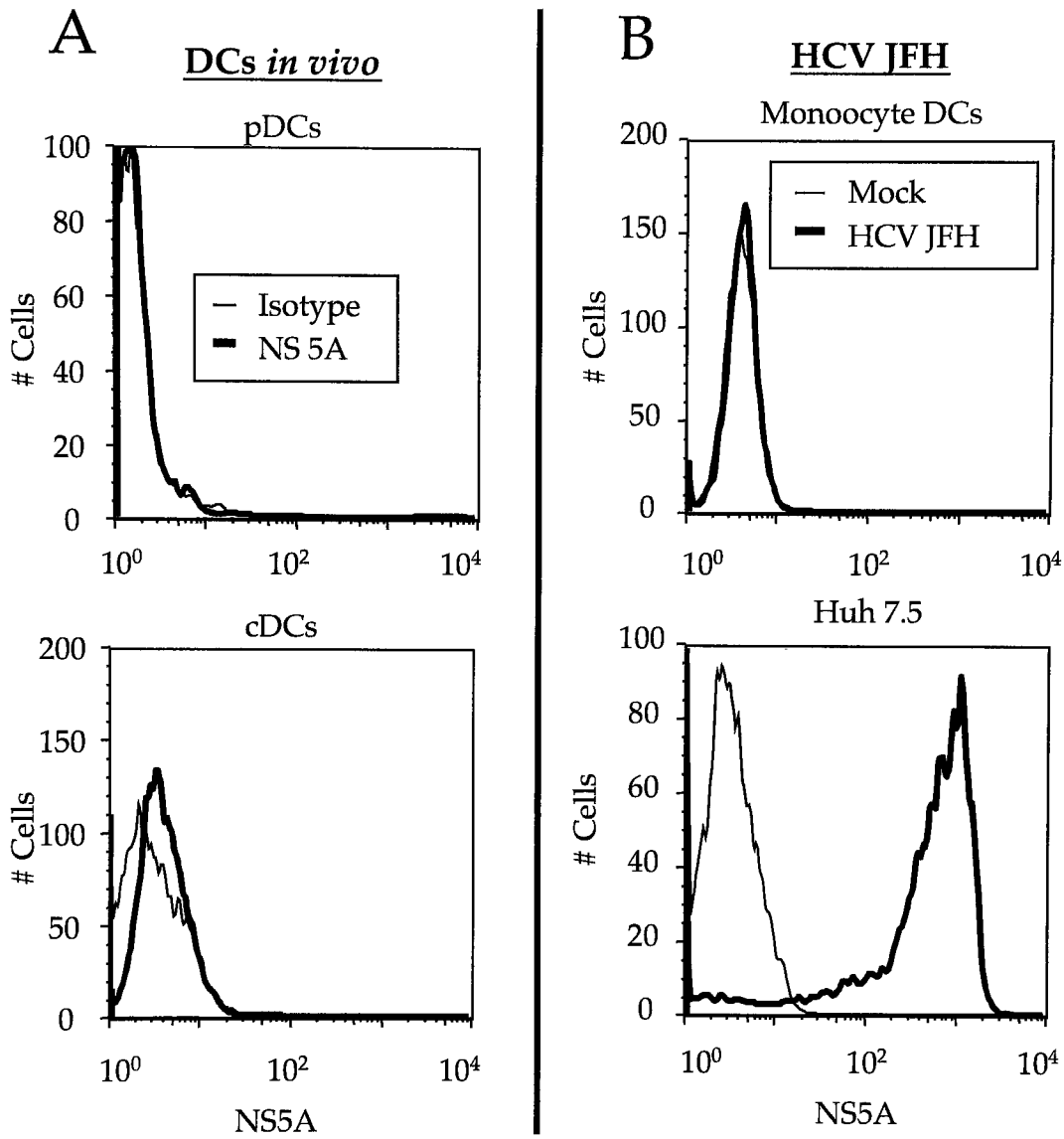

Figure 2.8. No robust replication of HCV in DCs *ex vivo* or *in vitro*. A. pDCs or BDCA-1+ myeloid DCs were purified from an HCV+ donor and stained for NS5A expression. **B.** Monocyte derived DCs or Huh7.5 hepatoma cells were exposed to J6/JFH chimeric virus. NS5A expression was monitored after 3 days.

2.6 Discussion

Controversial results in monocyte-derived DCs

In this study, we have characterized HCV patient DCs, demonstrating phenotypically normal and functionally active mDCs. This finding differs from previous reports[159-161], which we suggest is due to differences in culturing conditions. It is possible that incomplete maturation resulted in a perceived functional defect in the study by Auffermann-Gretzinger et al., as suggested by the presence of contaminating $CD83^-$ cells in the FACS plots of normal individuals mDC cultures[160]. Specifically, we do not find evidence for a defect in TNFα mediated DC maturation as demonstrated by a clear shift in allo-stimulatory capacity when iDC are effectively matured (Fig. 2.4A). T cell priming with mDCs (Fig. 2.4A, B) contrasts other studies where reported thymidine incorporation was 10-fold lower than typically observed[159], again reflecting poor growth conditions for DCs and or T cells in allo-MLR cultures. In our study, using adequate conditions for DC maturation and T cell priming, monocyte-derived DCs from chronic HCV patients are found to be both phenotypically normal and functionally active. Although these findings are from *in vitro* derived DCs, the presence of robust influenza specific T cell responses suggests that *in vivo*, chronic HCV patients maintain the ability to support central memory T cells. It also offers additional evidence for functionally competent mDCs in chronic HCV patients, and illustrates that the observed defect in the HCV-specific T cell repertoire does not extend to other viral antigens (Fig. 2.4C). These data are consistent with the current understanding of immune function in and pathogenesis of HCV, which suggests that the immune defect is HCV-specific. These results are also consistent with the growing belief that *in vivo*,

PBMCs are not typically infected by HCV ([141] and data not shown). Moreover, they offer crucial support for DC-based immunotherapy strategies for HCV.

Despite our description of functional monocyte-derived DCs in chronic HCV, this data does not rule out a possible *in vivo* DC (or DC subset) defect. For example, as mentioned in chapter 1, HCV may directly interact with DCs via DC-SIGN[94,95] to modulate DC function in an HCV-specific fashion. Initial reports evaluating circulating cDCs suggest that functional defects in DCs are associated with HCV infection, but no direct mechanism for the putative DC dysfunction has been demonstrated[173,174]. Furthermore, these reports do not account for the HCV-specificity of the immune defect. In order to evaluate these results in circulating DCs, we designed and inititated a clinical trial, CRI-0505, which allowed for the procurement of large numbers of PBMCs for BDC analysis.

Lower numbers of circulating DCs in chronic HCV patients

Similar to several recent studies[162,174], our data support the finding of lower percentages of circulating cDC and pDCs in PBMCs from chronic HCV patients (Fig. 2.5). However, these findings do not necessarily seem specific for HCV—SVRs and patients with non-HCV liver disease show similarly lower numbers. Further, HIV patients are reported to have lower circulating numbers of pDCs, which may suggest a general characteristic of chronic infection [175]. Importantly, lower numbers may reflect relocalization of cDCs or pDCs to inflamed tissue or lymph node following activation [155] or dowregulation of BDC specific markers used to identify these populations in blood. Several forms of liver disease may cause decrease in circulating levels of cDCs due to migration to inflamed liver [176]. Analysis of both infected tissue as well as draining lymph

nodes for pDCs and IFNα+ cells may offer insight into this question; however, access to high quality tissue is often limited.

Functional pDCs in chronic HCV infection

Consistent with previous reports [162], our data suggest that PBMCs from chronic HCV patients produce less total IFNα when stimulated with pDC specific agonist to TLR9 (Fig. 2.7A). However, in contrast to the interpretation by others, we believe that this decrease reflects a decrease in absolute pDC numbers and not inhibition of TLR9 specific signaling. First, we show that influenza, acting as an agonist for TLR7 (and possibly TLR3), produces similar levels of IFNα (Fig. 2.7A). Second, when we normalize the data for total IFNα production per pDC, no statistical difference is seen between healthy controls and chronic HCV infected donors, suggesting that lower numbers of pDCs in the bulk PBMC cultures accounts for decreased IFNα production (Fig. 2.7B).

These findings suggest normal function of pDCs and functionally intact TLR7 and TLR9-signaling pathways. In order to evaluate this formally, IFNα production in pDCs was directly assayed using FACS-based ICCS (Fig. 2.7C, D). Even in patients with low numbers of DCs, the same percentage of cells respond to TLR7 and TLR9 agonists to produce IFNα.

Important implications for immunotherapy

Strong CD8+ T cell response are associated with successful resolution of HCV infection and protection[41]. These findings provide a rationale for immunotherapy as well as vaccination strategies to augment HCV-specific CD8+ T cell response. Autologous DC immunotherapy has been used to induce CTLs against tumor antigens[177]. Despite the limited success of DC vaccines in tumor immunotherapy, infectious diseases may be more amenable to this type of treatment. Our findings along with similar results in the chimpanzee model [178] support the use of autologous monocyte derived or circulating cDCs loaded with HCV antigen for these trials.

IFNα is the currently approved treatment for chronic HCV infection. Although it is effective in approximately 50% of patients (depending on the HCV genotype), treatment is associated with significant side effects including nausea, dizziness, and flu-like symptoms which may persists for the six month course of treatment. TLR7 (Anadys Pharmaceuticals/Novartis) and TLR9 (Coley Pharmaceutical Group/Pfizer) agonist therapy for chronic infection are currently in clinical trials with multiple aims. First and foremost, it is necessary to evaluate the efficacy of these therapies in achieving an antiviral effect (i.e. a reduction in viral load). Second, it is hypothesized that the specific and localized triggering of endogenous IFNα may provide more effective delivery as well as avoid many of the side effects associated with systemic IFNα treatment. Our data suggest that pDCs during chronic HCV are competent to respond to these types of agonists; however, the effects of lower absolute pDC numbers need to be evaluated. An

additional benefit of TLR7/9 agonist therapy may involve pDCs role in HCV-specific T cell priming which will be addressed in chapter 4.

HCV antigen presentation in the absence of direct infection

Although the data regarding infection of PBMCs and DCs, in particular, is controversial[141,163], our results suggest that circulating DCs are not robustly infected (Fig. 2.8A). These findings are supported by the lack of robust infection of monocyte derived DCs *in vitro* by the recently described J6/JFH virus (Fig. 2.8B). One explanation for this difference may be that our conclusions are mainly based on intracellular staining for NS5A while contrasting data is based on RT-PCR data[163]. Importantly, DCs do interact with E2 via DC-SIGN[95]. Thus, PCR based assays for DC infection must differentiate between positive and negative strand to conclusively show infection. Alternatively, cells should be washed with EDTA before RNA is prepared to dissociate bound viral particles.

Although CD81, a key component required for HCV infection[12,16,18,179], is expressed on many cell types, the availability of particular cellular proteins required for HCV replication may account for cell tropism. For example, certain hepatocyte cell machinery may provide for efficient replication not available in DCs. These cellular factors required for efficient HCV replication are currently under investigation. Alternatively, the production of and response to antiviral cytokines such as IFNα may play an important role in cell tropism.

In the absence of direct infection, DCs may still acquire antigen for presentation to T cells via cross-presentation. A recent study has shown that DCs can cross-present viral particles for stimulation of HCV-specific T cells against structural proteins epitopes carried in the viral particles[180]. In light of the

presence of CD8+ T cells reactive against NS protein epitopes[41], cross-presentation of infected hepatocytes may likely play a key role in generating CD8+ T cell immunity. Thus, a detailed understanding of the mechanisms regulating immunologic outcome of cross-presentation may offer insight into the HCV immune response.

Finally, our data support the investigation of alternate roles for the DC in HCV pathogenesis as we must account for the HCV-selective defect in the T cell repertoire of chronic HCV patients. In the next two chapters, I consider two important factors associated with HCV infection that may influence the immunologic outcome of DC / CD8+ T cell engagement—namely, IFNα/β and CD4+ T cell 'help'.

Chapter 3: DC maturation alters intracellular signaling networks enabling differential effects of IFNα/β on antigen cross-presentation

3.1 Summary

Type I interferons (IFN), produced largely by plasmacytoid dendritic cells (pDCs), bridge innate and adaptive immune responses and modulate immune function in complex ways. Here, we show that pDC-derived IFNα/β induces opposing effects on the immunologic outcome of cross-presentation. Specifically, IFNα/β acts on immature conventional dendritic cells (DCs) during antigen capture to block IL-12 production and inhibit subsequent $CD8^+$ T cell activation. In contrast, exposure of mature DCs to IFNα/β enhances T cell activation. Further investigation revealed a molecular switch in intracellular signaling pathways, which occurs during DC maturation and accounts for the opposing effects of IFNα/β on antigen cross-presentation. The alteration of signaling networks offers a novel mechanism by which DCs modulate the integration of signals from the surrounding environment and helps to explain the paradoxical effects of IFNα/β on adaptive immunity.

3. 2 Introduction

HCV infection, in contrast to other viral liver infections including HBV, induces robust IFN response genes upon infection[139]. Although this interferon gene signature is not predictive of clinical outcome, HCV replication is sensitive to IFNα and pegylated-IFNα / ribavirin is a highly effective therapy for HCV[101]. The difficulty in understanding the complex actions of IFNα/β during HCV pathogenesis is reflective of the pleiotropic effects of IFNα/β[140]. Although most widely known for its antiviral activity, IFNα/β is also a potent immunomodulator. For example, exposure of natural killer (NK) cells and naïve T cells to IFNα/β during viral infection inhibits their production of IFNγ[181]; however, IFNα/β has also been shown to trigger NK cell cytotoxicity and stimulate proliferation of certain T cell subsets[182,183]. These polarized physiologic and pharmacologic effects are mediated by the same heterodimeric IFNα receptor.

This complexity extends to the effects of IFNα/β on conventional dendritic cells (DCs). Considered the 'sentinels' of the immune system, DCs are responsible for integrating immune responses. Tissue DCs, which exist in an immature state of differentiation, are responsible for capturing and processing antigen, as well as transmitting pro-inflammatory and immune regulatory signals to cells within draining lymph nodes[52]. DCs also possess a unique ability to cross-present exogenous antigen, offering a mechanism for priming $CD8^+$ T cells specific for viruses that do not directly infect DCs. Recent data suggests that type I IFNs may serve as a 'danger' signal during this process, facilitating DC activation[184] and licensing DCs to cross-prime[185]; however, IFNα/β has also been

shown to inhibit the production of IL-12 by DCs[186], and may also inhibit DCs from stimulating T_H1 cell differentiation[187].

pDCs are widely recognized for their role in sensing viral and bacterial components and producing significant amounts of IFNα/β [188]. In humans, pDCs express TLR7 and TLR9, receptors for ssRNA[126] and CpG[189], respectively. Upon activation, pDCs migrate to lymph nodes and produce IFNα/β, thus influencing the afferent phase of T cell activation [155,156]. During chronic inflammation and infection, pDCs may also traffic to affected tissues[190,191]. Although activated pDCs may serve as a strong adjuvant for adaptive immune responses[189], the immunologic significance of pDC-derived IFNα/β in distinct spatial locations has not been carefully evaluated.

Here, we evaluated the effects of IFNα/β on the cross-presentation of antigen. Importantly, our consideration of the distinct compartmentalization of IFNα/β production, in peripheral tissue *versus* the lymph node, led to a surprising result. Immature DCs (iDCs) exposed to IFNα/β (referred to herein as 'IFN-early')—mimicking the micro-environment of a tissue DC—were impaired in their ability to activate $CD8^+$ T cells via the cross-presentation pathway. In contrast, mature DCs (mDCs) exposed to IFNα/β (referred to herein as 'IFN-late')—mimicking the micro-environment of DC : T engagement within the lymph node—demonstrated enhanced T cell activation. Investigation into the regulation of these opposing effects revealed a molecular switch in IFNα/β signaling networks that is a consequence of DC maturation. Our findings offer insight into how IFNα/β shapes the adaptive immune responses to cross-

presented antigen and uncover a novel mechanism by which DCs modulate their response to cytokine signals from the surrounding environment.

3.3 Materials and Methods

Isolation and preparation of cells

Peripheral blood mononuclear cells (PBMCs), DCs, and T cells were prepared as previously described[168]. PBMCs were isolated from whole blood by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ). iDCs were prepared from the T cell depleted fraction by culturing cells in the presence of 1000 U/mL GM-CSF (Berlex, Seattle, WA) and 500-1000 U/mL IL-4 (R&D Systems, Minneapolis, MN) for 6 days[169,170]. To generate mature DCs, cultures were stimulated on day 6 with 50 ng/mL TNFα (Alexis Biochemicals) and 0.1mM PGE_2 (Sigma) or 10ng/mL LPS (Sigma) for 36-48hrs[171]. At days 6–7, >95% of the cells were $CD14^-CD83^-HLA\text{-}DR^{lo}$ DCs. After maturation, on days 8–9, 70–95% of the cells were of the mature $CD14^-CD83^+HLA\text{-}DR^{hi}$ phenotype. $CD4^+$ and $CD8^+$ T cells were purified to >99% purity by positive selection with the MACS column purification system (Miltenyi Biotech, Auburn, CA). pDCs were also purified by MACS with BDCA-4 microbeads. pDCs were stimulated with CpG (2216: 5'-GGGGGACGATCGTCGGGGGG-3') in 1% single donor plasma. After 24 hrs, cultures were harvested and supernatants purified by centrifugation.

Induction of apoptotic death

The mouse lymphoma cell line EL4 (number TIB-39, ATCC, Rockville, MD) was used as a source of apoptotic cells because they can be efficiently infected with influenza virus and do not induce marked background T cell activation to mouse antigens. EL4 cells were infected with influenza and apoptosis was triggered using a 60UVB lamp calibrated to provide 2 mJ/cm2/s.

Endogenous and exogenous loading of DCs with antigen

For endogenous loading, mDCs were infected with 0.002 HAU influenza A/PR8/1976 (Charles River, Spafas Conn) per cell. Virus was quenched and removed with 2 washes of media with 5% pooled AB human serum (Labquip, NY). For loading DCs with exogenous antigen, iDCs were co-cultured with influenza infected EL4s that had been exposed to UVB[62]. Co-cultures were incubated in the presence of TNFα / PGE_2 to allow for phagocytosis of the apoptotic EL4 cells, antigen processing and DC maturation to occur. mDCs were then used for functional assays.

Detection of antigen-specific T cells: ELISPOT assay for IFNγ release

Antigen loaded DCs were collected, counted and added to purified T cell populations in plates that had been coated with 10μg/ml of anti-IFNγ mAb (clone Mab-1-D1K, Mabtech, Cincinnati, OH). In all experiments, 2×10^5 T cells were added to 6.6×10^3 DCs to give a 30:1 T cell:DC ratio. The cultures were incubated for 40–44 h at 37°C. Cells were washed out with mild detergent (0.5% Tween-20 in PBS) and the ELISPOT plate was incubated with 1μg/ml of a biotin-

conjugated IFNγ mAb (clone Mab 7BG-1, Mabtech) for 2 hrs at 37°C. ELISPOT plate was then washed with 0.1% Tween-20 in PBS and developed using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (AEC) substrate. Colored spots indicate the cells that had released IFNγ and data is reported as spot forming cells/10^6 $CD8^+$ T cells. Data from triplicate wells were averaged and mean data is reported. Error bars represent one standard deviation.

Analysis of STAT phosphorylation

DCs were harvested, washed with PBS, resuspended in 10% FBS containing media and stimulated with indicated concentration of rIFNα, purified IFNα/β or IFNγ (Sigma, St. Louis, MO) for 30 minutes at 37°C. For western blot analysis and immunoprecipitation, lysates were prepared in RIPA lysis buffer (150mM NaCl, 50mM Tris-Cl pH 8, 1% NP40, 0.5% DOC, 0.03% SDS, 2mM EDTA) with 1mM Vanadate (Sigma), 1mM PMSF, and Complete anti-protease tablet (Roche). Total protein was determined using the Bradford assay (Biorad). Immunoprecipitation was performed with 400μg of total protein and 0.2μg of anti-IFNAR1 (CD118) overnight at 4°C. Protein A and G beads were mixed 1:1 and incubated 4°C for 1 hour followed by three RIPA washes. Beads were boiled at 95°C in 2x loading buffer and run on 8% SDS-PAGE. For IFNAR2 western blot, 20μg of total protein was loaded on to 8% SDS-PAGE. Proteins were transferred to PVDF membrane and blotted with indicated antibodies (Cell Signaling Technologies). Blots were probed first with phospho-specific STAT antibodies, stripped with low pH, and reprobed with total STAT antibodies to

ensure p-STAT is monitored in the context of total STAT. For intracellular FACS analysis, samples were fixed, permeabilized with Cytoperm/Cytofix (BD Pharmingen). Cells were stained with STAT1-PE, STAT3-PE, and STAT4-APC (Pharmingen) at 1:50 final dilution as indicated.

Affymetrix Microarray U133A (see Appendix 2)

Biotinylated cRNA fragments were prepared from monocyte derived DCs as per affymetrix protocol[192]. 10^7 cells were washed in PBS and lysed using Qiashredder (Qiagen, Valencia, CA). Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA and fragmented biotinylated labeled cRNA were subsequently prepared as per manufacturer's instructions (Affymetrix and Enzo Life Sciences) and 20μg of fragmented cRNA was loaded onto Affymetrix U133A. Data was analyzed using Microarray Suite 5.0 (Affymetrix, Santa Clara, CA), and compiled using a Filemaker Pro-based customized database (adapted from a database that was generously provided by Dr. Nir Hacohen, Harvard University). Absolute expression, p-call, and *p*-value as calculated by MAS 5.0 software was exported to Excel spreadsheet and converted into a Filemaker Pro document. Data from multiple runs was linked via Affy ID number.

IL-12 and IFNα ELISAs

IL-12p70 levels were confirmed in triplicate using IL-12 ELISA (R&D systems). IFNα ELISA (PBL Biomedical, Piscataway, NJ) was performed in triplicate and data from two donors was averaged.

Analysis of IL-12 production in MCMV infected mice

C57BL/6 were obtained from Charles River (France); STAT1-/- (129SVE) from Taconic; and STAT4-/- (Balb/c) from Jackson. IFNAR-/- mice on the C57BL/6 background were provided by D. Braun, Institut Pasteur. RB6-8C5 (anti-GR-1) bioreactor supernatant was obtained from monoclonal antibody facility (MSKCC) and mice were depleted with 500μg at 36 hrs and 12 hrs before infection. Mice were infected with 4×10^5 PFU/mL of salivary gland prepared MCMV. 36 hours after infection, spleens were harvested, incubated 1:50 dilution of anti-FcγR (BD Pharmingen) to block non-specific antibody interaction in mouse FACS wash buffer. Cells were surface stained with CD8 FITC (1:150 dilution, BD Pharmingen) and CD11c PE (1:150 dilution, BD Pharmingen), fixed and permeabilized with cytoperm/cytofix (BD Pharmingen), and stained with IL-12 APC (1:150, BD Pharmingen).

3. 4 Results

Maturation prevents IFNα/β mediated inhibition of cross-presenting DCs

In order to explore the effects of IFNα/β on antigen cross-presentation, we employed an experimental system based on the activation of primary human memory $CD8^+$ T cells (Fig. 3.1). DCs cross-presenting antigen were generated by co-culturing iDCs with apoptotic, influenza (flu)-infected MHC-mismatched cells in the presence of maturation stimuli[62]. Antigen-loaded mDCs were then cultured with purified $CD8^+$ T cells and the activation of influenza specific IFNγ-producing cells was monitored using ELISPOT. Maximal $CD8^+$ T cell activation by cross-presentation requires CD40L engagement, mimicking the effect of $CD4^+$

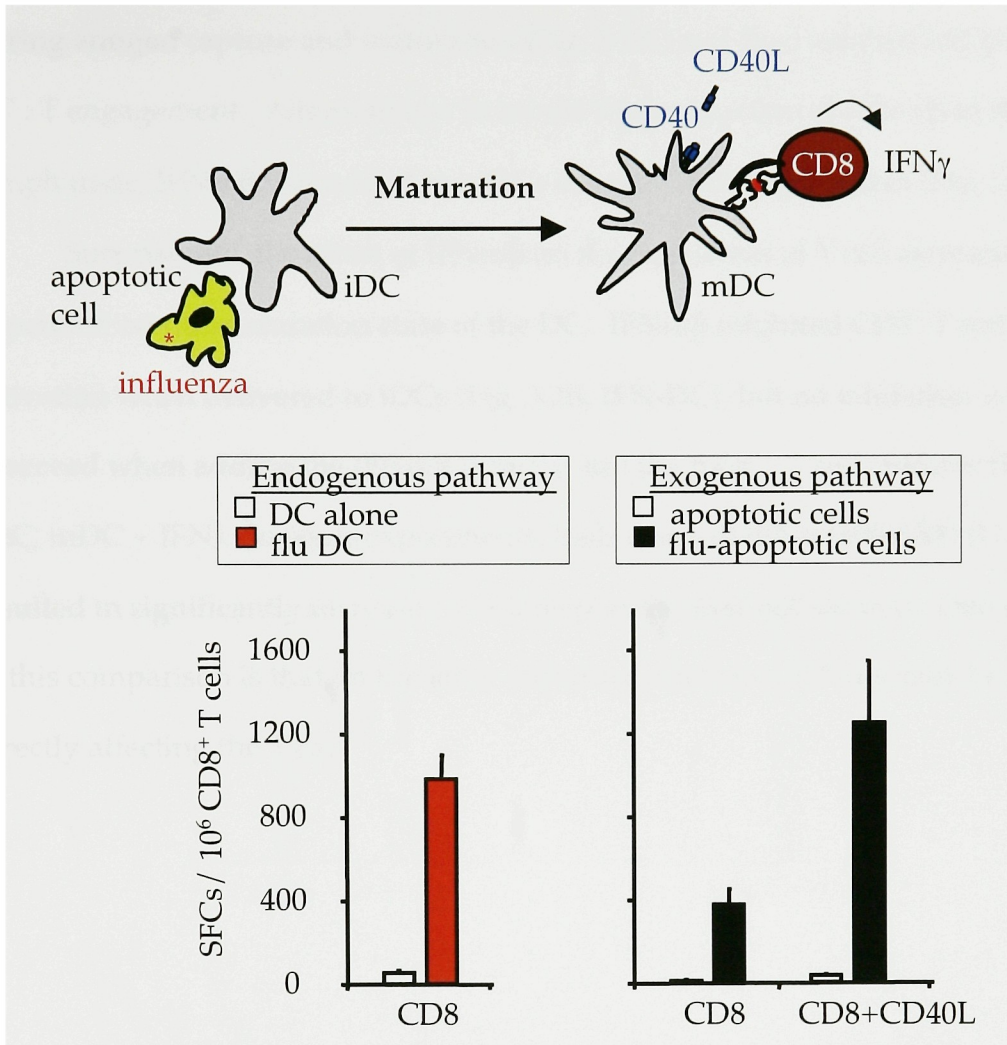

Figure 3.1. ***In vitro*** **system for monitoring CD8+ T cell activation requires CD40 ligation.** DCs loaded with endogenously processed antigen were prepared by direct infection with influenza (flu DC) and compared to uninfected DCs (DC alone). DCs loaded with exogenous antigen were prepared by cross-presentation of influenza infected apoptotic cells (flu-apoptotic cells) and compared to uninfected apoptotic cells (apoptotic cells). DC groups were cultured with syngeneic purified $CD8^+$ T cells. T cell activation was monitored by IFNγ ELISPOT and displayed as spot forming cell (SFCs) / 10^6 $CD8^+$ T cells. Recombinant CD40L was added to cultures as indicated.

T cell / DC interactions[62] (Fig. 3.1). In order to model IFNα/β production in peripheral tissue, leukocyte derived or recombinant IFNα was added to iDCs during antigen capture and maturation (Fig. 3.2A) and then washed out prior to DC : T engagement. Alternatively, to model the production of IFNα/β in the lymph node, IFNα/β was added to mDCs during T cell engagement (Fig. 3.2A).

Surprisingly, the effect of IFNα/β on the regulation of T cell activation depended on the maturation state of the DC. IFNα/β inhibited $CD8^+$ T cell activation when delivered to iDCs (Fig. 3.2B, IFN-DC), but no inhibition was observed when adding the IFNα/β directly into the mDC : T cell cultures (Fig. 3.2C, mDC + IFN). In some experiments, high doses of IFNα (600-6000 IU/ml) resulted in significantly increased T cell responses (data not shown). One caveat in this comparison is that, in the latter culturing condition, IFNα/β may be directly affecting the T cell.

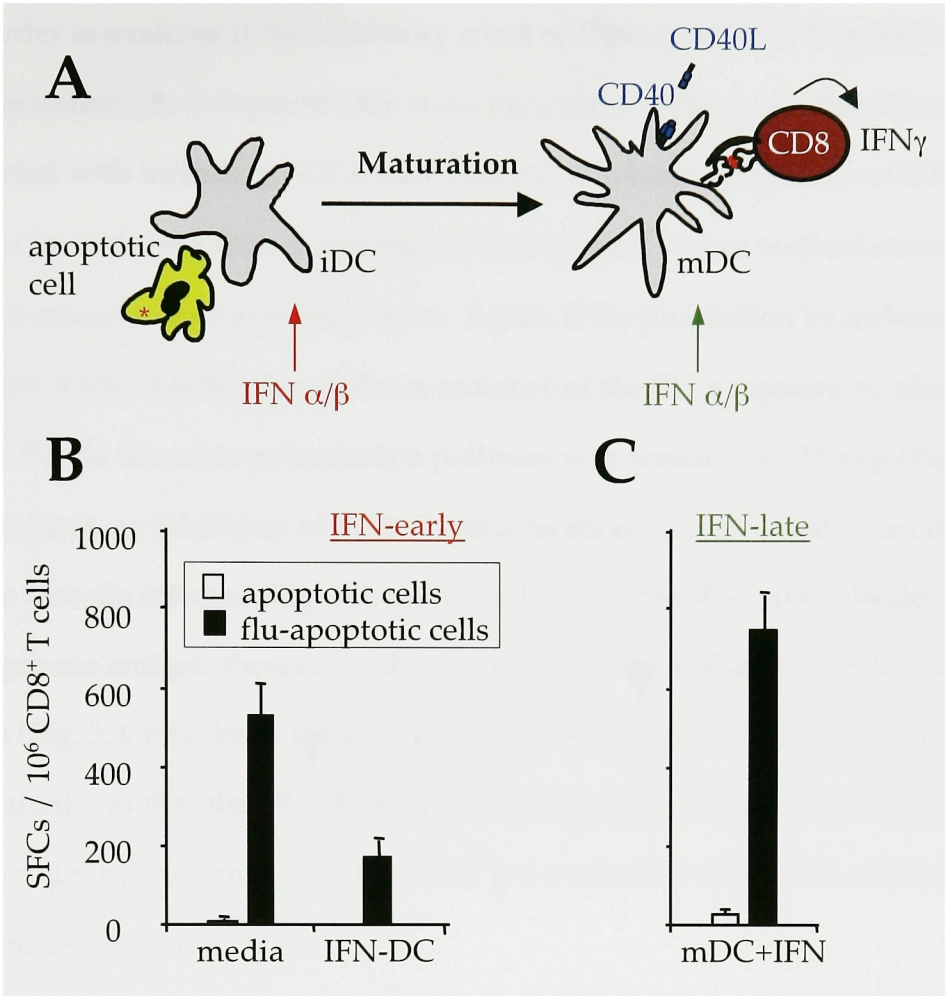

Figure 3.2. Maturation prevents IFNα mediated inhibition of cross-presenting DCs. A. Schematic depicting experimental setup. IFNα/β was either added to iDCs during antigen capture and maturation (IFN-early) or during DC:T cell engagement (IFNα-late). **B,C.** (B)IFNγ ELISPOT with DCs cross-presenting uninfected apoptotic cells (white bars) or DCs cross-presenting influenza antigen-expressing apoptotic cells (black bars) exposed to 60 IU/mL IFNα/β during antigen capture (IFN-early: IFN-DCs) or (C) during DC:T cell engagement (IFN-late: mDC + IFN) cultured with purified CD8+ T cells. Data are representative of ten experiments and similar results were obtained when using recombinant IFNα (data not shown).

The inhibitory effect of IFNα/β is selective for CD40-dependent cross-presentation

In order to evaluate if the inhibitory effect of IFNα/β on iDCs is specific for cross-presentation, we compared DCs cross-presenting antigen to those directly infected with influenza. iDCs were treated with increasing doses of IFNα/β (10-60 IU/mL) during their maturation. These IFN-DCs were washed extensively and exposed to live influenza virus. Again, IFNγ production by influenza-specific $CD8^+$ T cells was used as a measure of the DC's capacity to stimulate T cells. While the cross-presentation pathway was sensitive to IFNα/β (Fig. 3.3, black line), no inhibition of $CD8^+$ T cell activation was observed when IFN-DCs were directly infected (Fig. 3.3, red line). Further, the IFN-mDCs loaded with exogenous antigen showed no defect in stimulating influenza-reactive $CD4^+$ T cells (Fig. 3.3, blue line), thus providing an internal control that antigen had been captured and that the DCs were capable of engaging T cells. From these experiments, we conclude that IFNα/β has a selective effect on T cell activation by cross-presenting DCs.

This selective effect may be due to decreased antigen capture, inefficient DC maturation, or the skewing of activation signals specific for T cell activation by cross-presentation (e.g. CD40 engagement). In order to directly evaluate antigen capture, apoptotic cells and iDCs were labeled with fluorescent lipophilic dyes as described[193]. Capture of the red-labeled apoptotic cells by the green-labeled DCs was monitored by flow cytometry. After 5hr, ~40% of the iDCs had captured an apoptotic cell body / bleb (Fig. 3.4A). Addition of IFNα/β to the co-cultures did not inhibit antigen capture (Fig. 3.4A), nor did it alter the kinetics of uptake (data not shown). We next evaluated the effect of IFNα/β on phenotypic

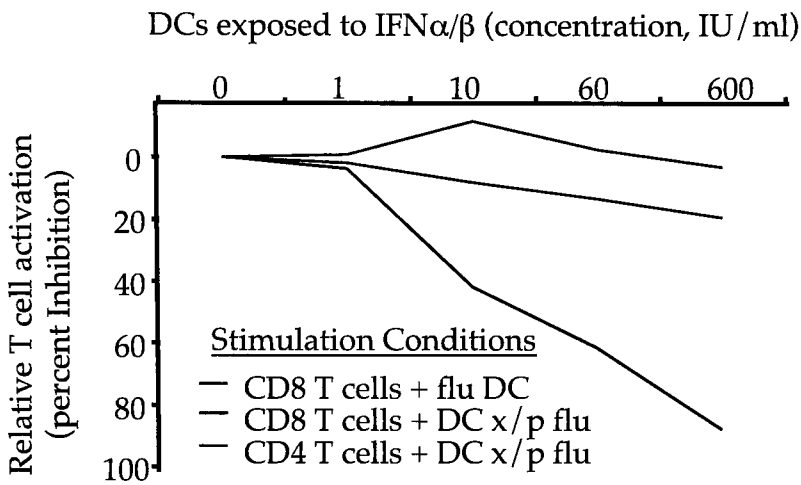

Figure 3.3. Inhibition by IFNα/β is selective for cross-presenting DCs. DCs were directly infected with influenza virus (flu DC) or co-cultured with apoptotic influenza-expressing apoptotic cells (DCs x/p flu-AC). In each, the iDCs were exposed to a dose range of IFNα/β iDCs (conc. 0-600 IU/ml). $CD8^+$ T cell and $CD4^+$ T cell activation were monitored by IFNγ ELISPOT. Percent inhibition of T cell activation is displayed. Maximal stimulation (no IFNα/β) in the experiment shown is equivalent to 400 spot forming cells / million T cells.

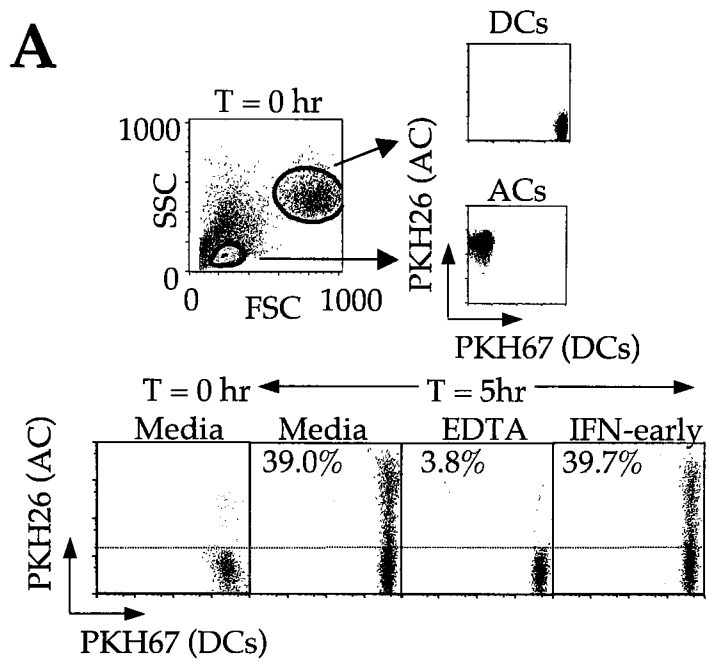

Figure 3.4. IFNα/β does not alter phagocytosis, phenotypic or functional maturation of DCs. (Continued on following page)

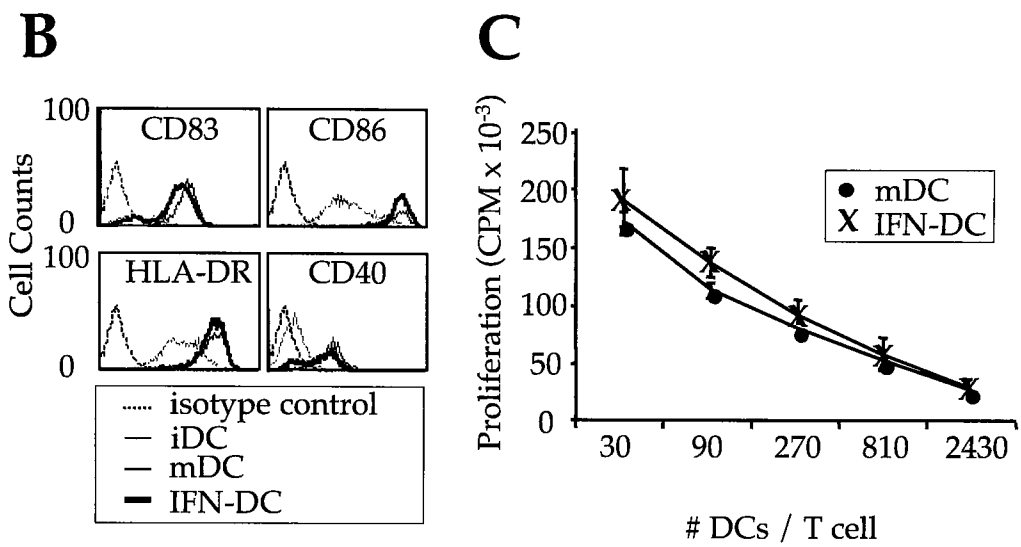

Figure 3.4. IFNα/β does not alter phagocytosis, phenotypic or functional maturation of DCs. A. Phagocytosis of PKH26 (red dye)-labeled apoptotic cells (AC) by PKH67 (green dye)-labeled iDCs was monitored by flow cytometry. iDCs and apoptotic T cells were distinguished based on forward scatter (FSC) and side scatter (SSC) properties, as shown. Gating on iDCs using scatter and FL-1 criteria permitted monitoring of cells that engulfed a red-labeled dying cell, as based on their becoming double positive (lower FACS plots). Co-cultures were incubated in the presence of media, EDTA, or IFNα as indicated. Percent phagocytosis is indicated. **B.** FACS analysis of CD83, CD86, HLA-DR, and CD40 expression levels on iDCs, mDCs, or DCs matured in the presence of IFNα/β (IFN-DC). **C.** Allostimulatory potential of mDCs or DCs matured in the presence of IFNα/β (IFN-DC) was monitored by stimulation of T cell proliferation after 5 days of culture. Proliferation was monitored by incorporation of ^{3}H-thymidine. Triplicate wells are averaged and +/- standard deviation is represented by error bars.

and functional maturation of the DCs. Importantly, the addition of IFNα/β did not affect TNFα-mediated maturation as monitored by expression of CD40, CD83, HLA-DR and CD86 (Fig. 3.4B). Moreover, the allostimulatory capacity of IFNα-mDC was equivalent to mDCs, as measured using a standard allogeneic mixed lymphocyte reaction (Fig. 3.4C). The finding that antigen capture and DC maturation are intact is consistent with the observation that $CD4^+$ T cells may be activated by IFNα early-DCs (Fig. 3.3). These data also suggest that IFNα/β treatment of iDCs may be affecting the DCs ability to be 'licensed' by CD40, a feature unique to the cross-priming of exogenous antigen in our model system[62].

Maturation alters IFNα/β regulation of CD40L-induced IL-12

In order to evaluate the inhibitory effect of IFNα, we focused our attention on the effect of 'IFN-early' on CD40L-induced gene changes. In order to screen for these changes, we took advantage of the Affymetrix U133A microarray (see Appendix 2). Briefly, RNA was prepared from the samples of interest: iDCs or mDCs matured in the presence or absence of IFNα/β and stimulated with or without CD40L. RNA probes were prepared as per the Affymetrix protocol and the microarray data was analyzed with MAS5.0 statistical software. Our array data set was queried for gene changes in which: mDCs + CD40L $\geq$ 2x mDCs; AND mDCs + CD40L $\geq$ 2x IFNα-mDCs + CD40L ($n = 2$) (see Appendix 2). With such a Boolean search, we exclude gene changes that are simply a result of IFNα-treatment of the DCs. From the list generated, genes were excluded if they did not meet a 'p' call in mDCs or mDCs + CD40L in either or both of the experiments, as per the Affymetrix MAS5.0 statistical package. Data on iDCs,

mDCs, IFNα-mDCs, mDCs + CD40L and IFNα-mDCs + CD40L from the two individual donors were averaged, and means are graphically represented to reveal CD40L-induced transcription changes that distinguish treatment of mDCs (blue) *versus* IFNα-mDCs (red). This analysis yielded nine candidate genes that were inhibited by 'IFN-early' (Fig. 3.5).

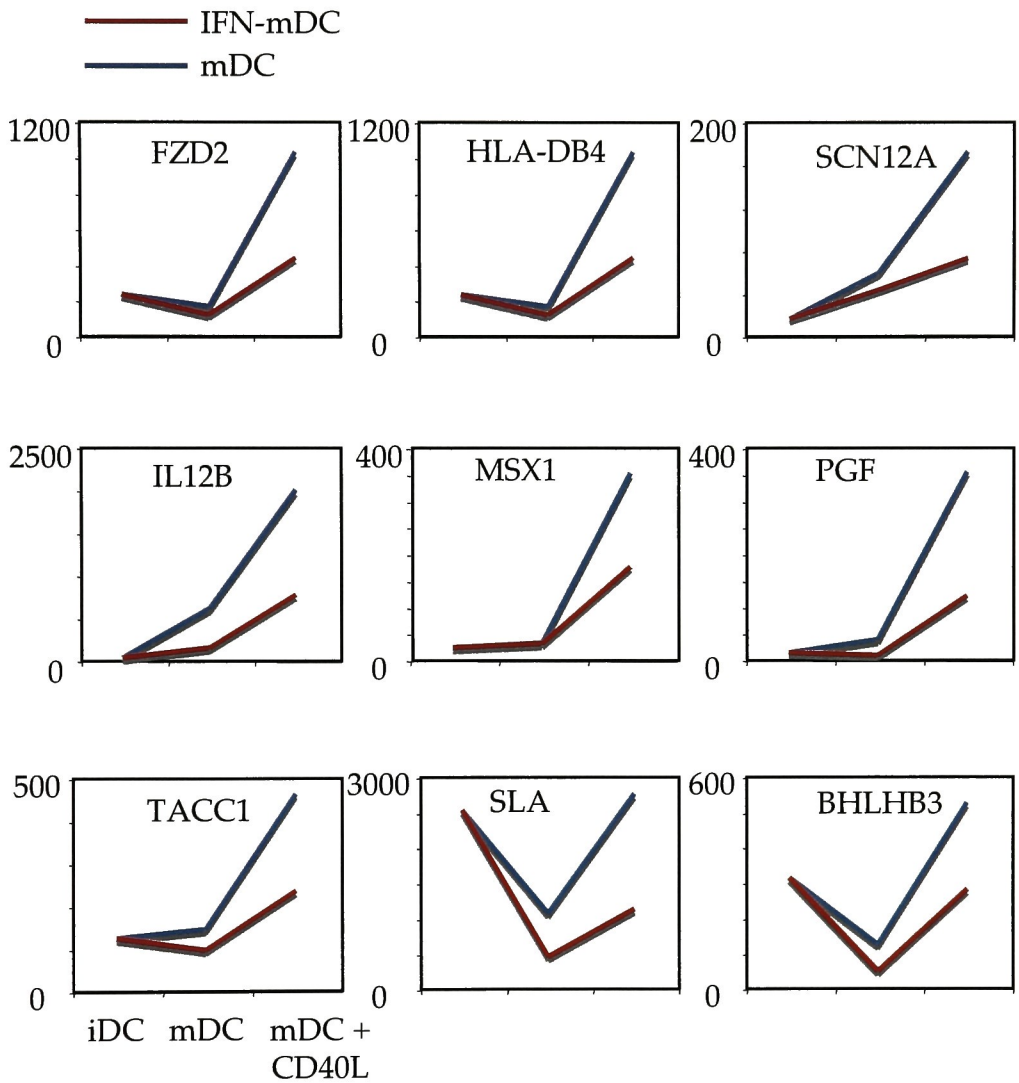

Figure 3.5. IFNα/β early alters CD40L gene induction profile. Affymetrix data was queried for genes induced by CD40L that were blunted by exposure of iDCs to IFNα/β during maturation. The top nine differentially regulated genes are displayed graphically by relative expression in iDCs, mDC, or mDCs exposed to CD40L (mDC + 40L). Blue line, untreated. Red line, IFNα/β treated.

In light of the important role that IL-12 plays in cross-priming[75,76,194], we further explored the observation of differential IL-12p40 mRNA expression (Fig. 3.6A). Using an ELISA, we confirmed that CD40L-induced production of bioactive IL-12p70 was inhibited by 'IFN-early' (Fig. 3.6B). In contrast, 'IFN-late' synergized with CD40L, as even higher levels of IL-12p70 were produced by mDCs under these conditions (Fig. 3.6B).

To determine the importance of IL-12 inhibition in the functional block of CD8+ T cell activation, we evaluated the ability to overcome the IFN-early block by adding rIL-12 to the IFN-DCs cross-presenting antigen. IFNγ ELISPOT results from three donors were normalized to the donor specific maximal T cell response and the percent maximal T cell response is shown (Fig. 3.7). Indeed, IL-12 rescued the ability to stimulate antigen-specific $CD8^+$ T cells via the cross-presentation pathway. While other factors may be involved, the decrease in CD40L-induced IL-12 seems to be a critical component of the inhibitory effects of IFNα/β. These data also suggest that cross-presentation *per se* remains intact in IFN-DCs, and that the dominant mechanism of regulation is via inhibition of CD40-dependent licensing of DCs. Thus, differential responsiveness of iDCs and mDCs to IFNα/β regulates the production of cytokines that determine the immunologic outcome of cross-presentation.

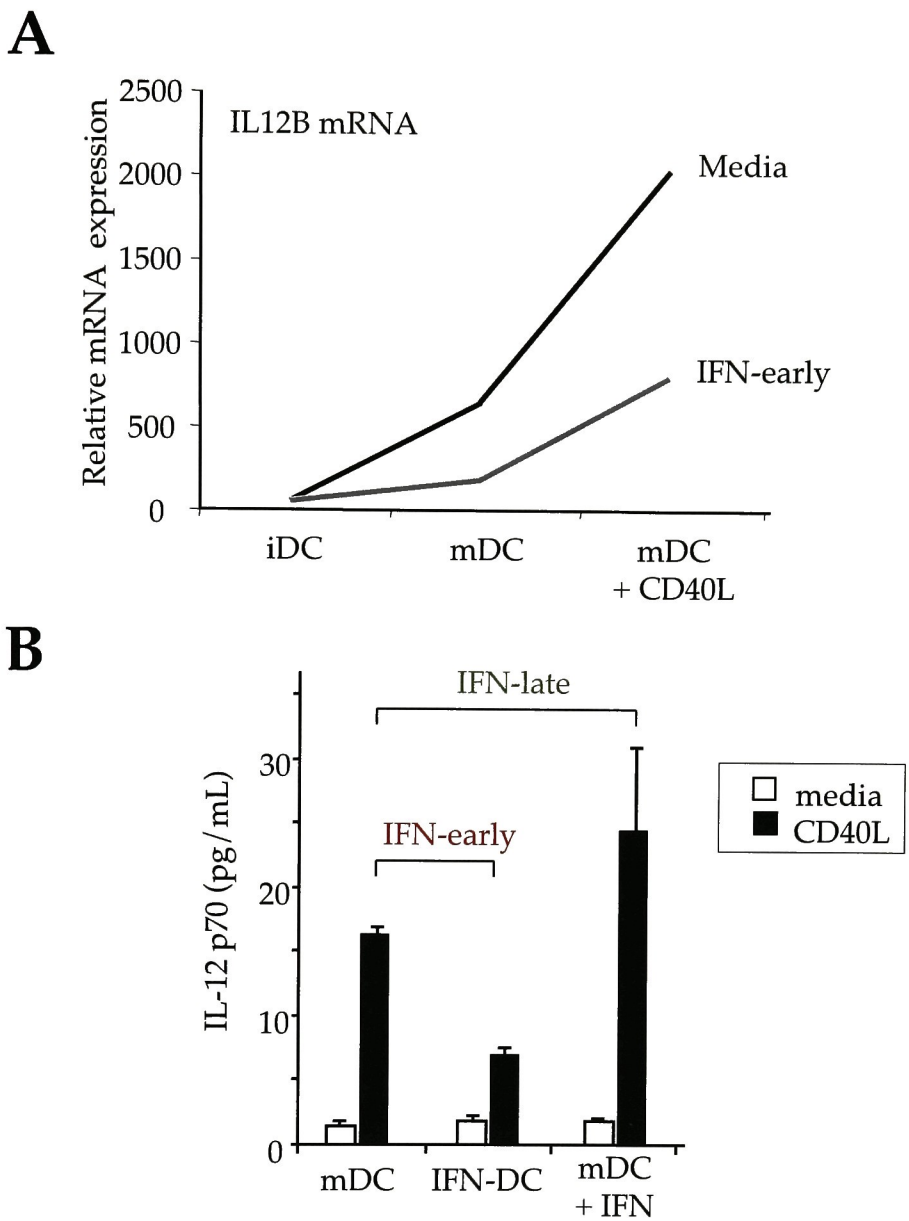

Figure 3.6. IFNα/β-early, not late, inhibits CD40L-triggered IL-12. A. Affymetrix analysis shows relative expression of IL-12B subunit in iDC, mDC, and mDC + CD40L. Untreated, black line. IFNα/β treated iDC, gray line. **B.** Supernatants from DCs matured alone (mDC), in the presence of IFNα/β (IFN-DC), or DCs exposed to IFNα/β after maturation (mDC + IFN) were treated with CD40L as indicated for 2 hours. IL-12p70 was measure by ELISA and the average of triplicate wells are reported.

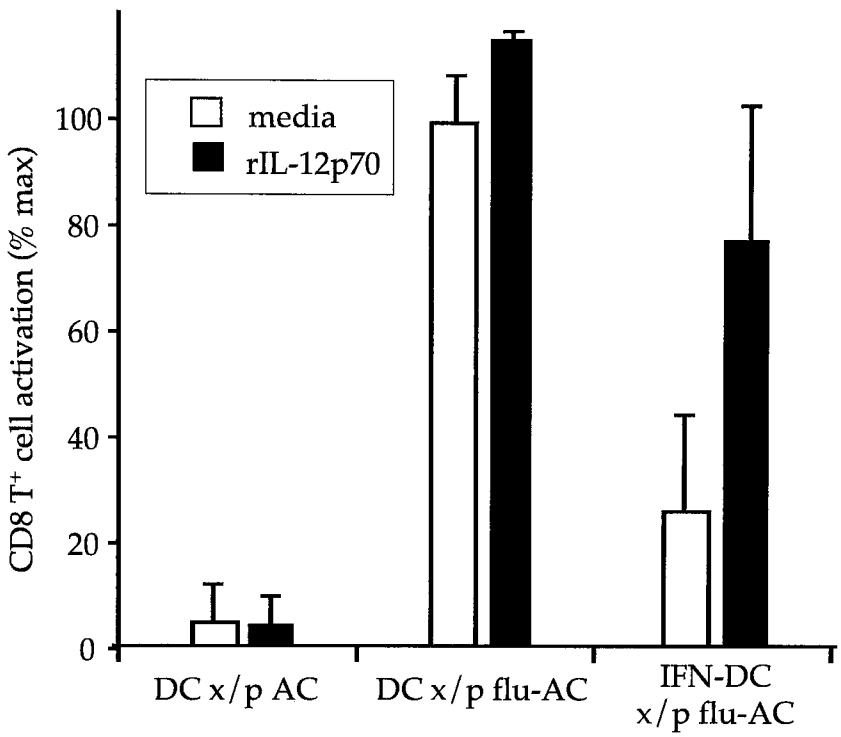

Figure 3.7. Exogenous IL-12 rescues inhibition by IFNα. T cell activation by DCs cross-presenting apoptotic cells (DC x/p AC), DCs cross-presenting apoptotic cells loaded with influenza antigen (DC x/p flu-AC) or DCs matured in the presence of IFNα/β cross-presenting influenza antigen (IFN DC x/p flu-AC) was monitored by IFNγ ELISPOT. In addition to CD40L alone (media), recombinant human IL-12 (rIL-12p70) was added to the DC:T cell cultures. Data from three donors were normalized to the donor-specific maximal T cell response and the percent maximal T cell response in the presence or absence of IFNα/β and IL-12p70 is shown.

DC maturation alters IFNα/β signaling by switching STAT utilization

In light of the correlation of distinct DC maturation states with the paradoxical effects of IFNα/β, we considered the possibility that intrinsic differences between iDC and mDC IFNα/β responsiveness could account for the opposing effect on T cell activation. Three possibilities were evaluated to directly test this hypothesis: (i) differences at the level of IFNα/β receptor (IFNAR) expression; (ii) utilization of distinct signaling molecules; and (iii) altered CD40-mediated transcriptional events, resulting from differences in IFNα/β signaling.

First, expression of IFNAR on iDCs and mDCs was evaluated by FACS. Surface expression of both subunits, IFNAR1 and IFNAR2, were equivalent in iDCs and mDCs (Fig. 3.8A). Immunoblot analysis of IFNAR1 and IFNAR2 confirmed that there are equivalent levels of both subunits in iDCs and mDCs (Fig. 3.8B). Based on these data, we conclude that differential receptor expression does not account for the distinct effects of IFNα/β on iDCs and mDCs.

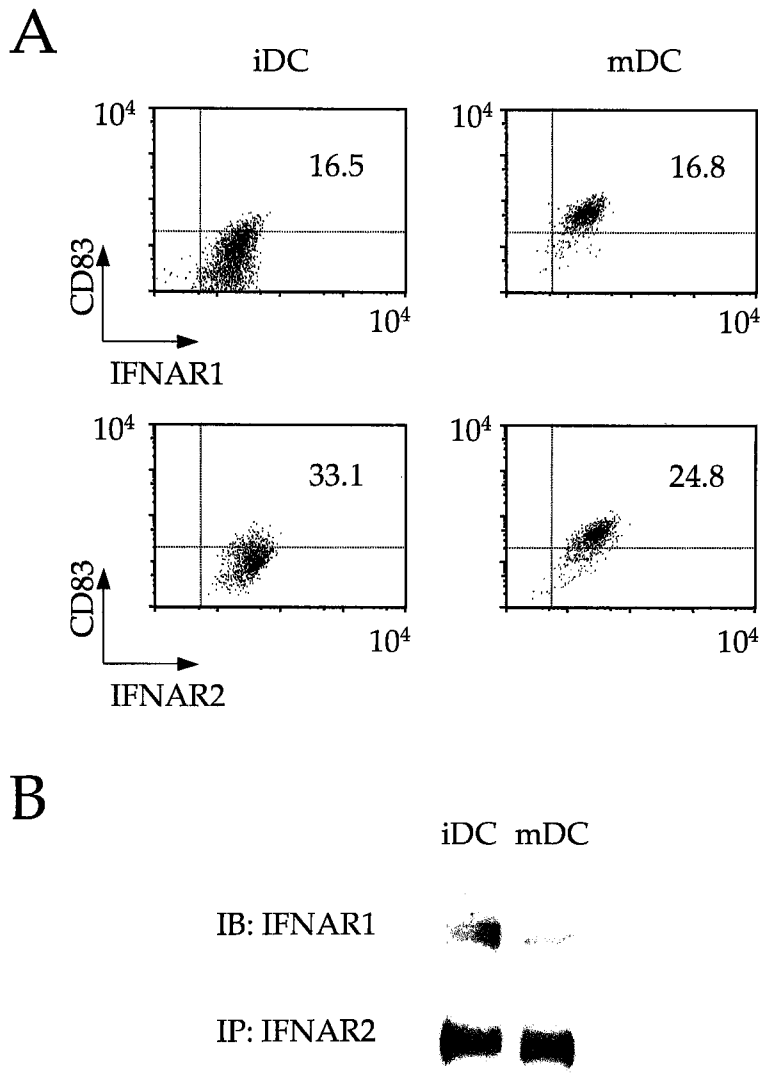

Figure 3.8. Maturation does not alter IFNAR surface expression. A. iDC and mDC were stained with anti-CD83-Cy5 and mAbs specific for IFNAR1 (AA3, Biogen) or IFNAR2 (MMHAR-2, Calbiochem). Anti-mouse IgG PE (Jackson) was used to visualize anti-IFNAR binding. Dot plots representative of a single donor are shown. Three individuals were evaluated and revealed similar results. MFI for x-axis is indicated. **B.** Immunoblot for IFNAR1 and IFNAR2 expression in iDC or mDCs by western and immunoprecipitation, respecitively. DCs were either unstimulated or treated with 100IU/mL IFNα for 30 minutes.

We next considered the possibility of differential signaling downstream of IFNAR. In order to screen for potential points of divergent regulation, we analyzed transcriptional profiles of iDCs and mDCs from four individuals using Affymetrix U133A microarrays. Analysis of genes known to be involved in IFNα/β-signaling revealed possible differences in IFN-response (Table 3.1). Our transcriptional analysis of DCs revealed a two-fold change in total Jak1 and Tyk2, the kinases associated with IFNAR and responsible for STAT phosphorylation. In addition, transcriptional screening showed a 2-fold downregulation of total STAT1 upon maturation and 2-fold upregulation of STAT4. Consistent with the skewing of STAT expression, Affymetrix analysis shows numerous differences in IFN-induced genes between iDCs and mDCs exposed to IFNα/β (data not shown).

In order to evaluate IFNα/β signaling in DCs, we first looked at kinase expression. As reviewed in chapter 1, IFNAR is constituitively associated with Jak1 and Tyk2. Engagement of IFNAR by IFNα/β induces dimerization and oligomerization of the receptor, which allows for Jak1 and Tyk2 transphosphorylation. Consistent with the transcriptional data, Jak1 protein expression is increased upon maturation (Fig. 3.9A). Although total changes in protein expression of Tyk2 are not apparent upon maturation, phosphorylation of Tyk2 in mature DCs is significantly decreased compared to iDCs in response to IFNα/β stimulation (Fig. 3.9A).

We next investigated the effect of this differential kinase expression and activation on STAT activation as monitored by phosphorylation of STAT proteins by Western blot. Classically, STAT1 and STAT2 are phosphorylated in

Table 3.1. Transcriptional profiles of IFNα/β associated genes

	Affy ID	iDC avg[¥]	A/P[*]	pval[†]	mDC avg[¥]	A/P[*]	pval[†]	Fold Change (mDC/iDC)[§]
IFNAR1	204191_at	124 (55 -188)	A/P	0.165	139 (73 -150)	A/P	0.133	1.1
IFNAR2	204786_s_at	126 (51 -279)	P	0.004	122 (57 -208)	P	0.003	1.0
JAK1	201648_at	1377(1227-1821)	P	<0.001	3782(2799-4632)	P	<0.001	2.7
TYK2	205546_s_at	491 (315 -880)	P	0.011	963 (605 -1229)	P	0.005	2.0
STAT1	200887_s_at	1306(892 -1597)	P	<0.001	730 (641 -908)	P	<0.001	0.6
STAT1	97935_3_at	720 (370 -924)	P	<0.001	474 (310 -616)	P	<0.001	0.7
STAT2	205170_at	164 (132 -185)	P	0.003	156 (109 -189)	P	0.009	0.9
STAT3	208991_at	928 (670 -1179)	P	<0.001	2084(1458 -2467)	P	<0.001	2.2
STAT4	206118_at	206 (126 -297)	A/P	0.092	430 (319 -507)	P	0.024	2.1
STAT5A	203010_at	492 (343 -675)	P	0.037	994 (772 -1143)	P	0.006	2.0
STAT5B	205026_at	110 (87 -139)	P	0.008	239 (205 -285)	P	0.002	2.2
STAT6	201331_s_at	1975(1773 -2188)	P	<0.001	1424(1252 -1681)	P	<0.001	0.7
STAT6	201332_s_at	368 (306 -428)	P	0.007	269 (183 -302)	A/P	0.044	0.7

¥ RNA from iDCs and mDCs was anlayzed by Affymetrix microarray U133A. Affymetrix data was compiled from four individual donors. Average relative mRNA expression for iDCs (iDC avg) and mDCs (mDC avg) are indicated with the range indicated in parentheses.

*, † Determination of the absence or presence (A/P) of a gene transcript as well as p-values were calculated using Affymetrix statistical package, MAS5.0

§ Fold change of average expression levels are expressed as a ratio of mDC avg / iDC avg.

response to IFNα/β, forming a heterotrimer with IRF-9, which binds to interferon sequence response element (ISRE) containing genes[118]. In certain cell types, STAT4 may be phosphorylated in response to IFNα/β, leading to transcription of distinct sets of genes[119]. Exposure of iDCs to IFNα/β, results in phosphorylation of STAT1 and STAT2 (Fig. 3.9B). In contrast, despite the presence of total STAT1 and IFNα/β-induced STAT2 phosphorylation, mDCs showed reduced levels of STAT1 phosphorylation. Notably, total STAT4 is significantly upregulated during maturation and exposure to IFNα/β resulted in its phosphorylation (Fig. 3.9B).

Intracellular FACS analysis confirmed, on a per cell basis, that differential IFNα/β-induced STAT-phosphorylation was dependent on the state of DC maturation. Using CD83 as a marker, analysis of STAT1 *versus* STAT4 phosphorylation was evaluated in both iDCs and mDCs. Consistent with Western blot analysis, iDCs phosphorylate STAT1 and not STAT4 in response to IFNα/β (Fig. 3.9C), while mDCs phosphorylate STAT4 and show a significantly reduced phosphorylation of STAT1. The qualitative shift in IFNα/β signaling is also reflected in the activation of STAT3. mDCs demonstrate increased STAT3 expression (Table 3.2), however, maturation results in a blunting of STAT3 phosphorylation in response to IFNα/β (Fig. 3.9D). Together, these results suggest that DC maturation alters IFNα/β signaling through the modulation of STAT utilization.

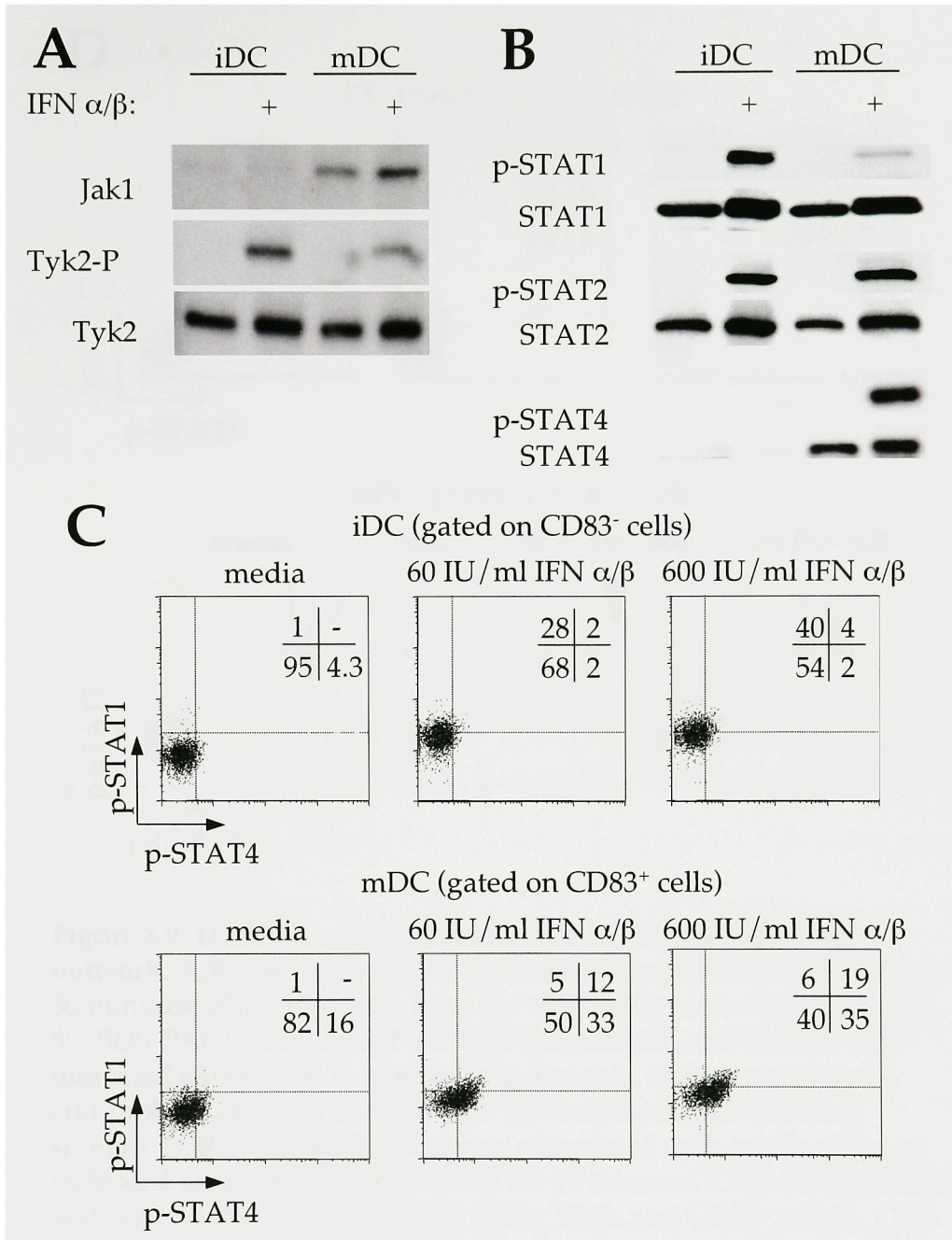

Figure 3.9. Dendritic cell maturation alters the IFNα/β signaling network. Continued on following page.

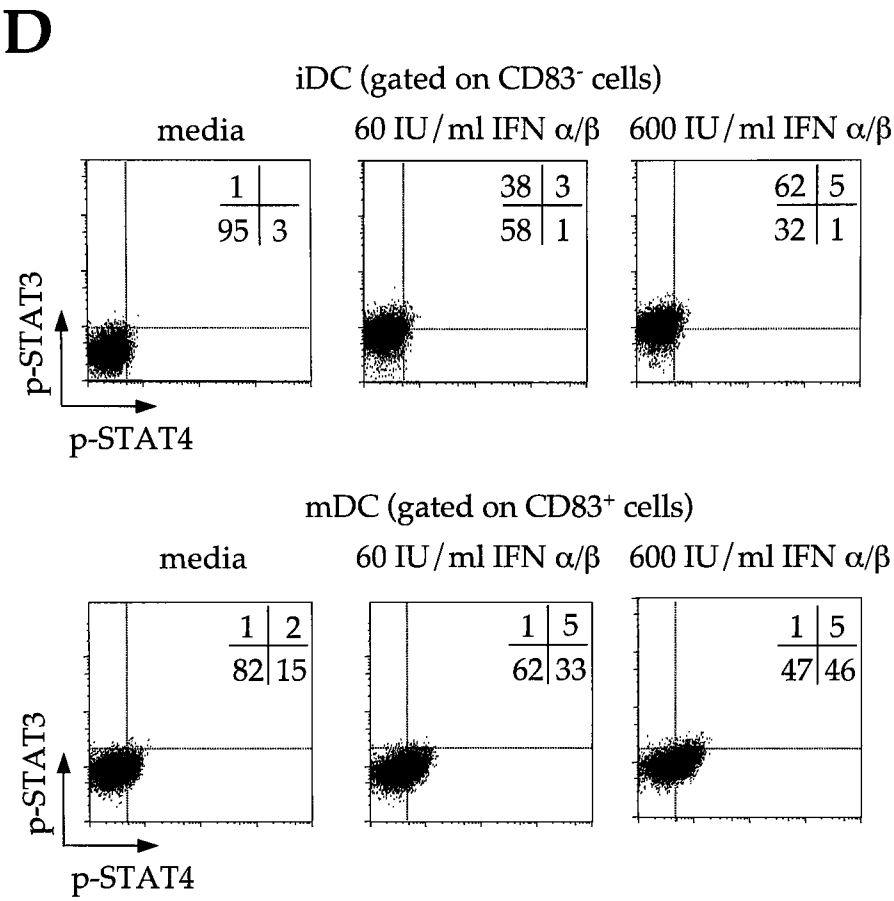

Figure 3.9. Dendritic cell maturation alters IFNα/β signaling network. A,B. iDC and mDCs were exposed to 60 IU/ml IFNα/β for 30 minutes. 20μg of protein from the total cell lysates were run on an 8% SDS-PAGE, transferred to PVDF membranes, and blotted with indicated antibodies. In selected experiments, blots were stripped and probed for β-actin as a control for loading the gel (data not shown). **C,D.** iDCs and mDCs were stimulated with media alone or indicated concentrations of IFNα/β for 30 minutes. Cells were stained with anti-CD83-FITC to identify $CD83^-$ iDCs and $CD83^+$ mDCs. Cells were fixed and permeabilized as described in Materials and methods and stained for p-STAT1-PE or p-STAT3-PE and p-STAT4-APC. Percentage of events in each quadrant is indicated on the respective plot. Data is representative of three independent experiments.

Importantly, this finding is not dependent on maturation conditions, as DCs matured with 10ng/mL of LPS also showed impaired STAT1 phosphorylation (Fig. 3.10A). Furthermore, the inhibition of STAT1 signaling is also observed after TNFα-mediated maturation of BDCA-1+ circulating DCs (Fig. 3.10A). In order to test if the inhibition of STAT1 phosphorylation in mDCs is intrinsically impaired, we evaluated the ability of IFNγ to trigger STAT1 activation. Notably, IFNγ-treatment of mDCs resulted in efficient phosphorylation of STAT1, indicating that inhibition of pSTAT1 is in fact specific to IFNα/β signaling (Fig. 3.10B).

STAT1 required for IFNα/β inhibition of IL-12

In order to evaluate the role of the STAT signaling switch in this dual regulation of IL-12 production, we took advantage of the fact that, similar to IFNα/β in our *in vitro* system, IFNα/β during MCMV infection significantly inhibits IL-12 production by CD11c+ DCs [195]. We confirmed these results showing significant increase in CD11c+/CD8- IL-12 production in both IFNAR-/- and pDC depleted mice (Fig. 3.11A). In order to evaluate that STAT-dependence of this effect, STAT1 and STAT4 KO mice, with 129SVE and Balb/c controls, respectively, were infected with MCMV Smith strain i.p. Analysis of IL-12 production by CD11c high DCs in the spleen 36 hours post-infection by FACS revealed that STAT1, but not STAT4 plays a crucial role in inhibiting the production of IL-12 by CD11c high DCs (Fig. 3.11B). These results suggest that the inhibitory effect of IFNα/β on IL-12 production by DCs is mediated by STAT1 and not STAT4.

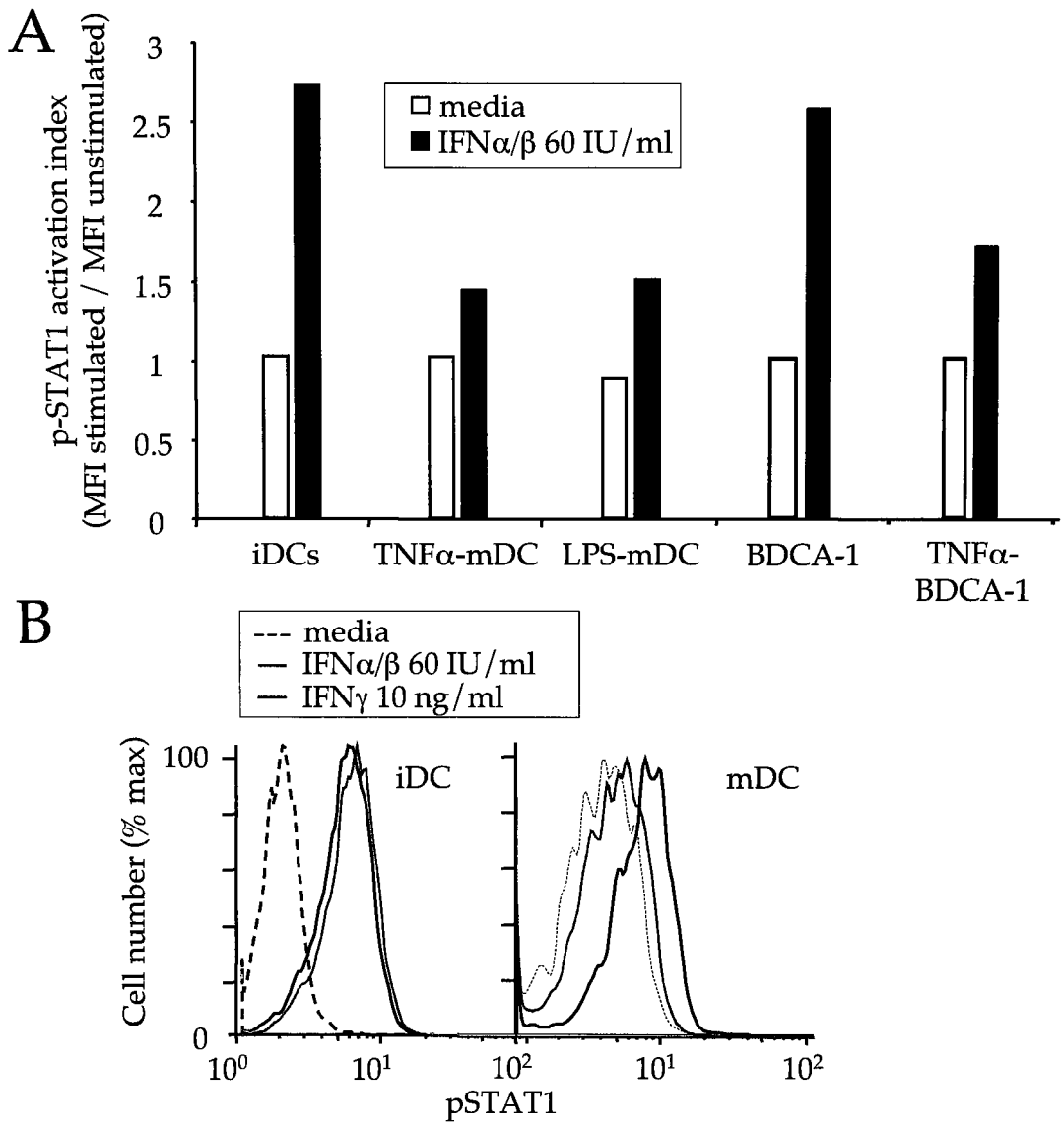

Figure 3.10. Failure to phosphorylate STAT1 is IFNα/β-specific and characteristic of mDCs. A. iDCs and mDCs were stimulated with media alone, 60 IU/ml IFNα/β, or 10 ng/ml IFNγ for 30 minutes as indicated. Cells were fixed, permeabilized, and stained for p-STAT1 (Pharmingen). **B.** iDCs, DCs matured with TNFα / PGE_2, or DCs matured with LPS were stimulated with media alone or IFNα/β as indicated. Geometric mean fluorescence intensity (MFI) of p-STAT1 was calculated using FloJo statistical analysis. p-STAT1 activation index is represented as a ratio of MFI IFN-stimulated / MFI media alone.

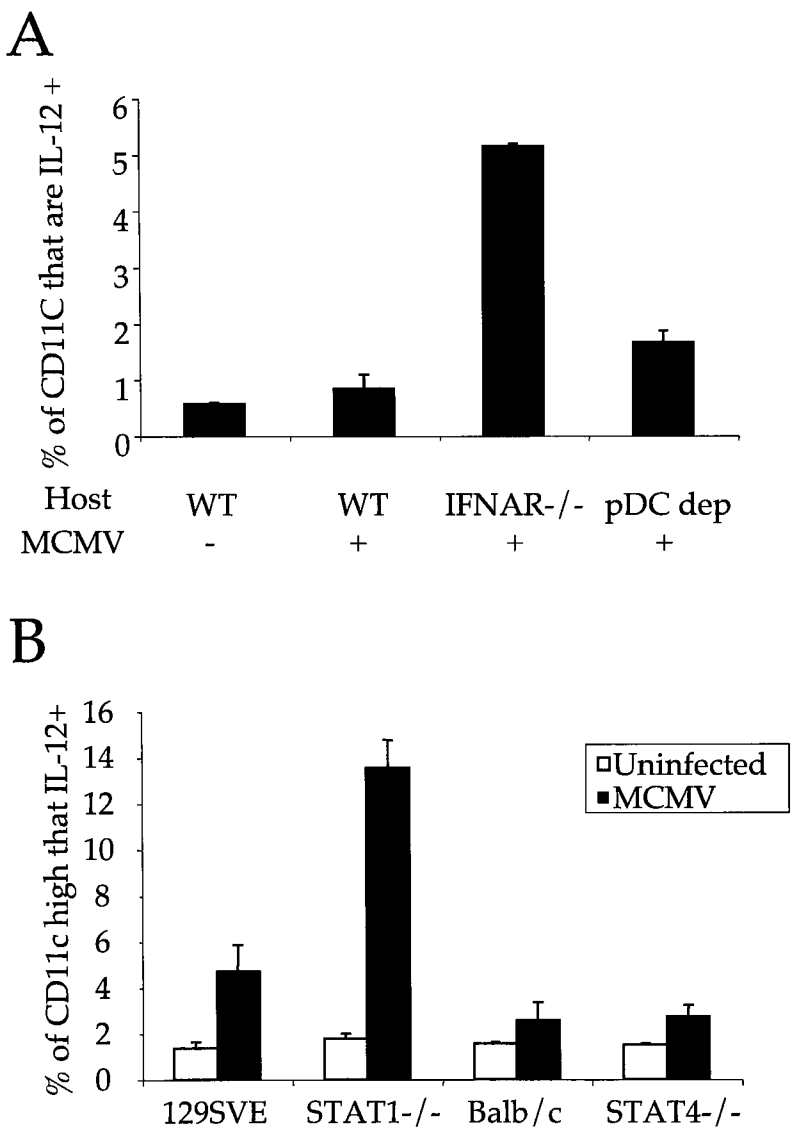

Figure 3.11. STAT1 is required for IFNα/β inhibition of IL-12 production by DCs. A. Mice were untreated or infected with MCMV. At 36 hours post-infection, spleens were harvested and stained with CD8 FITC, CD11c PE, and IL-12 as described in Materials and Methods. The percentage of CD11c+/CD8- DCs that stained positive for IL-12 is expressed as bar graph. **B.** STAT1-/- or STAT4-/- mice and their respective controls (129SVE and Balb/c) were infected with MCMV. Similarly, the percentage of CD11c+/CD8- DCs that stained positive for IL-12 is expressed as a bar graph.

pDC IFNα/β differentially regulates CD8⁺ T cell activation by cross-presenting DCs

In order to explore the effects of pDC-derived IFNα/β on antigen cross-presentation, supernatants from CpG-stimulated pDCs were added to iDCs during antigen capture and maturation (Fig. 3.12A, iDC + pDC IFNα/β), and then washed out prior to DC : T cell engagement. Alternatively, the pDC supernatant was added to mDCs during T cell engagement (Fig. 3.12A, mDCs + pDC IFNα/β). pDC-derived supernatants were prepared from BDCA-4 purified cells that had been stimulated with 20μg/mL CpG-2116 for 24 hours and the concentration of IFNα in the supernatant was quantified by ELISA (Fig. 3.12B). Similar to the effects of recombinant IFNα, the effect of pDC-derived cytokines on the regulation of T cell activation depended on the maturation state of the DC. iDCs exposed to pDC IFNα/β were inhibited in their ability to stimulate $CD8^+$ T cells (Fig. 3.12C). In contrast, mDCs demonstrated enhanced $CD8^+$ T cell activation in the presence of pDC IFNα/β (Fig. 3.12C). This increase in IFNγ production was antigen specific, as pDC-derived IFNα/β alone did not directly activate T cells. As a negative control for this experiment, supernatants from unstimulated pDCs were employed (Fig. 3.12C). Exposure of iDC co-cultures to CpG did not inhibit CD8+ T cell activation (data not shown).

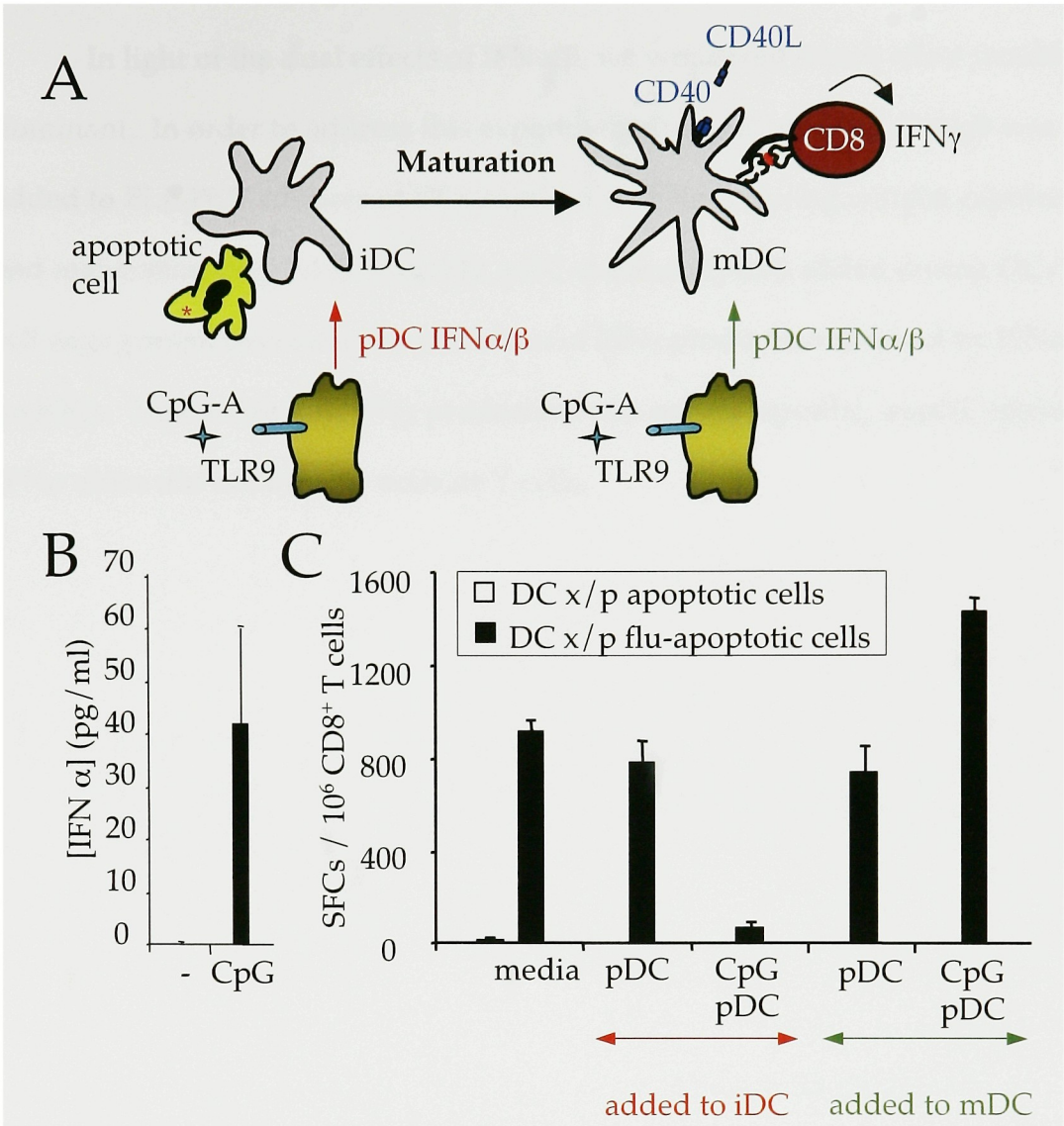

Figure 3.12. pDC-derived IFNα/β has a dual effect on cross-presenting myeloid DCs. A. pDC IFNα/β was generated from BDCA-4 purified pDCs. As illustrated, pDC IFNα/β was added to iDCs during antigen capture and maturation, or to mDCs during CD40 cross-linking and $CD8^+$ T cell engagement. **B.** Quantitation of IFNα concentration in pDC supernatants unstimulated (-) or stimulated with CpG. **C.** In all conditions, DCs cross-presenting (abbrev. x/p) antigen were cultured with purified $CD8^+$ T cells and CD40L. The activation of influenza-specific $CD8^+$ T cells was monitored by IFNγ ELISPOT and reported as spot forming cells (SFCs). DCs cross-presenting uninfected apoptotic cells (white bars) or DCs cross-presenting influenza antigen-expressing apoptotic cells (black bars). Unstimulated (pDC) or CpG-stimulated pDC (CpG-pDC) supernatant was added to the DCs as indicated. The data shown here is representative of two experiments. ELISPOT counts from triplicate wells were averaged and mean data is reported. Error bars indicate standard error of the mean.

In light of the dual effects of IFNα/β, we wondered which effect would be dominant. In order to address this experimentally, pDC-derived IFNα/β was added to ELISPOT cultures of DCs exposed to IFNα/β during antigen capture and maturation (Fig. 3.13). Notably, pDC-derived IFNα/β added during DC/T cell engagement overcame the inhibition of IFNγ production induced by IFNα/β on iDCs. This increase in IFNγ production was antigen specific, as pDC-derived IFNα alone did not directly activate T cells.

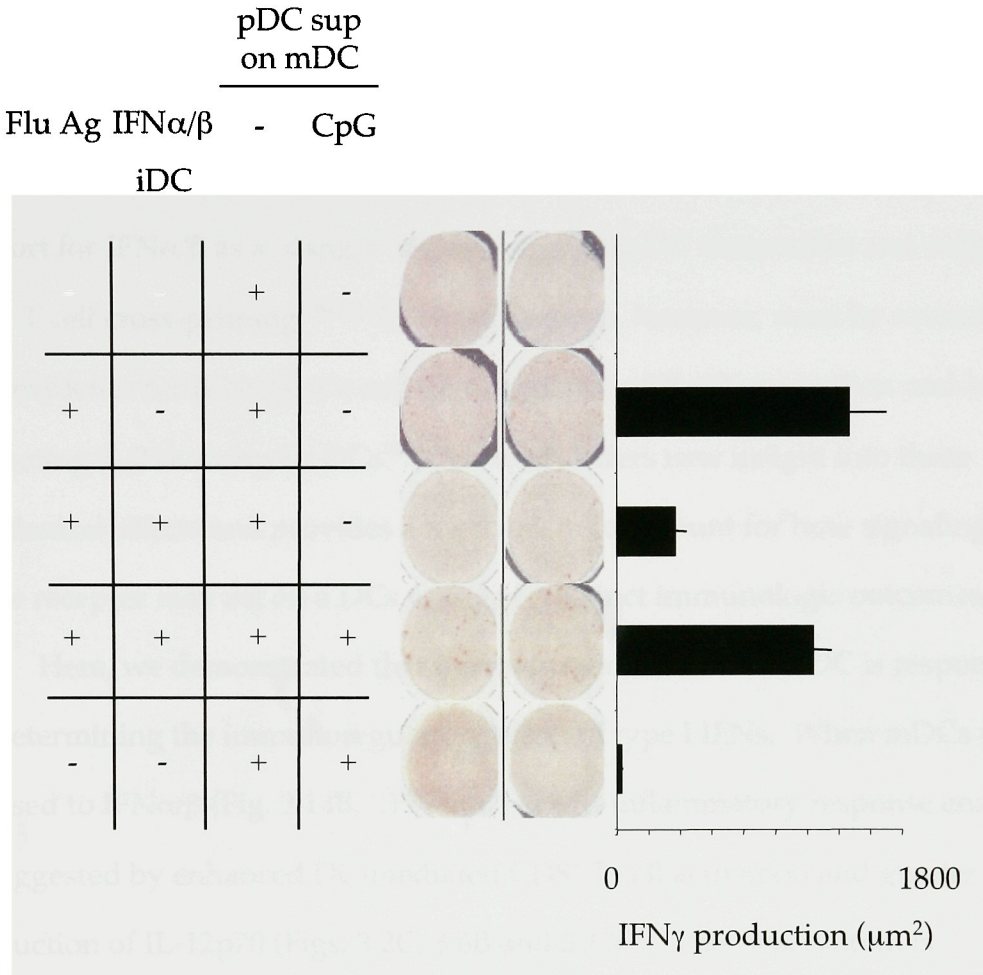

Figure 3.13. pDC-derived IFNα/β overcomes inhibitory effects of IFNα/β on iDCs. DCs cross-presenting influenza antigen (flu ag) exposed to IFNα/β during antigen capture (IFNα/β iDC) were cultured with purified $CD8^+$ T cells and CD40L in an IFNγ ELISPOT with pDC sup alone (-) or CpG-stimulated pDC (CpG pDC) as indicated. Pictures of duplicate wells are shown with corresponding bar graph indicating relative levels of IFNγ production as a function of total spot area (IFNγ production = spot number x average spot size).

3.5 Discussion

Dual regulation of cross-priming by pDC IFNα/β

While it is widely accepted that pDCs and type I IFNs offer an important link between innate and adaptive immune responses[188], there exist contrasting data regarding their immunoregulatory effects[108]. With respect to DC biology, there is support for IFNα/β as a 'danger' signal, triggering DC maturation and enhancing $CD8^+$ T cell cross-priming [196,184,185]. These findings, however, must be reconciled with evidence for IFNα/β as a negative regulator of IL-12 production and its promoting T_H2 skewing by DCs[187]. Our study offers new insight into these paradoxical effects and provides a mechanism to account for how signaling via a single receptor may act on a DCs to trigger distinct immunologic outcomes.

Here, we demonstrated that the maturation state of the DC is responsible for determining the immunoregulatory effect of type I IFNs. When mDCs were exposed to IFNα/β (Fig. 3.14B, 'IFN-late'), a pro-inflammatory response ensued, as suggested by enhanced DC-mediated $CD8^+$ T cell activation and greater production of IL-12p70 (Figs. 3.2C, 3.6B and 3.12C). This observation is consistent with the role of IFNα/β in enhancing TLR mediated cytokine production[197,198] and the 'licensing' of DCs for cross-priming[185]. In contrast, we discovered that iDCs exposed to IFNα/β during antigen capture and maturation (Fig. 3.14A, 'IFN-early') are impaired in their ability to activate $CD8^+$ T cells (Figs. 3.2B). This inhibitory effect of 'IFN-early' is specific for CD40-dependent $CD8^+$ T cell activation (Figs. 3.3 and 3.4), implicating the modulation of a 'third signal' produced by the DCs[76].

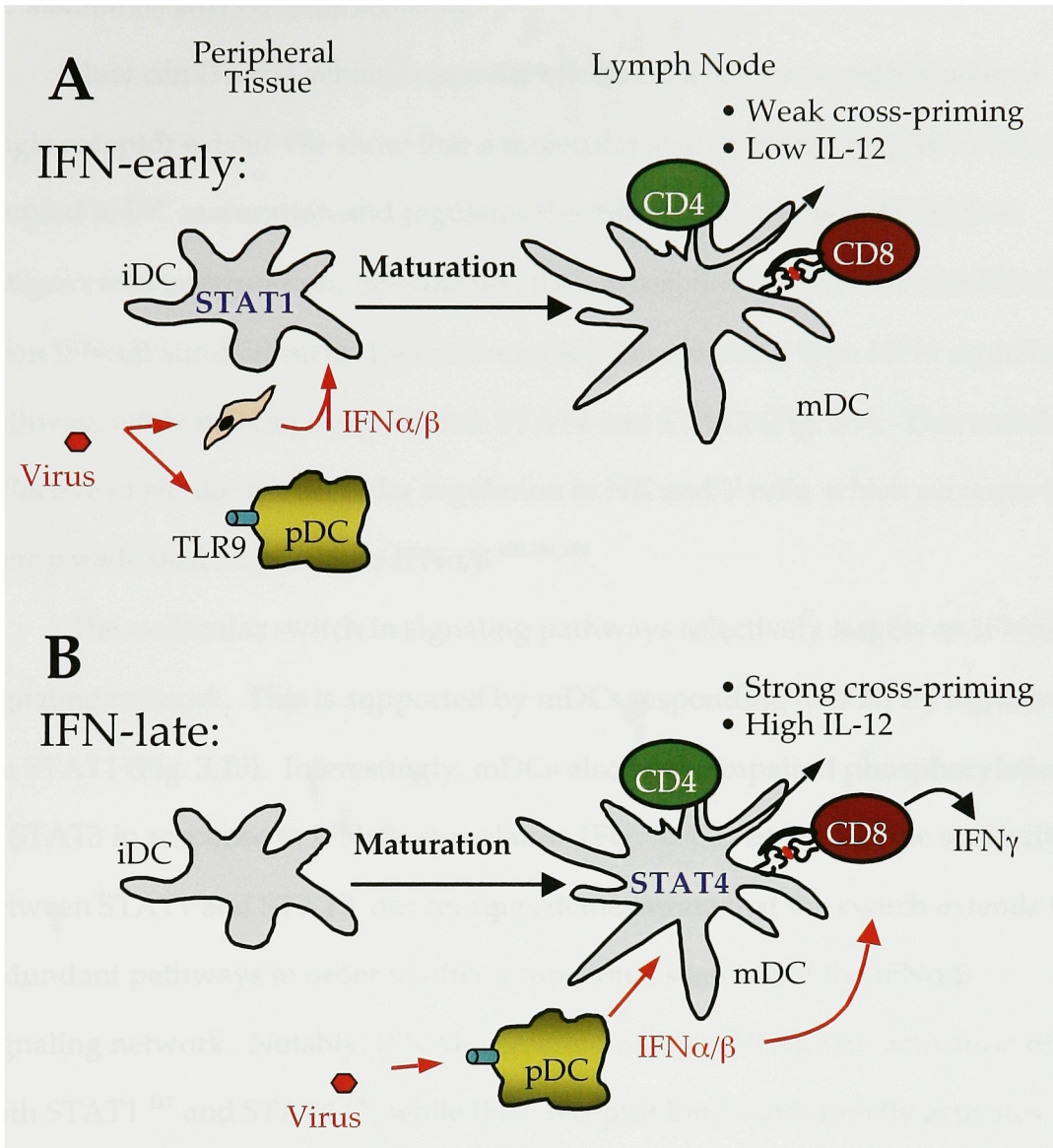

Figure 3.14. Spatial location of pDCs results in compartmentalization of type I IFNs, and differential effects on cross-presentation due to a DC maturation-induced switch in STAT utilization. This schematic represents our working model for how type I IFNs may exert their paradoxical effect on cross-presentation though the compartmentalization of IFNα/β. A. IFN-EARLY: IFNα/β produced in the tissue by infected cells or TLR engagement of pDCs activates STAT1 dependent signaling in immature myeloid DCs, which results in low IL-12p70 production and an impairment of helper-dependent cross-priming. B. IFN-LATE: Altered signaling networks in mDCs prevents STAT1-mediated signaling. Instead, IFNα/β produced in lymph organs results in STAT4 dependent signaling in mature DCs, which facilitates enhanced production of IL-12p70 upon CD40 engagement. IFNα/β may also act directly on CD8+ T cells.

DC maturation alters IFNα/β signaling

How can IFNα/β achieve opposite effects on iDCs *versus* mDCs when a single receptor exists? We show that a molecular switch in signaling networks is coupled to DC maturation and regulates the differential effects of IFNα/β on antigen cross-presentation. Specifically, iDCs phosphorylate STAT1 and STAT2 upon IFNα/β stimulation and seem to employ the canonical type I IFN signaling pathway, while mDCs phosphorylate STAT4 and STAT2 (Fig. 3.9). This switch is reflective of similar intracellular regulation in NK and T cells, which accounts for their paradoxical responses to IFNα/β [108,181,199].

The molecular switch in signaling pathways selectively targets an IFNα/β signaling network. This is supported by mDCs responding to IFNγ by signaling via STAT1 (Fig. 3.10). Interestingly, mDCs also show impaired phosphorylation of STAT3 in response to IFNα/β stimulation (Fig. 3.9D). In light of the similarity between STAT1 and STAT3, our findings demonstrate that the switch extends to redundant pathways in order to offer a functional skewing of the IFNα/β signaling network. Notably, IFNAR signaling requires STAT2 for activation of both STAT1 [117] and STAT4 [200], while IFNγ receptor binds and directly activates STAT1. These differences may account for the selective regulation of pSTAT1 downstream of IFNAR.

The master regulator of this switch in IFNα/β signaling remains unknown. Although quantitative shifts in STAT1 inhibit STAT4 activation[199], it is not clear if reciprocal increases in STAT4 are directly responsible for inhibiting STAT1 activation. Alternatively, regulation may occur at the JAK level. Increase in total Jak1 and the Jak1:Tyk2 ratio may skew downstream signaling (Fig. 3.9).

Alternatively, Tyk2 may play a dominant role in STAT1 phosphorylation[201]. Interestingly, transcriptional changes in suppressor of cytokine signaling-2 (SOCS-2) and protein tyrosine phosphatase N1 (PTPN1) show 10-fold and 5-fold increase upon maturation respectively (Fig. 3. 15). Although SOCS-2 has not been defined to play a role in the STAT signaling pathway, family members SOCS-1 and SOCS-3 negatively regulate the JAK-STAT pathway by directly inhibiting JAK activity[202]. PTPN1 may also play a role in skewing signaling by promoting STAT1 dephosphorylation. Experiments are currently underway to evaluate these candidate genes using retrovirally transduced shRNAs.

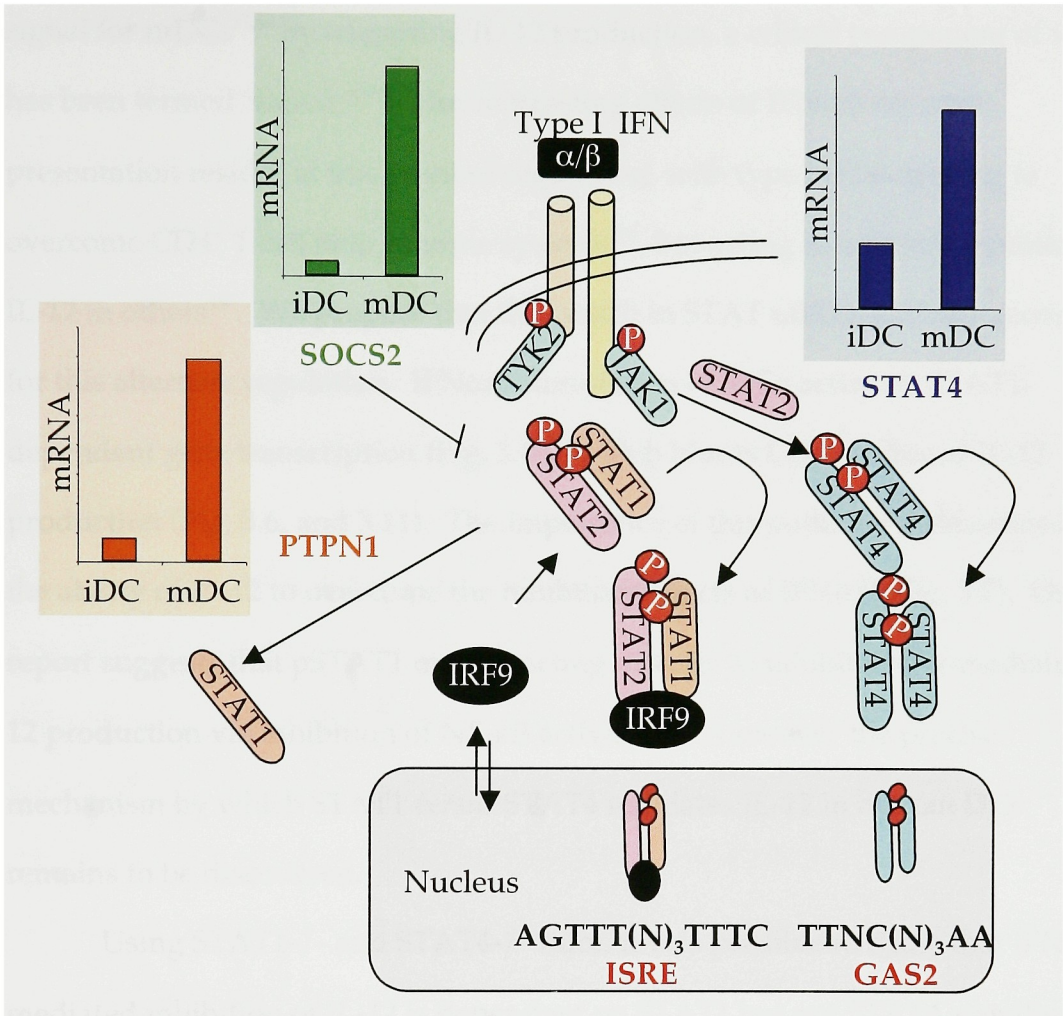

Figure 3.15. Maturation alters IFNα/β signaling in DCs. This schematic represents our working model for how type I IFNs signal in DCs. In iDCs, phosphorylation of STAT1 and STAT2 allows formation of ISGF3 and transcription of ISRE-dependent genes. In mDCs, striking upregulation of STAT4 correlates with impaired response of STAT1 phosphorylation. Formation of STAT4 homodimers leads to transcription of GAS-dependent genes. Upregulation of suppressor of cytokine signaling-2 (SOCS2), protein tyrosine phosphatase N1 (PTPN1), and STAT4 as a result of maturation may contribute to the signaling regulation. Histograms indicate relative mRNA expression determined by affymetrix microarray.

Altered signaling regulates outcome of CD40 engagement via IL-12

$CD4^+$ T cells, acting via CD40L : CD40 engagement, serve as a 'licensing' signal for mDCs[72,62] by triggering IL-12 production, a critical component of what has been termed 'signal 3'[76]. The contrasting effects of IFNα/β on cross-presentation resides at this point of regulation, with type I IFNs serving to overcome $CD4^+$ T cell help in some systems[185], but acting as a potent inhibitor of IL-12 in others[187]. We propose that the switch in STAT utilization may account for this alternate regulation. IFNα/β stimulation of iDCs activates STAT1-dependent gene transcription (Fig. 3.14), which blunts CD40-induced IL-12 production (Fig. 3.6, and 3.11). The importance of this pathway is illustrated by the ability of IL-12 to overcome the inhibitory effects of IFNα/β (Fig. 3.7). One report suggests that pSTAT1 may be acting directly to inhibit TNFα-mediated IL-12 production via inhibition of NF-κB activation[203], however the precise mechanism by which STAT1 *versus* STAT4 regulates IL-12 in human DCs remains to be discovered.

Using STAT1-/- and STAT4-/- mice, it was possible to show that IFNα/β mediated inhibition of IL-12 is dependent on STAT1 but not STAT4 signaling (Fig. 3.11). The inhibition of IL-12 by IFNα/β is arguably occurring in the spleen, but the DCs analyzed are mostly resident DCs of the spleen that exist in an immature state under steady state conditions[81]. In contrast, tissue DCs must undergo maturation before migration to the lymph node. As such, we hypothesize that inhibition of IL-12 production would not be seen in this population. However, the STAT1 to STAT4 switch in mouse DC maturation has not been fully analyzed. Similar to our human data, splenic DCs upregulate

STAT4 upon maturation[204]; however, direct STAT1 or STAT4 activation has not been evaluated. Although early work suggested that IFNα-dependent STAT4 activation does not occur in mice due to a STAT2 minisatellite insertions[200], more careful analysis revealed that the pathway for STAT4 activation is intact in mice[199], but variable expression may depend on genetic background[205]. STAT2 minisatellite insertion may still account for species specific STAT2-dependent IFNα/β signaling.

Location, location, location: Physiologic implications of spatial compartmentalization of IFNα/β production

Regarding the physiologic and pathologic relevance for our findings, we suggest that spatial compartmentalization of IFNα/β production may offer a resolution for its paradoxical immunoregulatory effects on conventional DCs. Immature and mature DCs reside in different physiologic locations, with the former residing within peripheral tissue and the latter homing to the T cell area of draining lymphoid organs[52]. In situations of chronic infection, type I IFNs in the tissue (produced by parenchymal cells, pDCs that have moved into sites of chronic inflammation[191] or by immature myeloid DCs themselves[206]) may exert a counter-inflammatory effect on iDCs (Fig. 3.14A). Specifically, we suggest IFNα/β produced in the tissue during antigen capture, inhibits subsequent T cell activation by cross-presenting DCs. The restriction of this inhibition to cross-presentation is likely due to the CD40-dependence in our model system and may be more broadly applicable to other IL12-dependent responses[68].

During some viral infections, PAMP engagement of pDC TLRs induces migration to the lymph node and production of robust amounts of type I IFNs (Fig. 3.14B). In this situation, IFNα/β would act on mDCs that had migrated from the peripheral tissue with captured antigen. The exposure of mDCs to IFNα/β may act directly on the DCs to synergize with other inflammatory stimuli and enhance IL-12 production. Our model does not rule out an additional, direct effect of IFNα/β or other pDC-derived cytokines on $CD8^+$ T cell activation. For example, recent data suggests that activation of STAT4 in antigen specific $CD8^+$ T cells may potentiate IFNγ production[207,208]. Notably, the exposure of mDCs to IFNα/β during DC : T cell engagement overcomes the inhibitory effect of IFNα/β (Fig. 3.13). Thus, even if the cross-presenting DC had captured antigen in the presence of IFNα/β, pDC IFNα/β in the lymph would enable that DC to prime a T cell response.

Why would the host employ IFNα/β production as an innate immune response if it interfered with the generation of adaptive immunity? The counter-inflammatory roles of IFNα/β may play an important role in tempering the immune response during acute periods of hyperactivity. For example, IFNα/β inhibits bystander T cell activation[209] as well as high levels of IL-12[186] preventing cytokine toxicity. The inhibition of CD8+ T cell activation by cross-presenting DCs may provide another level of regulation against non-specific activation during viral infection. Delivery of inflammatory cytokines to draining lymph nodes at the site of DC : T cell engagement by accessory cells such as pDCs, therefore, may play an important role in regulating activation.

Both pathogens and therapies may take advantage of the inhibitory effects of IFNα/β on cross-priming. HCV, for example, induces robust levels of IFNα/β production soon after infection[139]. Although it remains unclear whether DCs can be infected by HCV, the high levels of IFNα/β in the liver may play an important role in preventing effective $CD8^+$ T cell response in chronically infected patients[41]. Interestingly, the bacterial pathogen *L. monocytogenes* may also use the host's IFNα/β response to prevent an effective immune response[210]. In addition to its role in infectious immunity, the inhibitory effect of IFNα/β on tissue DCs may account for the recent observation of an inverse correlation between length of remission and pDC infiltration in early stage breast cancer[211]. Finally, the results presented here may offer a more detailed mechanism for the therapeutic use of type I IFN in multiple sclerosis to decrease T cell activation[212].

In contrast, the therapeutic effects of IFNα for HCV are thought to enhance T cell immunity to aid in viral clearance; however, recent reports suggest that this may not in fact be the case[101]. Despite sustained virologic response (SVR), T cell immunity to HCV may decrease with pegylated-IFNα / ribavirin[213]. The decreased T cell reactivity may be beneficial in reducing the hepatic inflammation and fibrosis during recovery. Several scenarios may account for these findings including a simple decrease in antigen load during SVR or the inhibitory effects of IFNα on iDCs when delivered systemically. In contrast, the therapeutic activation of pDCs may enhance IFNα/β production in lymph nodes and it will be interesting to see if comparative studies addressing the effects of pDC-derived IFNα/β on HCV-specific T cell immunity produce similar results.

Despite the potency of IFNα/β inhibitory effect on cross-presenting DCs, immune responses to viral infections do occur. As mentioned above, the inhibitory effects on cross-presenting DCs are most pronounced for immunity that depends on CD40 licensing of DCs and the production of IL-12 as a signal for T cell activation. Although these signals serve as an important component of the immune response in many infections, the number of infections that absolutely depend on these signals may be quite limited. Moreover, IFNα/β production is not restricted to the tissue in many viral infections that induce it. PAMPs, which trigger IFNα/β production in the tissue, may also trigger pDCs to produce IFNα/β at the DC/T cell interface, thereby overcoming the inhibitory effects of tissue IFNα/β. Nonetheless, these findings offer a more detailed understanding of the role of IFNα/β in both pathogenesis and therapy for all infections.

In all, these findings show that DC maturation alters signaling networks, enabling distinct effects of IFNα/β on the immunologic outcome of cross-presentation. This plasticity illustrates a novel mechanism by which DCs modulate the integration of signals from the surrounding environment. These findings suggest a rationale for spatial compartmentalization of pDCs during viral pathogenesis and offer insight into more effective therapeutic delivery of IFNα. As such, we turn in the next chapter to *in vivo* models to evaluate pDC-derived IFNα/β in CD8+ T cell immunity.

Chapter 4: pDC-derived IFNα/β skews DC / T cell engagement from tolerance to priming in lieu of CD4+ T cell 'help'

4.1 Summary

CD8+ T cell immunity is an important determinant in the clinical outcome of HCV infection and depends on CD4+ T cell 'help'. IFNα has been shown to play a role in overcoming the CD4 requirement for T cell priming; however, the role for pDCs in providing a signal sufficient for T cell activation in the absence of CD4+ T cells has not been demonstrated. Using the minor histocompatibility male antigen (H-Y) model for CD4-dependent CD8+ T cell priming, we evaluated the ability of MCMV- or CpG-mediated pDC activation to substitute for the CD4 'help' requirement in CD8+ T cell priming. The results show that pDC activation can offer priming signals. The pDC priming signal requires IFNAR but is independent of IL-12 production. In contrast, viral stimulation of non-pDC IFNα/β does not serve as a priming signal for CD8+ T cells. The model of pDC 'help' could also be extended to CD4-dependent HCV $NS3_{1073-1081}$-specific CD8+ T cell responses in transgenic mice expressing human HLA-A2.1. These findings offer a mechanism for the role of pDC-derived IFNα/β in host immunity, provide a rationale for therapeutic intervention to boost CD8+ T cell responses, and may offer insight into how some viruses evade immunostimulatory actions of IFNα/β.

4.2 Introduction

In light of the fact that we find no overt defect in DC function in chronic HCV donors, we hypothesized that other signals may act on DCs to regulate HCV specific CD8+ T cell priming. As discussed in chapter 1, several lines of evidence suggest that CD4+ T cells play an important role in generating robust HCV specific CD8+ T cell responses[41]. However, the absence of a small animal model and the paucity of T cell reagents have precluded detailed evaluation of the immunologic outcome of 'help'-less CD8+ T cells in an HCV specific model.

As detailed in the introduction, CD4+ T cells can directly support CD8+ T cell activation (via IL-2 production or CD40 engagement[214]) or indirectly via DC activation. This model for CD4 'help' affords a sense of specificity for CD8+ T cell priming and prevents autoimmunity in the absence of a specific CD4+ T cell response[215]. The flip side of this model, the absence of CD4 'help', affords a model for peripheral tolerance by which DC / T cell engagement leads to division, death, and consequent tolerance (see Fig. 1.5). Although the regulation of CD8+ T cell activation versus tolerance by CD4+ T cells offers a mechanism for antigen specific regulation, this model simply transfers the point of regulation to priming or tolerance of CD4+ T cells. In contrast, the "danger model" suggests that regulation of activation *versus* tolerance is controlled by inflammatory danger signals[66].

In light of our observation concerning spatial compartmentalization of IFNα/β on DC immunological skewing, we hypothesized that lymph node IFNα/β may act to enhance T cell immunity (Fig. 4.1). pDC activation by TLR9 agonists result in pDC accumulation and IFNα/β production in lymph nodes and

spleen[216]. The importance of this spatial localization in viral immunity is not clear. Some reports suggests that pDCs may prime T cells *in vivo* [217]; however, their movement from blood to lymph node does not make them well suited for trafficking tissue antigens. Furthermore, the reason for IFNα/β production in the lymph node when viral infection occurs in the periphery is not clear. Recent data shows that IFNα/β itself may act as a key 'danger' signal by activating DCs as well as serving as a third signal for T cell activation [185,207]. Not all IFNα/β, however, is produced by pDCs [195,206]. All cells have the capacity to produce type I IFNs and some (e.g. conventional DCs) will produce equally large amounts if triggered appropriately. The immunological effects of IFNα/β production by different cell types or by the same cell type in different locations is not clear.

The role for activated pDCs in substitituting for CD4+ T cell 'help' has not been evaluated. In order to address this question, I took advantage of two *in vivo* systems. The first system is based on the minor histocamptibility (H) antigens that are associated with the Y chromosome (i.e. H-Y antigens)[218]. In C57BL/6 mice, an immunodominant D^b-restricted Uty epitope has been well defined. This model is particularly fitting because the priming of Uty-specific CD8+ T cells is CD4-dependent[215] and transgenic T cells are available[219]. The second model is one that I developed for HCV specific priming in transgenic mice expressing the human HLA-A2.1 molecule. In both models, I have focused on the endogenous T cell repertoire against these antigens. The findings show that activated pDCs can offer priming signals for CD8+ T cell activation and suggest important therapeutic implications for pDCs.

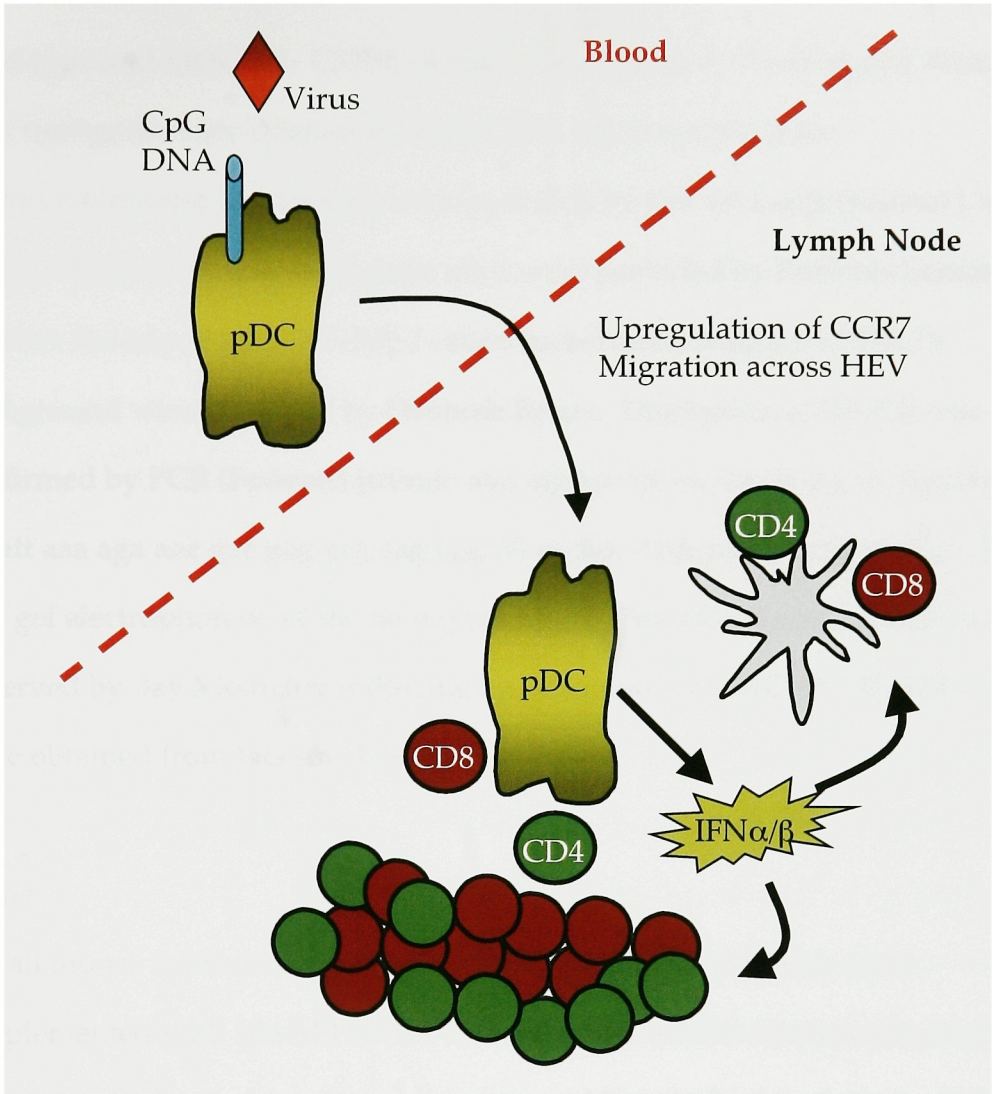

Figure 4.1. Immunological significance of pDC IFNα/β production in the lymph node. Following activation, pDC upregulated CCR7 and migrate across the HEV to T cell zones of the lymph node. pDC can prime CTLs *in vivo* but they can not cross-present antigen. The antiproliferative effects of IFNα/β may play a role in preventing bystander activation in the bulk T cell population. The interaction at the DC / T cell interface is not well defined.

4.3 Materials and Methods

Mice

Wild-type and MHCII-/- C57BL/6 mice were obtained from Jackson, Europe. TCR transgenic mice (Matahari) with CD8+ T cell reactivity for immunodominant Uty epitope were provided by Olivier Lantz (Institut Curie, Paris). HLA-A2.1 HHD transgenic mice were provided by Francois Lemonnier (Institut Pasteur, Paris). IFNAR-/- mice backcrossed onto the C57BL/6 background were provided by Deborah Braun. Disruption of IFNAR was confirmed by PCR (Forward primer: aag atg tgc tgt tcc ctt cct ctg ctc tga; Reverse: att att aaa aga aaa gac gag gcg aag tgg; 35 cycles; Annealing temperature: 55°C) and gel electrophoresis of the neomycin insert. Functional confirmation was also observed by day 5 lethality following i.p. infection with MCMV. IL-12B-/- mice were obtained from Jackson, USA.

Media

For all mouse cultures and BMDCs, 10% FBS in RPMI-1640 was used supplemented with 10mM HEPES, 100mM non-essential amino acids, 66μM β-mercaptoethanol, 1mM sodium pyruvate, and 2mg/mL of gentamicin (R-10). HCV cell lines were carried in 10% FBS in DMEM with 20μg/mL puromycin and tetracycline (complete media) to suppress expression of HCV. Expression of HCV was induced in complete media without tetracycline.

Peptides

For monitoring HY-specific CD8+ T cell responses, immunodominant Db-restricted $Uty_{246-254}$ peptide (WMHHNMDLI) was used. For MCMV T cell responses, immunodominant D^b-restricted M45 epitope (HGIRSNAFI) was used. For influenza ΔNS1 endogenous CD8+ T cell responses, D^b-restricted $NP_{366-374}$ (NMEAMDSNT) and Db-restricted $PA_{224-233}$ (SCLENFRAYV) were used. All peptides were synthesized by Genemed synthesis. For monitoring HLA-A2.1-restricted HCV-specific T cell responses, the following peptides were used: $Core_{35-44}$ (YLLPRRGPRL), $Core_{132-140}$ (DLMGYIPLV), $NS3_{1073-1081}$ (CVNGVCWTV), $NS3_{1406-1415B}$ (KLTGLGLNAV), $NS3_{1406-1415A}$ (KLVALGINAV).

Antibodies

PDCA-1 PE, CD11c (HL3) APC, Vβ8.3 PE, and CD8 FITC are from BD Pharmingen. GK1.5 hybridoma supernatant was prepared in low serum media (containing <1% FBS). Briefly, hybridoma was grown to confluency until 60-70% death. Cultures were harvested and supernatant prepared by filtration. RB6-8C5 (anti-GR-1) for *in vivo* depletion of pDCs cells was obtained from the monoclonal antibodies facility at MSKCC as bioreactor supernatant.

Cell lines

A human osteosarcomcell line with tetracycline-dependent full length HCV expression (HOS 57.3) was generated by Benno Wolk in the laboratory of Darius Moradpour (Basel, Switzerland). In order to induce HCV expression, cells were washed well with PBS (4-5 times) to remove residual tetracycline and plated at

20-30% confluency in complete media without tetracycline. Greater than 80% of cells expressed NS5A by FACS by 36hrs. 3T12 is a mouse fibroblast cell line obtained from Peggy MacDonald (Rice lab, RU). 3T12 cells were grown in DMEM with 10% FBS with 10mM HEPES, 100mM non-essential amino acids, and 100 units/mL penicillin and streptomycin.

Bone Marrow Derived DCs

Femurs and tibias were collected from C57BL/6 mice and bone marrow was harvested by flushing with PBS. Bone marrow cells were collected by centrifugation and lysed with 2.5mL 1.66% ammonium chloride for 5 minutes at 37°C. Cells were washed, filtered and resuspended at 10^6/mL in R-10 media with 1:30 dilution of J558 hybridoma supernatant (mouse GM-CSF). 3mLs were aliquoted per well into a 6-well dish and incubated at 37°C. On day 2, day 4, and day 6, media was removed and replaced with fresh R-10 with J558. For maturation, cells were harvested by blowing gently on the plate with a 5mL pipette to collect loosely adherent DC clusters. Cells were centrifuged and resuspended at 0.5 x 10^6/mL in R-10 media with 1:30 dilution of J558 hybridoma and 12.5ng/mL of mouse TNFα (R and D systems). 2mL were added per well and cells were allowed to mature for 36 hours. DC phenotype and maturation was assessed by FACS phenotype using CD11c, CD80/86, I-Ab, and B220.

In vivo depletions and immunizations

GK1.5 hybridoma supernatant was used to deplete CD4+ cells and RB6-8C5 (Monoclonal Antibody Facility, MSKCC) bioreactor supernatant was used to deplete GR-1+ pDCs. Mice were injected with 500μg i.p. at 36 hrs and 12 hrs

before immunization. Depletion of CD4+ T cell and pDCs was confirmed by FACS from both peripheral blood and spleen samples.

For H-Y priming, mice were immunized with male splenocytes as indicated. Spleens were harvested and mashed in ice cold PBS. Splenic debris was removed, cells were spun, and red blood cells were lysed with 1.66% ammonium chloride for 5 minutes at 37°C. Lysis was quenched with 20 volumes of PBS and cells were pelleted. Cells were filtered and washed again. Splenocytes were resuspended at 25 x 10^6/ mL and 200μL was injected i.d. on the belly just medial to the inguinal lymph nodes.

For peptide immunization, 10mg/mL of crude peptide dissolved in 10% DMSO was mixed 1:1 with titermax Gold (Sigma Aldrich). Mixture was vortexed for 45 minutes at room temperature to form an emulsion. 30μL was injected into the footpad.

For full length HCV cell line immunization, HOS57.3 were induced to express HCV proteins as described. After 36 hrs of induction, cells were harvested and washed well with PBS. Cells were resuspended at 20 x 10^6/mL and 250μL injected i.p.

Preparation of MCMV Smith strain from salivary gland

1.5 x 10^5 PFU tissue culture prepared MCMV was injected into 15 BALB/c. In susceptible mice, MCMV is cleared from tissues within a week but persists in the salivary glands for >3 weeks at high titers. Salivary glands were harvested at 17 days post infection and homogenized with a dounce homogenizer at 10% (w/v)

in DMEM with 10% FBS, 10% DMSO. Aliquots were frozen at –80°C for long term storage.

MCMV plaque assay

Mouse fibroblast cell 3T12 were plated as 70% confluence in 6-well dishes and allowed to adhere for 5-6 hours at 37°C in DMEM with 10% FBS. Media was removed from 6-well dishes and 200μl of serial 10-fold dilutions were added for 1 hr with shaking. During incubation 1% (2x) NOBLE agar (DIFCO) was prepared in water. Agar was autoclaved and allowed to cool in 56°C water bath. Just before overlaying, 2x cMEM (2x MEM, 10% FBS, 100units /ml penicillin / streptomycin) was mixed with 1% NOBLE agar solution. Agar was allowed to cool to 37°C and 3mL was added to 6-well dish just before congeling with swirling to evenly distribute. Plates were incubated upside at 37°C. An additional overlay of 2mL was added on day 3. On day 6, agar overlay was prepared with .0375% Neutral Red and 2mLs added for 16 hrs to allow for visualization. Plaques were counted with a light box and enumeration used to calculate PFU/mL.

Triggering and monitoring IFNα/β production in mouse serum

Various titrations of i.p. injection of both MCMV and influenza ΔNS1 were done to evaluate necessary PFU for maximal IFNα/β production. Based on these results, 5×10^3 PFU/mL of MCMV and 2×10^6 PFU/mL of ΔNS1 were used for stimulation of IFNα/β production. CpG motif was prepared as previously described [156]. Briefly, 5 μg of CpG motif (5'- tcattggaaaacgttcttcggggcg -3') with

phosphorothioate backbone was mixed with 170μL of PBS and 30μL of DOTAP Transfection Reagent (Roche Diagnostics) and delivered i.v. Retro-orbital eyebleeds were done to collect blood samples at the indicated times. Blood was allowed to clot at room temperature for 30 minutes and placed on ice for 10 minutes. Samples were spun at 13000 RPM 4°C in a micro-centrifuge for 10 minutes and serum supernatants were saved.

IFNγ ELISPOT analysis of antigen specific T cell responses

Spleens or lymph nodes were harvested 7 or 12 days post-immunization as indicated. CD8+ T cells were purified using MACS microbeads and plated with indicated target cells. Syngeneic splenocytes from naïve cells were used as target cells for ELISPOTs. Cells were pulsed 10mM of the indicated peptide at room temperature for 1 hr. Purified CD8+ T cells were resuspended at 4×10^6/mL and 50μL was added per well. Targets were resuspended at 10^6/mL and 100μL added per well (CD8+ T cell : Target ratio was 2:1).

Cells were plated in 96-well Millipore plates previously coated with 5μg/mL of anti-IFNγ mAb (Mabtech, Mab-1 AN18). Cultures were incubated for 24-36 hrs at 37°C, washed with mild detergent, and incubated with 1μg/mL biotin-conjugated anti-IFNγ mAb (Mabtech, Mab R4-6A2). Spots were visualized using Vectastain Elite Kit (Vector Laboratories) and AEC substrate. Evaluation was performed in a blinded fashion by an independent service (Zellnet Consulting, Inc., NY) using an automated ELISPOT reader (Carl Zeiss, Inc., NY). Spots represent IFNγ production by single cells and are reported as spot forming cells (SFCs)/10^6 cells.

In vivo proliferation

Matahari spleens were harvested and red blood cells were lysed as described above. Splenocytes were resuspended in PBS and labeled with 5mM CFSE for 5 minutes at 37°C. Cells were washed 3 times with PBS, resuspended in PBS at 10^7/mL, and 100μL was injected i.v. 6 hrs before immunization. 3 days after immunization, the draining inguinal lymph nodes and spleens were harvested. Matahari cells were identified by CD8, Vβ8.3, and CFSE staining described below.

In vivo CTL

Splenocytes were generated as above and resuspended at 10^7/mL in PBS with either 1 or 6mM CFSE for 10 minutes at 37°C. Cells were washed 2 times with PBS and incubated with or without 10mM of the indicated peptide for 1 hr at room temperature. Cells were washed twice with PBS and resuspended at 10^8/mL. Cells were mixed 1:1 and injected i.v. into immunized animals. Spleens were harvested after 16 hrs, red blood cells lysed, and splenocytes analyzed for ratio of CFSE low to CFSE high population. Percent killing = 1 - (sample peak pulsed targets/sample peak unpulsed targets)/(control peak pulsed targets/control peak unpulsed targets).

FACS analysis of HCV expression, CFSE proliferation, tetramers, and pDC accumulation

For HCV expression, cells were induced as above and resuspended at $2x10^6$/mL. Cells were washed with PBS and fixed/permeabilized with Cytoperm/Cytofix (BD Pharmingen) for 10-15 minutes. Cells were washed with permeabilization wash buffer (BD Pharmingen) and incubated with 1:200 dilution of mouse monoclonal against NS5A (9E10, generated by Tim Tellinghusien) or 1:100 NS3 (Maine Biotechnology). A 1:400 dilution of goat anti-mouse PE secondary (Jackson Immunoresearch) was used to detect HCV expression.

For CFSE proliferation and tetramer analysis, CD8 purified cells were incubated with 10μg/mL of FcγR-block (BD Pharmingen) to block non-specific antibody interaction in mouse FACS wash buffer (PBS with 5% FBS and 5% goat serum). Cells were stained with anti-CD8 FITC (1:150 dilution, BD Pharmingen), antiVβ8.3 PE (1:150 dilution, BD Pharmingen), $NS3_{1073-1081}$ HLA-A2.1 tetramer-PE (1:10 dilution, F. Lemaitre, Institut Pasteur, Paris), or $Core_{35-44}$ HLA-A2.1 tetramer-PE (1:10 dilution, F. Lemaitre), as indicated for 45 minutes at 4°C and analyzed using FACS analysis.

Spleens were harvested at indicated times following stimulation or infection. Spleens were homogenized by mashing and debris was removed. Cells were collected by centrifugation and red blood cells were lysed with 1.66% ammonium chloride for 5 minutes at 37°C. Following lysis, cells were washed and incubated with 10μg/mL of FcγR-block (BD Pharmingen) to block non-specific antibody interaction in mouse FACS wash buffer. Cells were stained

with anti-CD45.2 FITC (1:150 dilution, BD Pharmingen), anti-CD11c APC (1:150 dilution, BD Pharmingen), and anti-PDCA-1 PE (1:150, BD Pharmingen).

4.5 Results: H-Y model

CD4+ T cell 'help' is required for in vivo priming of H-Y specific CD8+ T cells by endogenously loaded DCs

In order to setup a model antigen system for CD4-dependent CD8+ T cell priming, we employed the male antigen (H-Y) system. Various routes of immunization with syngeneic male splenocytes were evaluated by assaying CD8+ T cell responsiveness to the immunodominant Uty epitope using IFNγ ELISPOT. Intradermal (i.d.) immunization yielded the most reproducible priming and was the route of immunization employed in all of these studies.

First, we verified previous results suggesting that CTL effector function required CD4+ T cell 'help' during priming [215]. In order to evaluate this *in vivo*, female WT or MHCII-/- C57BL/6 mice were immunized i.d. with 5×10^6 syngeneic male splenocytes. Spleens were harvested at 12 days and CD8+ T cells were purified with MACS magnetic beads. IFNγ ELISPOT analysis using Uty pulsed splenocytes as targets showed WT but not MHCII-/- mice could generate Uty-specific CD8+ T cell IFNγ-production (Fig. 4.2). Importantly, Uty-specific responses generated by immunization of male splenocytes vary significantly from mouse to mouse, but Uty-specific IFNγ responses were never seen in MHCII-/- animals. Notably, MHCII-/- females were primed with MHCII-/- male splenocytes to eliminate allo-reactivity. In order to confirm that this CD4-dependence is not merely the result of differential expression of H-Y antigens in

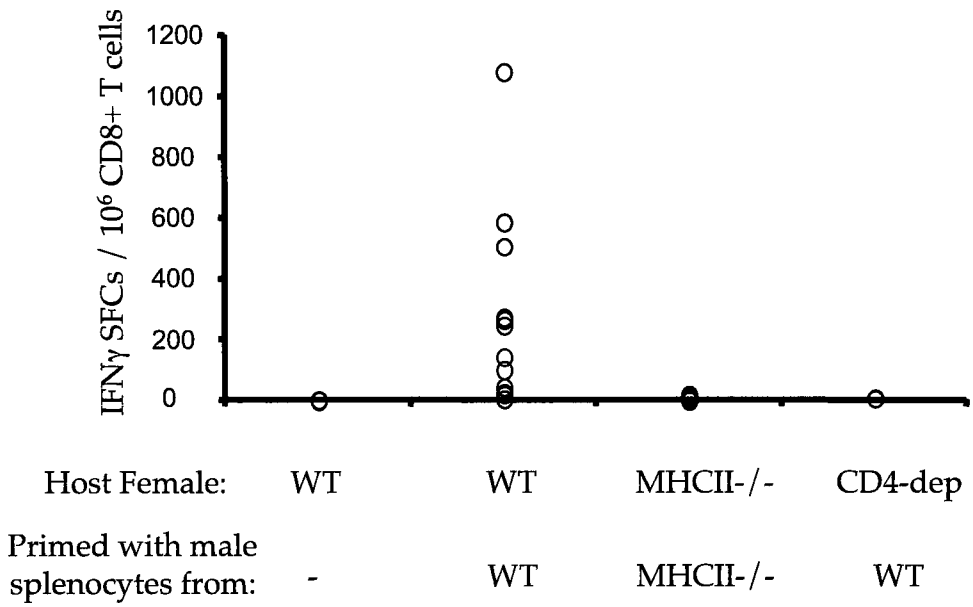

Figure 4.2 Uty-specific CD8+ T cell IFNγ production requires CD4 help during priming. Host C57BL/6 females were primed with male splenocytes from indicated C57BL/6 mice. The table shows spot forming cells (SFCs)/10^6 CD8+ T cells measured by IFNγ ELISPOT 12 day post-immunization. Each circle represents an individual mouse. CD4 depletion indicates GK1.5 depletion as described in Materials and Methods.

MHCII-/- male mice, female mice depleted of CD4+ T cells with GK1.5 (anti-CD4) were similarly immunized with male splenocytes. IFNγ ELISPOT shows that CD4 depletion also prevents priming of IFNγ-producing Uty-specific CD8+ T cell responses (Fig. 4.2).

In order to evaluate if IFNγ ELISPOT was representative of CTL function, specific killing *in vivo* was evaluated using Uty pulsed and unpulsed splenocytes that were differentially labeled with CFSE and injected 12 days following immunization (Fig. 4.3A). Similar to ELISPOT results, i.d. immunization with syngeneic male splenocytes induces robust Uty specific CTL activity (Fig. 4.3B, C). Priming of MHCII-/- females abgrogates *in vivo* killing suggesting that CD4 'help' is required for CTL function. CD4 depletion also abrogates *in vivo* CTL function confirming the CD4-dependence of CTL priming.

Further characterization of this system was done to evaluate the nature of the antigen presenting cell involved in Uty-specific T cell priming by male splenocytes. In order to test the contribution of endogenously loaded male DCs in Uty-specific priming during i.d. male spenocyte immunization, splenocytes were compared to CD11c-enriched or depleted splenocytes. CD11c+ enrichment enhanced Uty-specific CD8+ T cell IFNγ production 12 days post-immunization while CD11c-depletion virtually abrogated the response(Fig. 4.4). Consistent with other reports[218], this suggest that direct presentation is the dominant mechanism for Uty-specific priming.

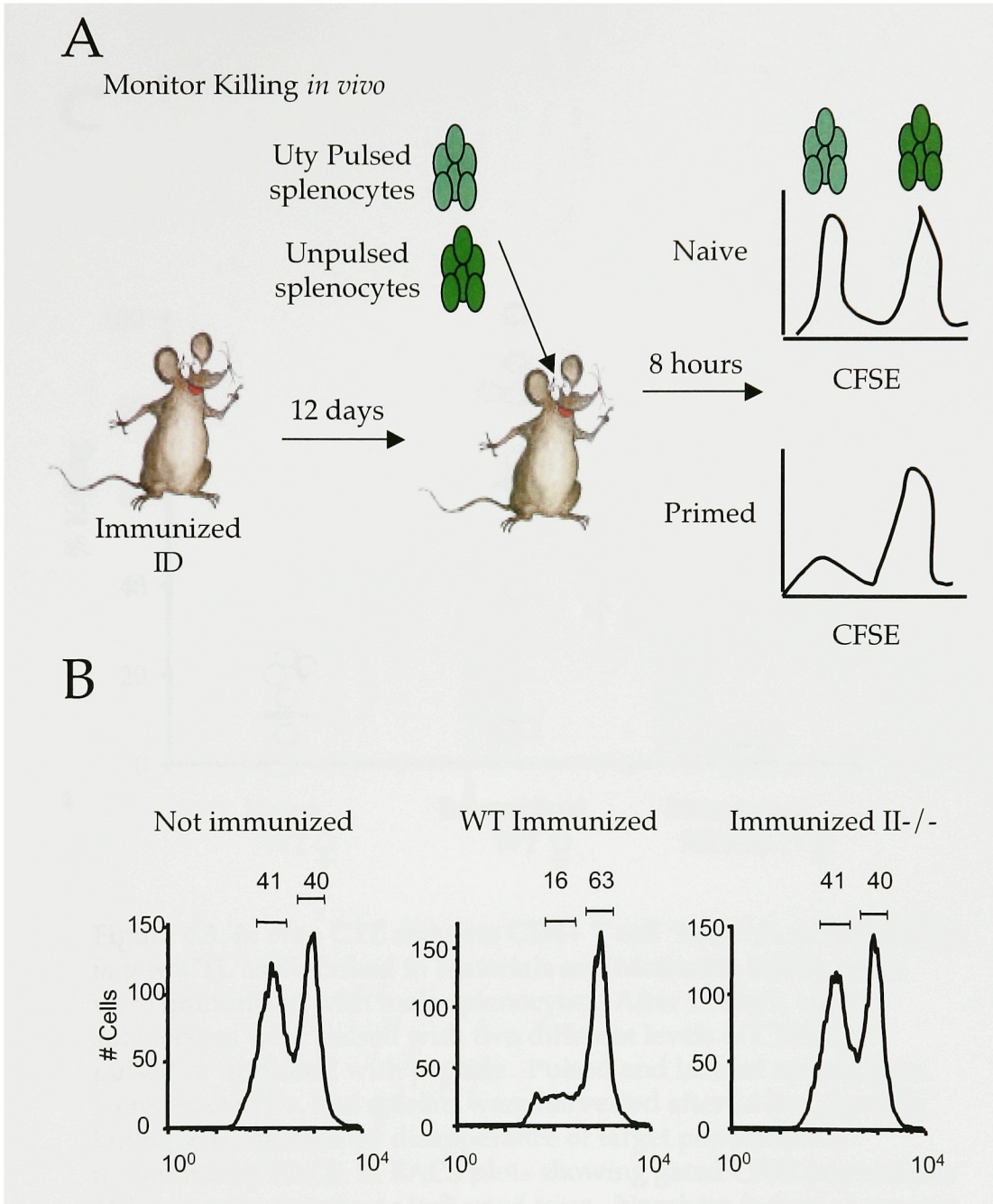

Figure 4.3. ***In vivo*** **CTL requires CD4+ T cell 'help'.** Continued on following page.

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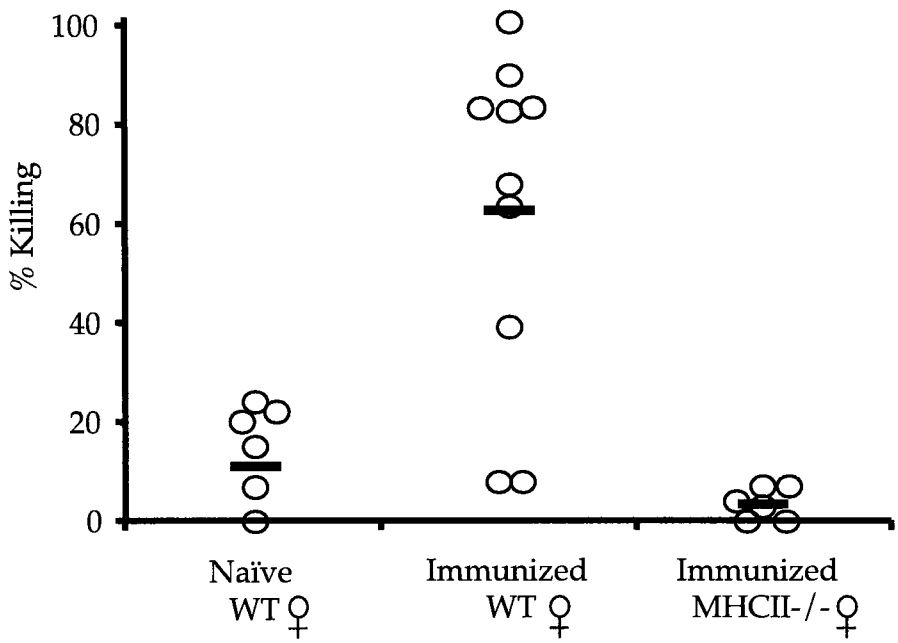

Figure 4.3. ***In vivo*** **CTL requires CD4+ T cell 'help'. A.** Schematic for *in vivo* CTL as described in Materials and Methods. Briefly, mice were immunized with male splenocytes. After 12 days, target splenocytes were pulsed with two different levels of CFSE and pulsed or unpulsed with peptide. Pulsed and labeled splenocytes were injected i.v. and spleens were harvested after 16 hrs. Specific killing was assessed by disapperance of target population as monitored by FACS. **B.** FACS plots showing gated CFSE populations 16 hours after transfer to indicated mice. Numbers indicated percent of total CFSE positive cells. **C.** Percent killing in indicated mice was calculated as described in Materials and Methods in the indicated mice and is displayed as a dot plot. Each dot represent an individual mouse.

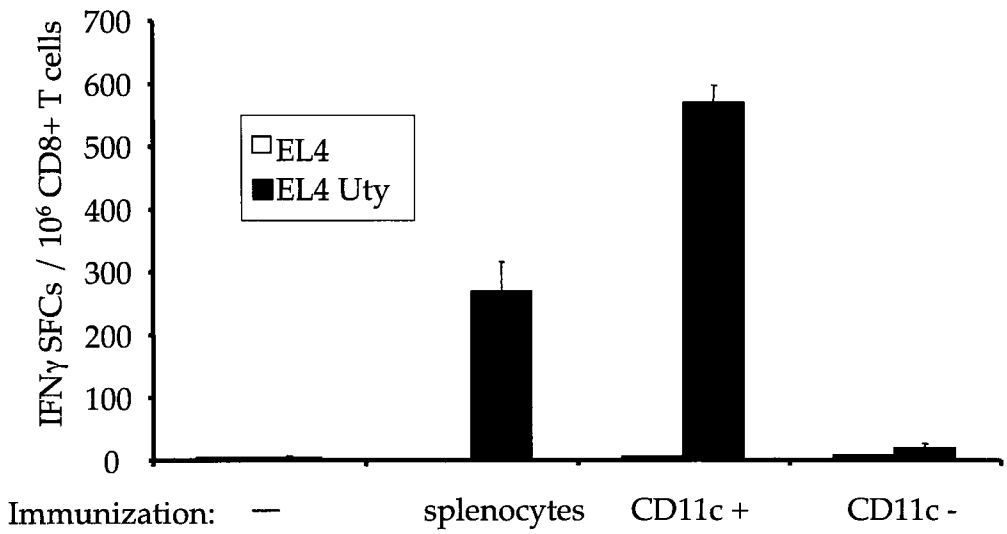

Figure 4.4. CD11c+ DCs required for efficient Uty-specific priming by male splenocytes. C57BL/6 WT female mice were either not immunized or immunized with total splenocytes, CD11c+ cell purified by two rounds of positive selection (CD11c+), or CD11c depleted cells remaining after CD11c+ purification (CD11c-) from syngeneic males. Spleens were harvested after 12 days and the T cell response monitored by IFNγ ELISPOT. Irradiated mouse thymoma cell line (EL4) was pulsed with Uty peptide and used as targets for T cells.

The absence of CD4+ T cell 'help' leads to antigen specific CD8+ T cell tolerance

In light of the role of CD4+ T cell 'help' for CTL function, I wanted to address the question of what happens to antigen specific CD8+ T cells in the absence of CD4 'help' signals. The majority of assays designed to monitor CD8+ T cell function focus solely on the absence or presence of a response. These assays do not evaluate what happens to the CD8+ T cells when no response is present. Thus, in this system, I asked if the lack of response in the absence of CD4+ T cells was simply the result of ignorance or the absence of a priming signal.

To address this question, I used TCR transgenic Uty-specific CD8+ (Matahari) T cells to monitor division in the presence or absence of CD4+ T cells. Matahari cells were CFSE labeled and adoptively transferred i.v. 6 hrs before immunization (Fig. 4.5A). In addition to the CFSE label, Matahari T cells can be identified by antibodies to TCR Vβ8.3. Lymph nodes and spleens were harvested 3 days post-immunization and division of CD8+/Vβ8.3+ CFSE labeled cells was analyzed by flow cytometry. In the presence of CD4+ T cell, CFSE division and expansion of Matahari cells was seen at day 3 in inguinal lymph nodes (Fig. 4.5B). Expansion was restricted to the draining lymph node and not seen in the spleen as primed T cells do not begin to circulate until day 4 post-immunization[78]. Similarly, in MHCII-/- animals, CFSE division and expansion of Matahari was apparent after 3 days suggesting that antigen presentation and T cell engagement was intact in the absence of CD4+ T cells; however, the number of divisions lagged slightly behind wild type hosts and expansion was not as robust. Although we did not directly evaluate death in the 'unhelped' dividing

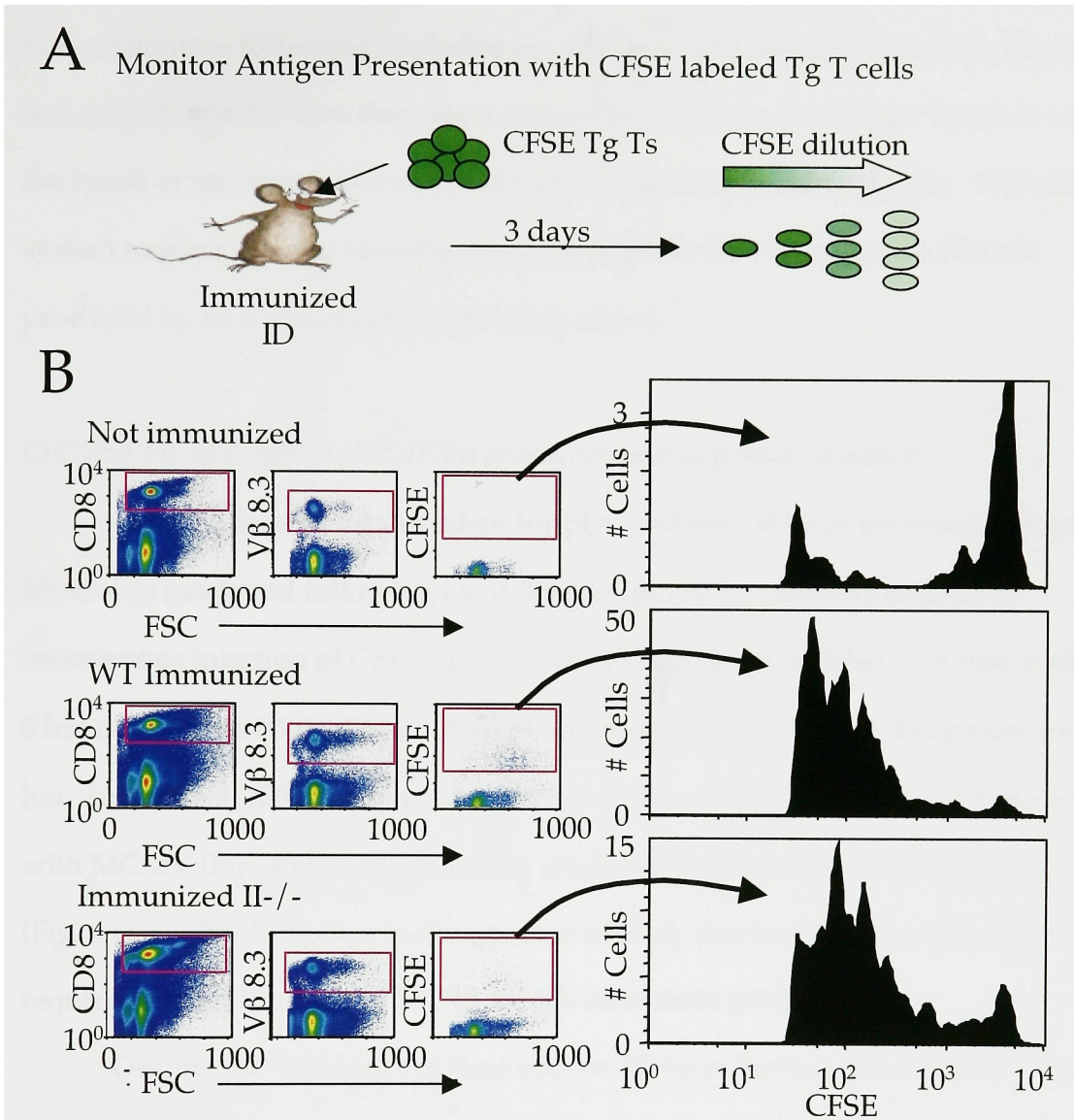

Figure 4.5. CD8+ T cells divide in response to immunization in the absence of CD4+ T cells. A. A schematic representation describing the use of CFSE staining to monitor cell division TCR transgenic Matahari cells are CFSE labeled and injected into a female mouse 6-12 hrs before immunization. 3 days after immunization proliferation of cells is monitored by CFSE dilution and increase in cell numbers. **B.** CFSE labeled Matahari cells were transferred to WT or MHCII-/- female mice and either not immunized or immunized with male splenocytes as indicated. Lymph nodes were harvested at day 3 post-immunization and Matahari cells were idenitified by CD8, Vβ8.3, and CFSE staining. Histograms illustrate dilution of CFSE in each case.

CD8+ T cells, animals primed in the absence of CD4+ T cells were functionally non-responsive following re-challenge. These results suggest, therefore, that the lack of CTL effector function when primed in the absence of CD4+ T cells is not the result of ignorance, but rather the lack of specific priming signals. With this system in place, I setup to evaluate my hypothesis that lymph node IFNα/β produced by pDCs may act as a priming signal.

CpG and MCMV induce pDC IFNα production and migration to spleen

In order to establish a system for pDC-induced IFNα/β secretion, serum IFNα was measured following i.v. delivery CpG or i.p. delivery of MCMV. Intravenous injection of CpG rapidly induces high level of IFNα in serum within 6 hrs of treatment (Fig. 4.6A). IFNα production is transient and disappears by 24 hrs. In contrast, IFNα serum levels do not peak until 36 hrs after i.p. infection with MCMV (Fig. 4.6B) with maximal production seen with 10^4 PFU/injection (Fig. 4.6C). The difference in time course is likely due to the replication time required to amplify the viral DNA which stimulates pDCs[125].

Despite the difference in time course, IFNα induction in both settings is dependent on pDCs[156,195,220]. In order to evaluate this in our system, serum IFNα for both CpG and MCMV stimulated animals was evaluated in pDC depleted mice. As reported previously, pDC depletion abrogates the IFNα response to both stimuli (Fig. 4.6D). In order to determine the requirement for the IFNα-dependent priming loop discussed in chapter 1, IFNα production was also monitored in IFNAR-/- mice challenged with CpG and MCMV. Interestingly, although CpG induced IFNα production requires IFNAR, MCMV triggers robust

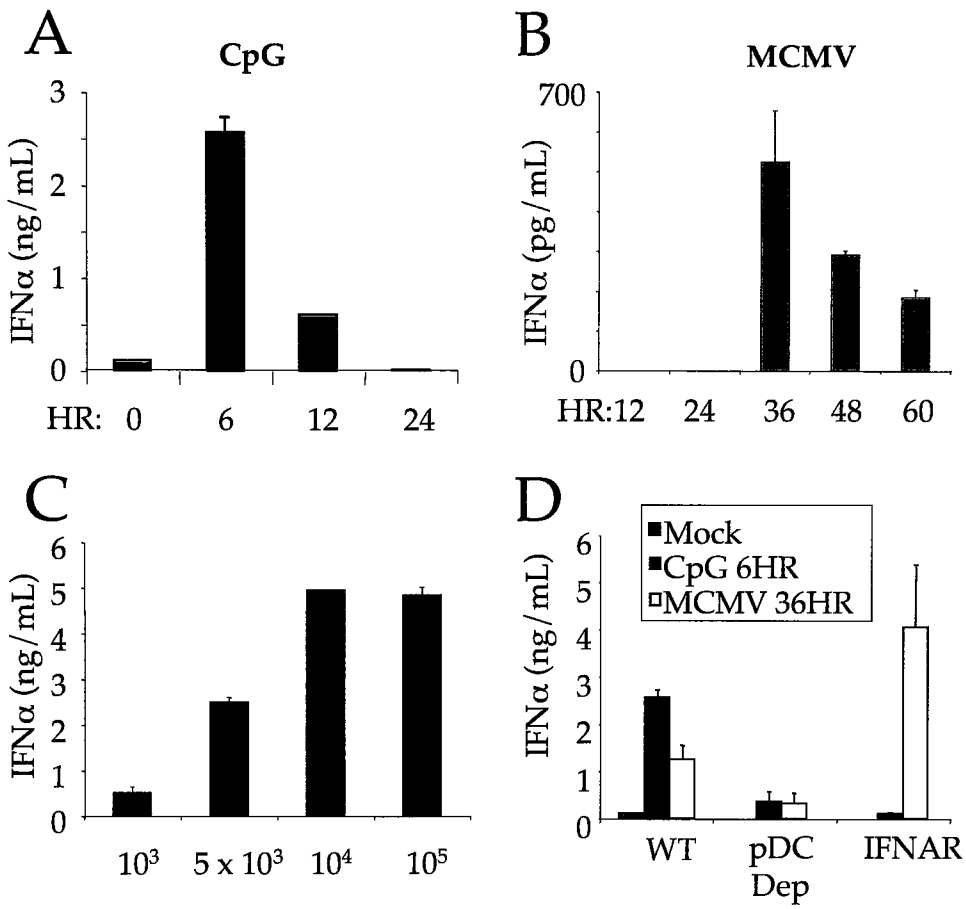

Figure 4.6. Kinetics of pDC IFNα production following stimulation with CpG or MCMV. **A.** Serum IFNα is shown at indicated hour (HR) following i.v. delivery of 5μg of CpG/DOTAP. **B.** Serum IFNα is shown at indicated hour following i.p. delivery of 10^3 PFU of MCMV. **C.** Serum IFNα is shown 36 hours following intraperitoneal delivery of indicated PFU of MCMV. **D.** Serum IFNα is shown at indicated time following CpG or 5 x 10^3 PFU of MCMV in either WT, pDC depleted (dep), or IFNAR-/- C57BL/6 mice.

IFNα even in the absence of IFNAR. Although the IFNAR-/- mice are on the MCMV-resistant C57BL/6 background[221], challenge with 3 x 10^5 PFU of MCMV i.p. was lethal in 5 out of 5 mice (data not shown) and subsequently could not be used for evaluation of adaptive immunity. Evaluation of splenocytes following stimulation confirmed accumulation of pDCs in the spleen. Using a pDC-specific marker, PDCA-1, and CD11c to identify pDCs, five to six-fold expansion of pDCs was seen by FACS at 6 hrs following CpG injection (Fig. 4.7A). CpG stimulation did not alter the percentage of $CD11c^{high}$ cells in the spleen. Similarly, as reported previously[222], MCMV infection dramatically increases the percent of PDCA-1+ CD11c+ cells in the spleen at 36 hrs (Fig. 4.7B). FACS staining confirmed that RB6-8C5 treatment depleted splenic PDCA-1+ pDCs. RB6-8C5 depletion does not affect $CD11c^{high}$ DCs (data not shown).

pDC activation by MCMV can offer priming signals for Uty-specific CD8+ T cells

Due to the accumulation of pDCs in the lymph nodes during DC / T cell engagement and the possible role of IFNα in substituting for CD4+ T cell signals, we next tested if pDC activation using a viral stimulus could provide priming signals in the absence of CD4s. Using the H-Y system described above, we evaluated the ability of MCMV infection to facilitate the priming of Uty-specific CD8+ T cells in MHCII-/- mice. Briefly, MHCII-/- females were immunized with male splenocytes 36 hrs after MCMV infection to maximize pDC activation and accumulation in spleen and lymph nodes (Fig. 4.8A). After 12 days, Uty-specific CD8+ T cell responses were monitored by IFNγ ELISPOT. Infection with

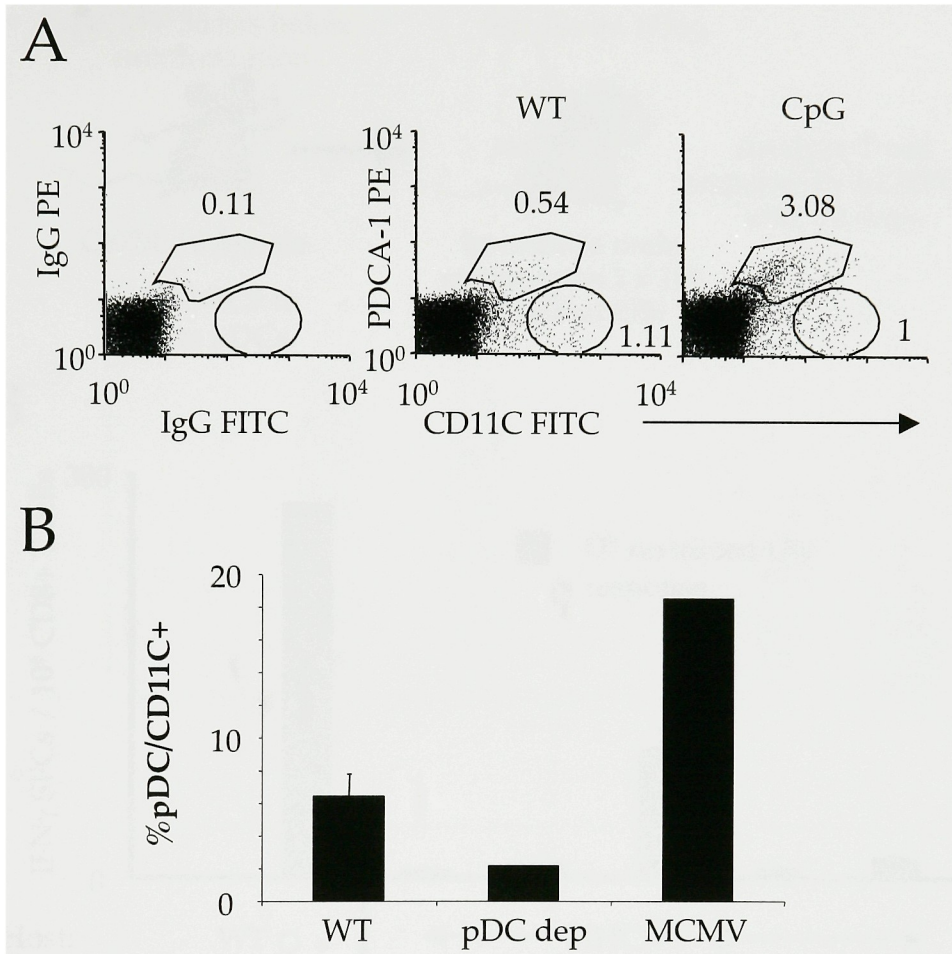

Figure 4.7. pDCs accumulate in spleen following CpG and MCMV activation. A. FACS staining of splenocytes from C57BL/6 mice 6 hrs following CpG or no treatement with indicated antibodies. Autofluorescent cells were gated out the population depicted. Numbers represent percent of total splenocytes within the indicated gates. **B.** The percentage of PDCA-1 positive cells per CD11c positive cells (%pDC/CD11c+) in total splenocytes was evaluated by FACS for WT mice, pDC depleted mice, or MCMV challenged mice and represented as a histogram.

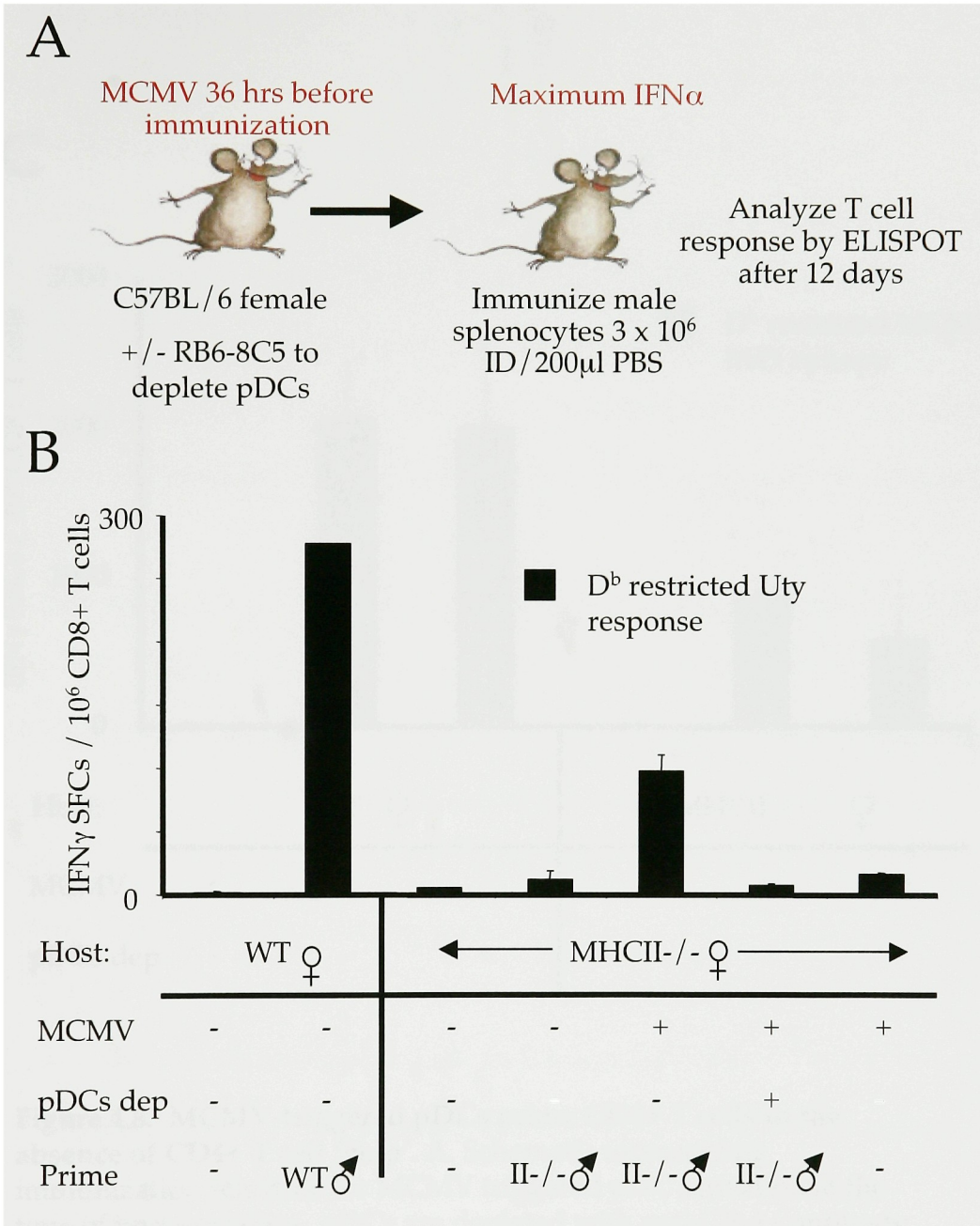

Figure 4.8. MCMV-triggered pDCs prime CD8+ T cells in the absence of CD4+ T cell 'help'. Continued on the following page.

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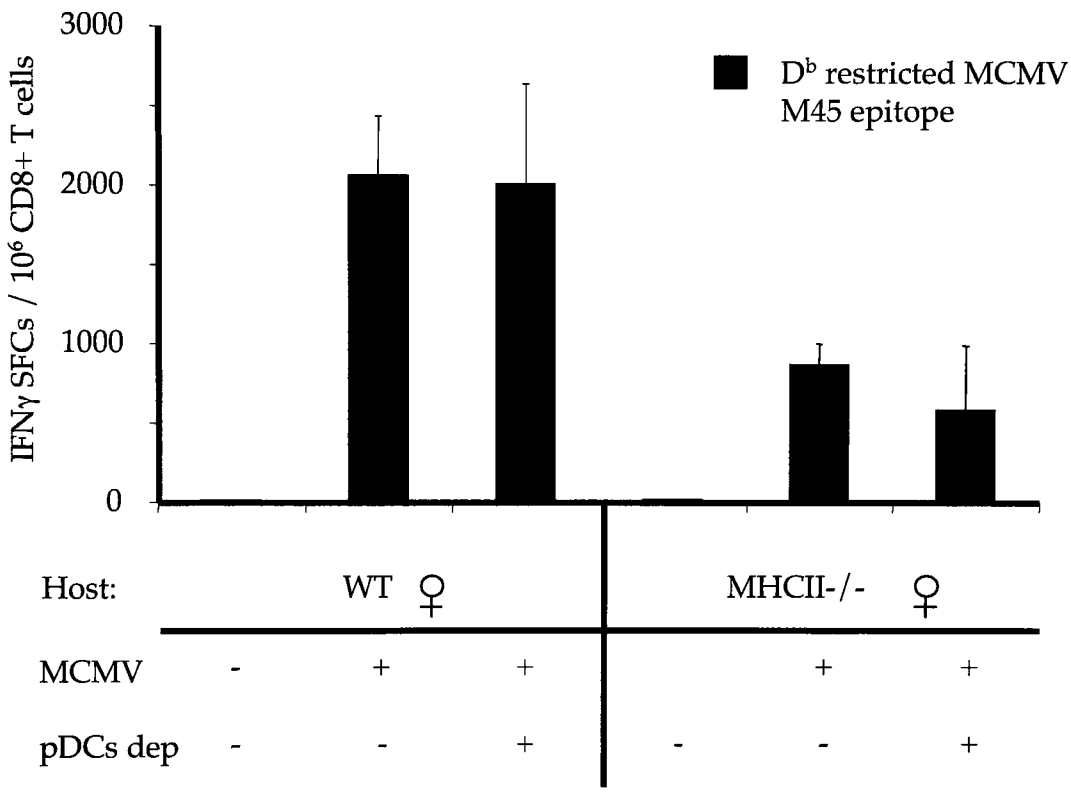

Figure 4.8. MCMV-triggered pDCs prime CD8+ T cells in the absence of CD4+ T cell 'help'. A. Schematic representing immunization schedule for MCMV triggered pDC activation at the time of immunization. pDCs are depleted with anti-GR-1+ antibody RB6-8C5 before infection with MCMV. **B.** IFNγ ELISPOT results of indicated host females immunized or not with male splenocytes from syngeneic animals in the presence of MCMV infection with or without pDC depletion (dep). **C.** IFNγ ELISPOT for D^b restricted MCMV M45 epitope in WT or MHCII-/- host infected with MCMV or depleted of pDCs analyzed in part B.

MCMV enabled the generation of Uty-specific CD8+ T cells in MHCII-/- animals (Fig. 4.8B). Although the absolute number of spot forming cells was less than wild type animals immunized with male splenocytes, the CD8+ T cell responsein MCMV treated MHCII-/- was antigen specific and significantly above background. In order to evaluate if this effect was dependent on pDC activation, pDCs were depeleted prior to MCMV infection using RB6-8C5 (anti-GR-1). As depicted in the figure, pDC depletion prevented the generation of Uty-specific CD8+ T cells in response to MCMV infection. Infection with MCMV alone in the absence of male splenocyte immunization did not induce any Uty-specific cross-reactivity.

In contrast to a recent report describing the importance of pDCs in HSV-specific CTL priming[223], pDC depletion during MCMV infection did not effect CD8+ T cell IFNγ production to the immunodominant M45 epitope as monitored by ELISPOT (Fig. 4.8). M45 reactivity was dependent on MHCII, however, a strong response was still present in the absence of CD4+ T cells and pDC depletion did not abrograte this response.

CpG stimulation also offers priming signals for Uty-specific CD8s

In order to confirm that priming signals offered by MCMV-stimulated pDCs were not unique to MCMV, the ability of CpG stimulated pDCs to substitute for CD4+ T cells was also evaluated. Due to the difference in time course of IFNα/β production, CpG was delivered at the same time as immunization with male splenocytes and Uty-specific immune responses were monitored after 12 days. Similar to the results with MCMV, CpG stimulation in

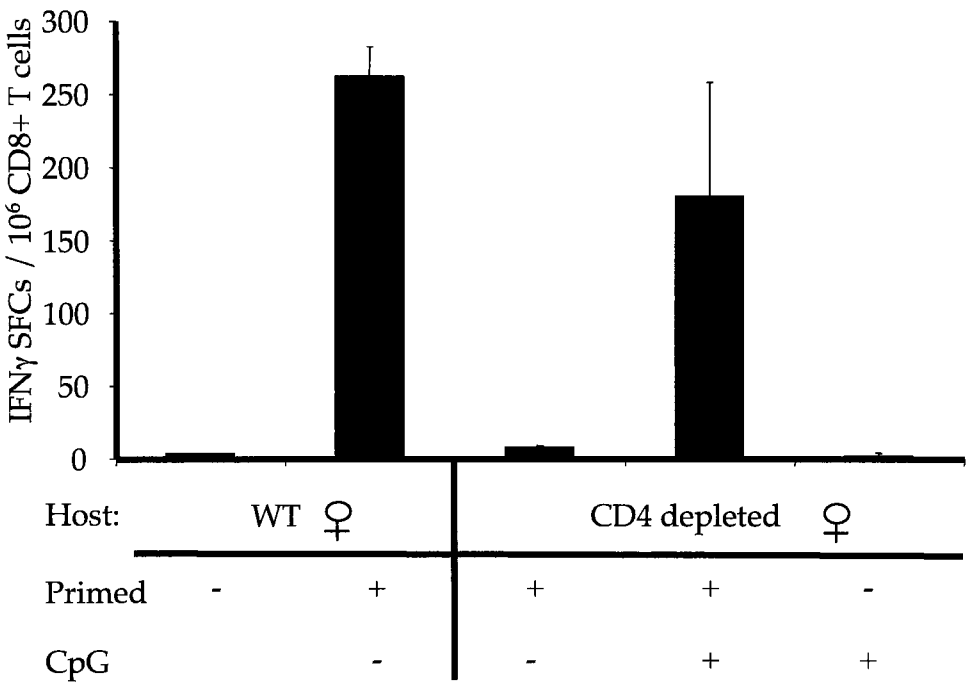

Figure 4.9. CpG triggered pDCs substitute for CD4+ T cells in priming CD8+ T cells. WT or CD4 depleted female mice were primed with syngeneic male splenocytes in the presence or absence of i.v.CpG as indicated. IFNγ ELISPOT of D^b-restricted Uty specific CD8+ T cells was performed at 12 days post-immunization. Bars represent averages of two mice.

CD4-depleted females immunized with male splenocytes primed Uty-specific CD8+ T cells as monitored by IFNγ ELISPOT (Fig. 4.9).

Importantly, some reports suggest that mouse pDCs may have low level of CD4 expression; therefore, we evaluated the effect of CD4 depletion on pDC numbers and IFNα production following stimulation. CD4 depletion did slightly decrease the absolute numbers of PDCA-1+ cells in spleen and total IFNα levels in the serum (Fig. 4.10A,B), but it did not abrogate the response to CpG as IFNα production was still significant and pDC migration to the spleen remained intact (Fig. 4.10B,C). Moreover, the slight decrease in pDC numbers and IFNα levels was seen in MHCII-/- as well as CD4 depleted mice (Fig. 4.10A) suggesting that these findings may be secondary to changes induced by lack of CD4s (i.e. lymph node architecture changes). Despite these differences, the functional ability of CpG to provide 'help' following CD4 depletion remains intact.

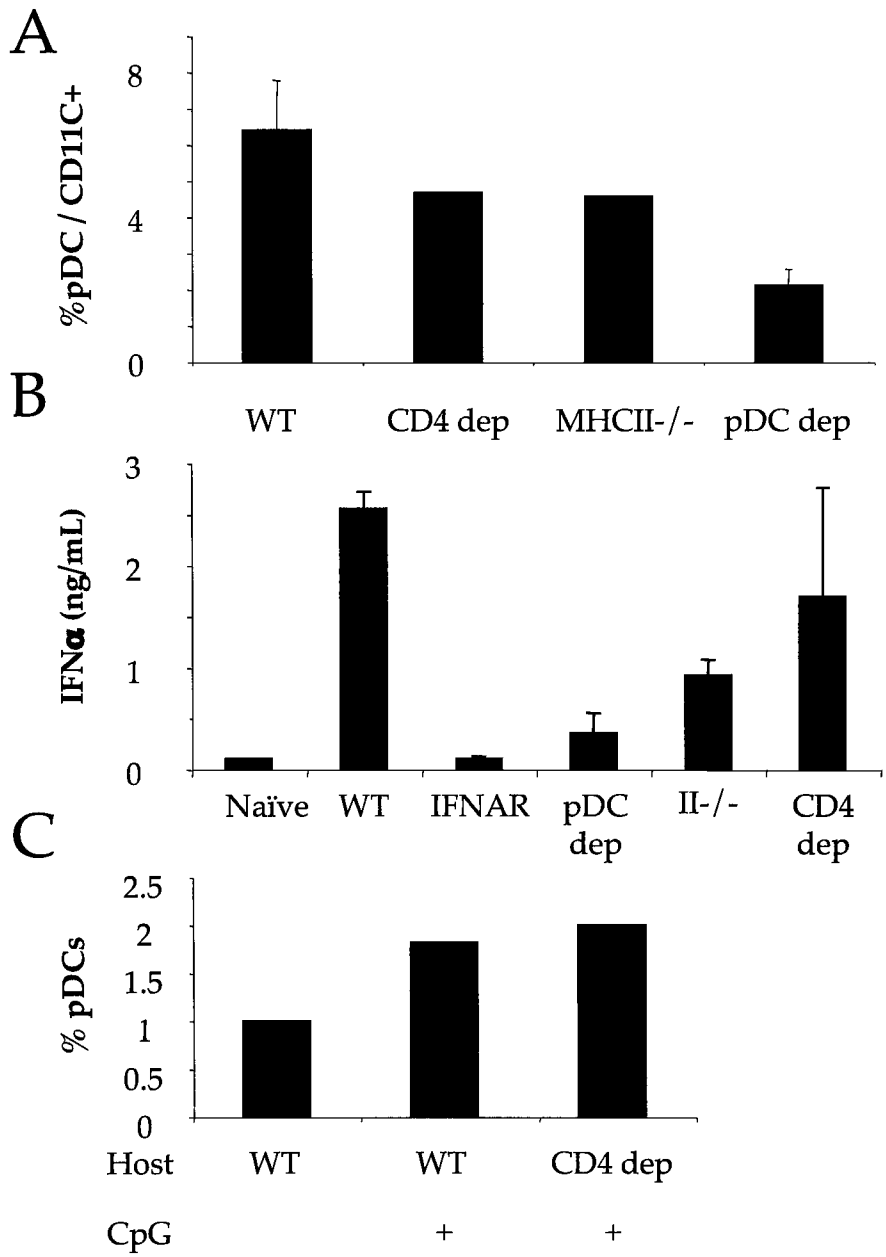

Figure 4.10. Effects of CD4 depletion on pDC number and function. **A.** Spleens from the indicated mice were harvested and the percent of pDC per CD11c+ cell (%pDC/CD11c+) assayed by FACS analysis. **B.** IFNα ELISA was used to quantitate the amount of IFNα in the serum 6 hrs after CpG administration in the indicated hosts. **C.** Spleens from the indicated hosts were harvested 6 hrs after CpG stimulation and the percent of pDCs was assessed by B220 and pDC specific marker PDCA-1.

pDC-dependent CD8+ T cell priming requires IFNAR but not IL-12

In mice, pDCs can secrete IL-12 in addition to IFNα[216]. Given the potent role of IL-12 acting as a third signal for CD8+ T cell priming, we wanted to evaluate the respective roles for IL-12 and IFNα in the ability of pDCs to substitute for CD4+ T cell priming cells. CD4-depleted IFNAR-/- or IL-12B-/- (p40 subunit of IL-12 and IL-23) mice were immunized with male splenocytes with or without CpG. IFNγ ELISPOT showed that immunization of IFNAR-/- females with WT male splenocytes was capable of priming Uty-specific T cell responses in a CD4-dependent fashion(Fig. 4.11); however, CpG stimulated pDCs were not able to prime in the absence of CD4 'help' suggesting that IFNAR is required for CpG to act as an adjuvant.

In contrast to IFNAR, IL-12B was not required for CpG to trigger priming signals in the absence of CD4+ T cells. Immunization of female IL-12B-/- mice with male IL-12B-/- splenocytes primed Uty-specific T cells response in a CD4-dependent fashion similar to other experiments, but CpG stimulation enabled Uty-specific priming, suggesting that IL-12 production by pDCs is not a critical component of this priming signal(Fig. 4.11). Notably, however, the ability of IL12B-/- male splenocytes to prime IL-12B-/- females suggests also that IL-12/23 is not a crucial component of helper-dependent Uty-specific priming.

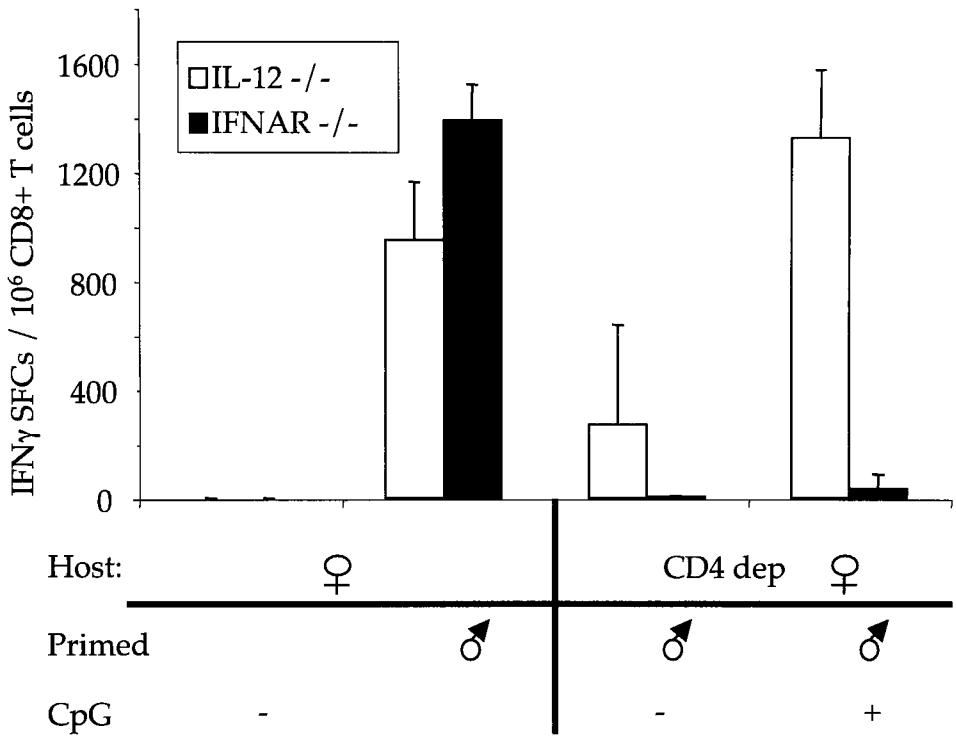

Figure 4.11. The ability of CpG stimulation to offer 'help' signals requires IFNAR but not IL-12. IFNAR-/- or IL-12-/- female mice with or without CD4 depletion as indicated were primed with syngeneic male splenocytes with or without i.v. CpG. The results of Uty-specific responses monitored by IFNγ ELISPOT 12 days post-immunization are shown. Bars represent averages of three mice.

IFNα production by influenza ΔNS1 does not enable CD8+ T cell priming

In efforts to examine the effects of non-pDC derived IFNα on CD4-dependent immunity, we examined induction of IFNα following influenza ΔNS1 infection. The NS1 protein of influenza binds dsRNA and prevents IFNα production in mice infected with wild type influenza; however, infection with influenza ΔNS1 triggers IFNα production[224]. The kinetics of IFNα production is faster than MCMV and peaks around 12 hrs post-infection, consistent with the faster replication time of influenza (Fig. 4.12A). In contrast to MCMV, pDC depletion did not significantly inhibit IFNα production suggesting that a non-pDC is responsible for the IFNα production (Fig. 4.12B). The IFNα production, however, was dependent on IFNAR.

In light of the different cell type responsible for IFNα production, we evaluated if infection with influenza ΔNS1 is capable of providing priming signals for CD8+ T cells. Similar to the experimental setup for the MCMV experiment, MHCII-/- female mice were infected with influenza ΔNS1 12 hrs before immunization with male splenocytes. IFNγ ELISPOT shows that, in contrast to MCMV, influenza ΔNS1 does not enable priming of Uty-specific CD8+ T cells (Fig. 4.12C). Responses against endogenous MCMV (M45) or influenza (PA) epitopes were monitored to provide an internal control for exposure to virus. Despite the differential adjuvant effects on Uty-specific priming, both MCMV and influenza ΔNS1 generated antigen specific T cell responses against their respective endogenous epitopes. These data suggest that IFNα production by a pDC is an important component of this priming signal.

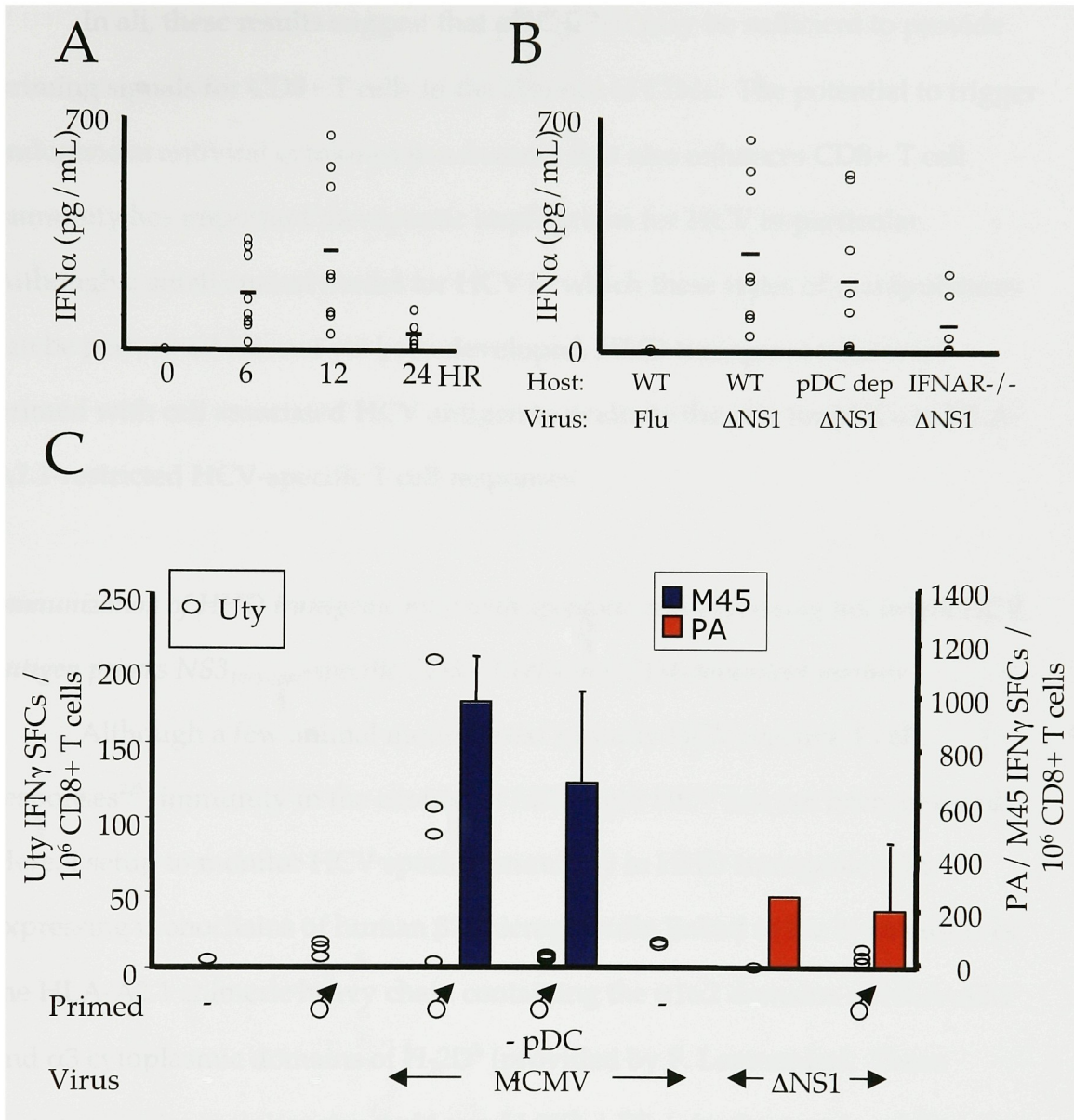

Figure 4.12. Non-pDC triggered IFNα by Influenza ΔNS1 does not provide 'help' signals for CTL priming. A. Levels of IFNα in serum at indicated time following IP challenge with influenza ΔNS1 were monitored by IFNα ELISA. Each circle represents an individual mouse. **B.** Levels of IFNα in serum at 12 hours post-infection were monitored for WT influenza (Flu) or influenza ΔNS1 (ΔNS1) in WT, pDC depleted (dep), IFNAR-/- mice. **C.** MHCII-/- females with or without pDC depletion (dep) were primed with male splenocytes with or without MCMV or influenza ΔNS1 (ΔNS1) infection. IFNγ ELISPOT for Uty, PA (influenza polymerase epitope), or M45 (MCMV epitope)-specific responses was performed 12 days after priming.

4.6 Results: HCV HHD transgenic mouse model

In all, these results suggest that pDC IFNα may be sufficient to provide priming signals for CD8+ T cells in the absence of CD4s. The potential to trigger endogenous antiviral cytokines in a manner that also enhances CD8+ T cell immunity has important therapeutic implications for HCV in particular. Although a small animal model for HCV in which these types of manipulations can be performed has not yet been developed, HHD transgenic mice were primed with cell associated HCV antigen to evaluate the role for pDCs in HLA-A2.1-restricted HCV-specific T cell responses.

Immunization of HHD transgenic mice with apoptotic cells expressing full length HCV antigen primes $NS3_{1073-1081}$-specific CD8+ T cells in a CD4-dependent manner

Although a few animal models exist to study HCV reactive T cell responses[225], immunity in the context of full length HCV has not been assessed. Here, I setup to monitor HCV specific immunity in HHD transgenic mice expressing monochains of human β2-microglobulin linked to the N-terminus of the HLA-A2.1 chimeric heavy chain containing the α1α2 domains of HLA-A2.1 and α3 cytoplasmic domains of H-2D^b (provided by F. Lemonnier). These transgenic mice were constructed in an H-2K^b-/-D^b-/- background and therefore express no H-2 class Ia molecules. The expression of HLA-A2.1 allows for a normally diversified TCR repertoire and the absence of MHCIa molecules enables a better CTL response to HLA-A2.1 restricted epitopes[226]. Several HLA-A2.1-restricted epitopes for HCV are known, but the relative importance of each

for antigen processing[227], immunity[49], cross-reactivity[228], or disease progression[229] is not well understood.

In order to evaluate HCV specific T cell priming *in vivo*, a xenogeneic osteosarcoma cell line expressing full length HCV under a tetracycline inducible promoter (provided by B. Wolk and D. Moradpour) was employed to immunize HHD transgenic mice. HCV specific immunity was evaluated using 4 well characterized HLA-A2.1 epitopes: $Core_{35-43}$, $Core_{132-140}$, $NS3_{1073-1081}$, and $NS3_{1406-1414}$. In order to evaluate the presence of HCV reactive T cells in the endogenous repertoire, HHD transgenic mice were primed with a 1:1 mixture of Titermax (Sigma) and the respective peptide (Fig. 4.13A). Similar to other studies, our results showed antigen specific CD8+ T cell responses in the spleen and draining lymph nodes to all of the indicated epitopes after 7 days. In contrast to peptide priming, immunization with 5×10^6 irradiated cells expressing full length HCV primed $NS3_{1073-1081}$ reactive T cells most efficiently(Fig. 4.13B).

We considered the possibility that subcellular localization of cell associated HCV played a role in epitope usage. Expression of HCV in cell culture results in the formation of a "membranous web" that is tethered to the endoplasmic reticulum (ER)[230]. In order to evaluate if this membranous web and ER tethering were important for epitope selectivity, we employed a cell line expressing NS3-4A, which associates with ER, or NS3 alone, which is diffusely spread around the cytoplasm (Fig. 4.14A)[230]. Expression of equivalent levels of NS3 protein in both cell lines was confirmed by FACS analysis. IFNγ ELISPOT two weeks post-immunization revealed equivalent $NS3_{1073-1081}$ responses suggesting that cellular localization was not crucial for epitope selectivity (Fig. 4.14B).

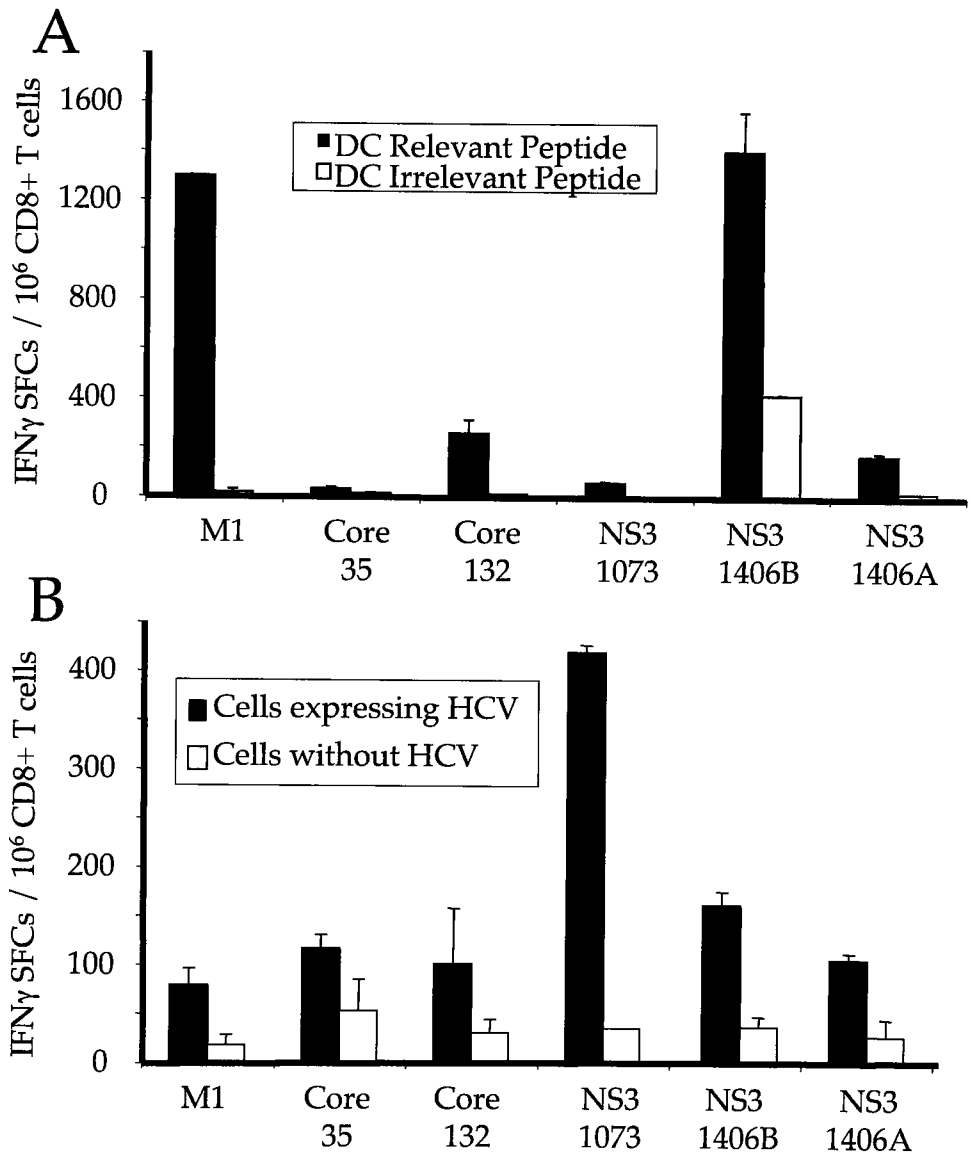

Figure 4.13. Immunization of HHD transgenic mice with full length cell associated HCV results in $NS3_{1073-1081}$ dominant CD8+ T cell responses. A. Lymph nodes of HHD mice immunized with peptide and titermax were harvested after 7 days and antigen specific CD8+ T cell activity assessed by IFNγ ELISPOT with DCs pulsed with relevant peptides *versus* DCs pulsed with irrelevant peptides as targets. **B.** Spleens from HHD mice immunized with cell associated HCV or cells not expressing HCV were harvested after 12 days and antigen specific CD8+ T cell activity was assessed by IFNγ ELISPOT with DCs pulsed with the indicated peptides as targets.

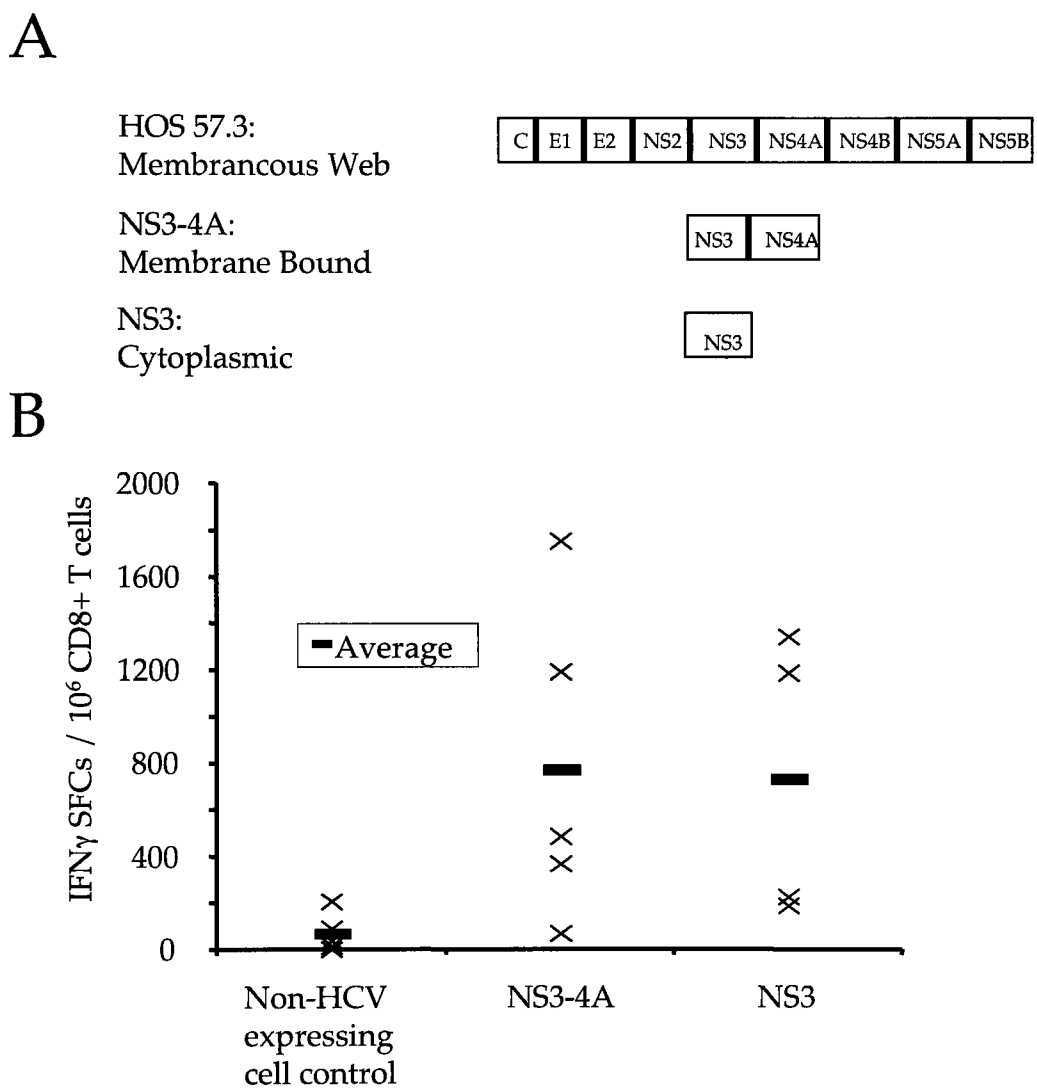

Figure 4.14. Alteration of subcellular localization of cell associated HCV does not change $NS3_{1073-1081}$ immunogenicity. A. A schematic showing the region of HCV expressed by the indicated cell lines and the consequent subcellular localization of NS3 in these cells (adapted from Woelk, B et.al J Virol 2000). **B.** T cell immunity to $NS3_{1073-1081}$ was monitored in HHD transgenic mice immunized with NS3-4A, NS3, or non HCV expressing cell lines after 12 days by IFNγ ELISPOT.

CD4+ T cells regulate priming versus tolerance of HCV-specific CD8s

In order to evaluate the role for CD4+ T cells in regulating the immunologic outcome of HCV-specific CD8+ T cell responses to $NS3_{1073\text{-}1081}$, CD4-depleted HHD mice were immunized with cell associated HCV antigen. Similar to our findings for endogenous presentation in the H-Y system, CD4-depletion reduced $NS3_{1073\text{-}1081}$ specific T cells responses as monitored by IFNγ ELISPOT (Fig.4.15). Tetramer analysis was also done to confirm these results. Using CD3 and CD8 antibodies to gate on the CD8+ T cell population, the percentage of tetramer positive cells in the the CD8+ T cell population was calculated. Consistent with the ELISPOT results, $NS3_{1073\text{-}1081}$ antigen specific T cells expanded when immunized in the presence, but not in the absence of CD4+ T cells.

Next, we evaluated the immunologic significance of the lack of CD8+ T cell response in the absence of CD4+ T cells. In other words, is the lack of response a result of ignorance or antigen specific tolerance? In order to do this in the HCV system, we boosted immunized mice to evaluate the functional state of the CD8+ HCV-specific T cells. IFNγ ELISPOT seven days after boost shows an increase number of CD8+ $NS3_{1073\text{-}1081}$ reactive cells in mice primed and boosted compared to boost alone or primed with a non-HCV control cell line (Fig. 4.16). In contrast, CD4 depletion during priming resulted in a decrease in IFNγ producing $NS3_{1073\text{-}1081}$ reactive CD8+ T cells after boost. Mice immunized with peptide alone were used as a positive control for tolerance induction[76]. These results were also confirmed using HLA-A2.1 tetramers for $NS3_{1073\text{-}1081}$ (Fig. 4.16). Consistent with the ELISPOT results, an increase in $NS3_{1073\text{-}1081}$ tetramer positive

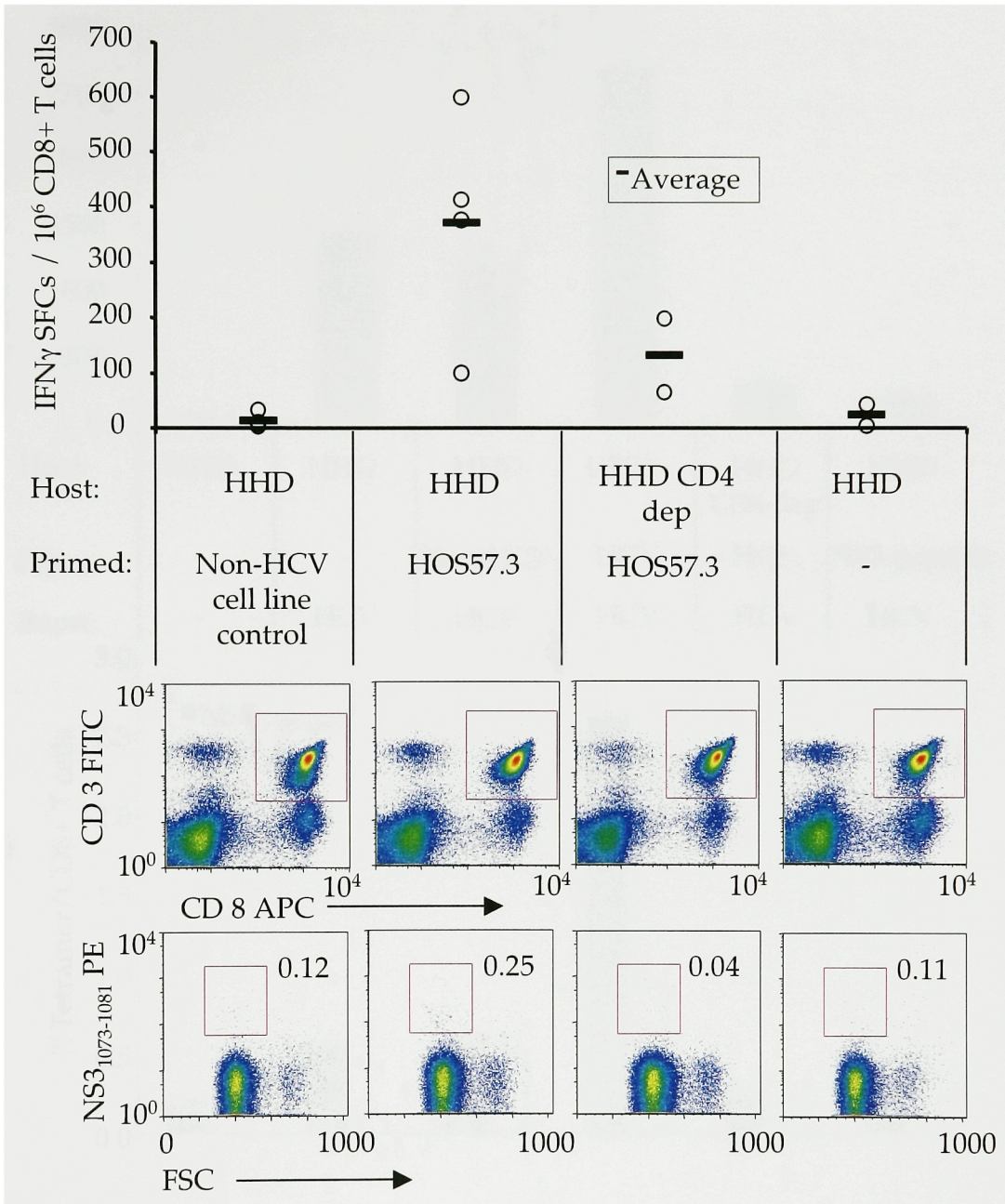

Figure 4.15. $NS3_{1073-1081}$ priming by cell associated antigen requires CD4+ T cells. $NS3_{1073-1081}$ specific T cell responses were monitored in HHD transgenic mice with or without CD4 depletion (dep) by IFNγ ELISPOT at 12 days after immunization with cell associated antigen. Each circle represents an individual mouse. $NS3_{1073-1081}$ specific T cell responses in corresponding samples were also evaluated with tetramer reagents by FACS. CD8+/CD3+ T cells were gated and used to identify the percentage of $NS3_{1073-1081}$ specific T cells indicated on the plots.

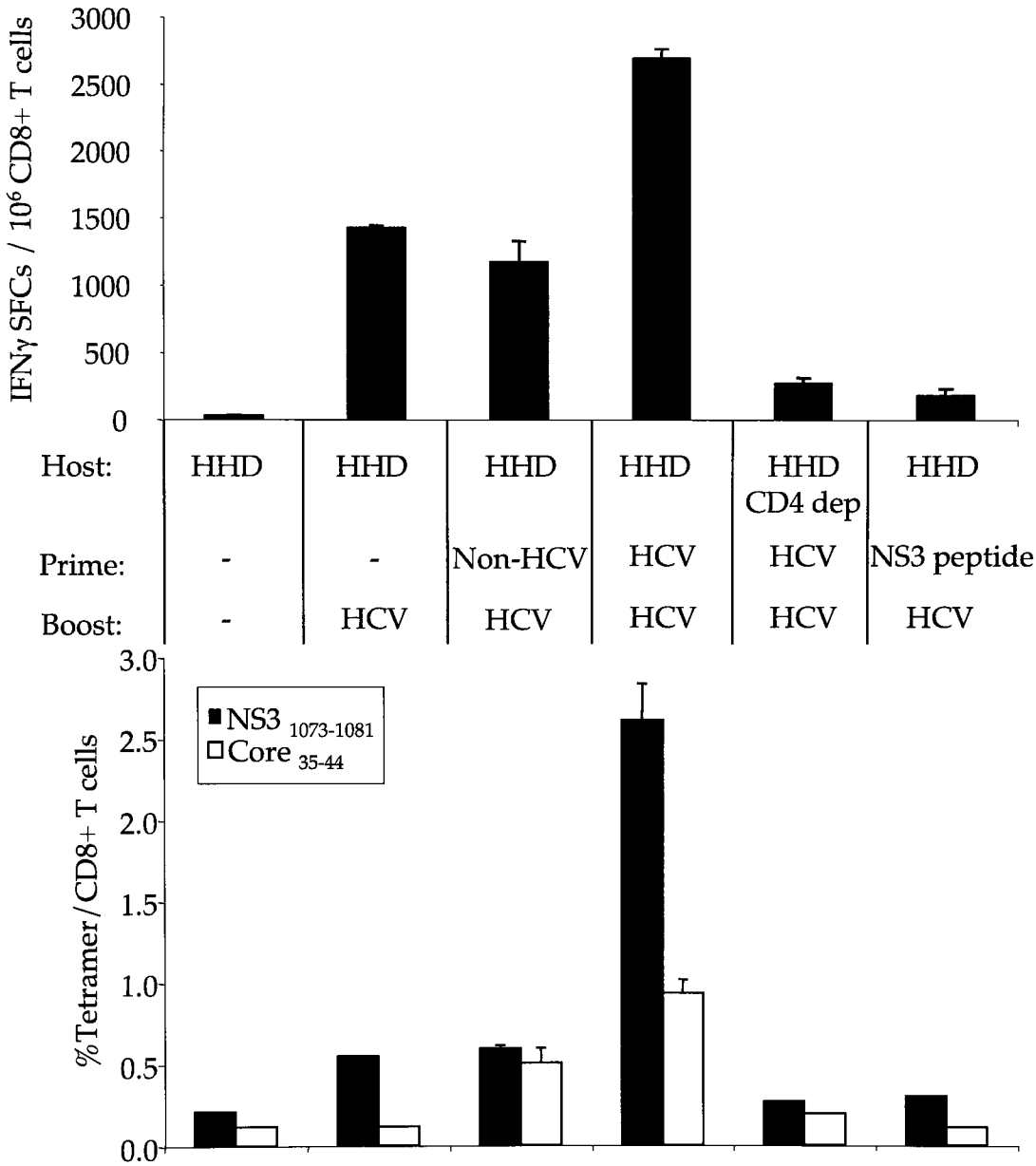

Figure 4.16. CD4+ T cell 'help' regulates priming *versus* tolerance of $NS3_{1073-1081}$ CD8+ T cell responses. HHD transgenic mice with or without CD4 depletion (dep) were primed and boosted after two weeks with HCV expressing cell lines (HCV) or non-HCV cell line controls (non-HCV). T cell immunity to $NS3_{1073-1081}$ was monitored 7 days after boost by IFNγ ELISPOT and tetramer staining. Bars represent averages of three mice.

CD8+ T cells were seen in animals primed and boosted compared to boost alone or primed with non-HCV control cell line. CD4 depletion during priming inhibits the expansion of $NS3_{1073-1081}$ tetramer positive cells comparable to immunization with peptide alone. $Core_{35-44}$ specific tetramer staining is shown to illustrate the dominance of the $NS3_{1073-1081}$ response.

CpG overcomes the requirement for CD4 'help' in $NS3_{1073-1081}$ T cell priming

Finally, we evaluated if CpG could overcome the requirement for CD4 'help' for $NS3_{1073-1081}$-specific CD8+ T cell priming. Briefly, mice were either untreated or CD4 depleted and immunized with either HCV expressing cell lines alone or with CpG. Analysis of $NS3_{1073-1081}$-specific CD8+ T cell responses after 2 weeks by IFNγ ELISPOT shows that CpG stimulation enables HCV-specific T cell priming in the absence of CD4 'help' (Fig. 4.17).

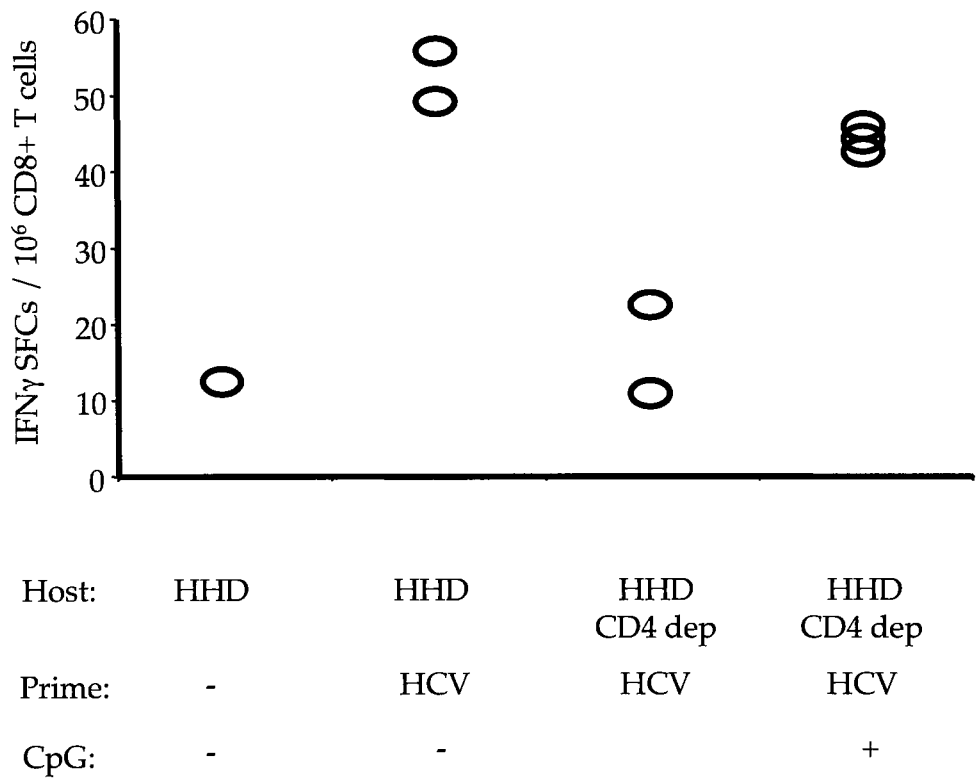

Figure 4.17. CpG can substitute for the absence of CD4+ T cells in priming NS3 $_{1073\text{-}1081}$-specific CD8+ T cells. HHD transgenic mice with or without CD4 depletion (dep) were immunized with HCV-expressing cell lines (HCV) with or without i.v. CpG. $NS3_{1073\text{-}1081}$ T cell immunity was evaluated after 12 days by IFNγ ELISPOT. Circles represent individual mice.

4.7 Discussion

pDC IFNα/β substitutes for CD4+ T cell 'help' in CD8+ T cell priming

Despite the potent clinical efficacy of IFNα in the treatment of HCV, the effects on HCV specific immunity have been contradictory[213,231]. IFNα can serve as a 'danger' signal in certain system to activate DCs[184] or provide help directly to CD8+ T cells[207]. In some systems, IFNα may even act directly on DCs to overcome CD4+ T cell requirements for CTL priming[185]. However, the experiments discussed in the last chapters suggest that spatial compartmentalization of IFNα may be an important component in achieving this immunological effect. Although pDCs are recognized as the main producers of IFNα/β, their role in providing these signals at the DC / T cell interface is not well defined. Here, using viral and non-viral triggers for pDC- and non-pDC-derived IFNα, we evaluated the ability of pDC IFNα in providing priming signals for CTLs in the absence of CD4 'help'.

Our results show that both MCMV and CpG can overcome the need for CD4 'help' in generating Uty-specific IFNγ producing CD8+ T cells (Fig. 4.8 and 4.9). Although MCMV and CpG trigger IFNα production with different kinetics (Fig. 4.6), IFNα production by both stimuli requires pDCs. pDC depletion of female mice before immunization abrogates the adjuvant effects of MCMV suggesting that host pDCs are required for this effect (Fig. 4.8). Since donor DCs are the main stimulators of CD8+ T cell immunity (Fig. 4.4) and pDCs are not efficient at cross-presenting antigen[232], our data suggest that host pDCs collaborate with immunizing male DCs to prime host CD8+ T cells (Fig. 4.18). Although this model lacks the antigen specificity afforded by CD4 engagement

of MHCII:peptide complexes, activated pDCs accumulate in the spleen following activation (Fig. 4.7A). This migration may put them in prime location to interact at the conventional DC / T cell interface, influencing priming and tolerance.

The pDC-dependent CD8+ T cell priming requires IFNAR in the host (Fig. 4.11). This may suggest that IFNα is acting directly on the CD8+ T cell to provide a third signal, similar to *in vitro* studies [207]; however, IFNAR is also required for IFNα production in response to CpG stimulation (Fig. 4.6D) and therefore effectively blocks potential effects on the immunizing WT DC as well. Preliminary data suggests that IFNAR is also required on the immunizing male splenocytes for CpG-dependent priming indicating that IFNα/β may also play an important role on the DC at the DC / T cell interface (Fig. 4.18). Although mouse pDCs may be capable of producing IL-12, this pDC-dependent priming effect is independent of IL-12p40, which excludes a role for both IL-12 and IL-23 (Fig. 4.11). Notably, Uty-specific priming in this system does not require IL-12 and, as such, these results cannot rule out a role for pDC produced IL-12 on CTL priming in systems that are more sensitive to IL-12.

As such, there are two notable differences between the *in vitro* human and *in vivo* mouse systems. First, IFNα is sufficient to allow for priming in the absence of CD4+ T cell 'help' in the mouse whereas IFNα synergizes with CD40L or TNF signaling to enhance priming in the human. It is possible that this reflects species specific differences in IFNα signaling[200]. Alternatively, signals provided by non-CD4+ T cells *in vivo* may synergize with IFNα/β to account for its ability to promote priming. Second, while IL-12 is sufficient to rescue the

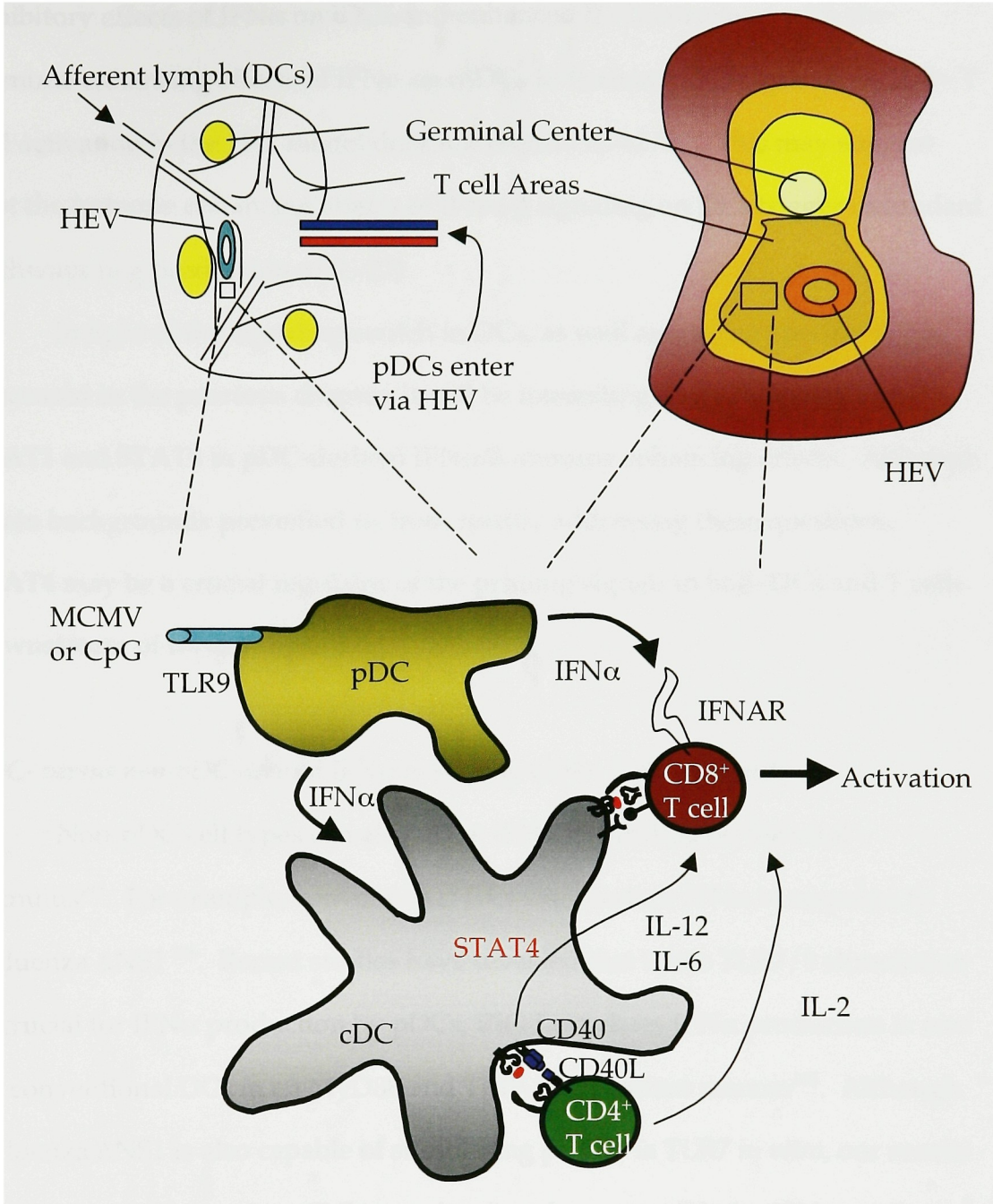

Figure 4.18. pDCs collaborate with DCs at the DC / T cell interface to provide IFNα/β-dependent 'helper' signals for CTL priming. pDCs enter secondary lymph organs and the spleen from the blood across high endothelial venules (HEV). pDCs may migrate into T cell zones and collaborate with conventional DCs (cDC) for T cell priming via IFNα. Notably, IFNα from the pDCs may act directly on the CD8+ T cells or on the DCs to activate STAT4 and promote T cell activation.

inhibitory effects of IFNα on iDCs and enhanced IL-12 correlates with the immune enhancing effects of IFNα on mDCs in human, pDC-dependent CD8+ T cell activation in the H-Y model does not require IL-12/23. This may suggest that the immune enhancing effects of IFNα/β signaling on DCs trigger redundant pathways (e.g production of IL-27).

In light of the signaling switch in DCs, as well as antigen specific T cells[208], discussed in the previous chapter, it will be interesting to assess the role of STAT1 and STAT4 in pDC-derived IFNα/β immune enhancing effects. Although strain backgrounds prevented us from readily addressing these questions, STAT4 may be a crucial regulator of the priming signals in both DCs and T cells downstream of IFNα/β signaling.

pDC- versus non-pDC-derived IFNα in shaping CD8+ T cell immunity

Non-pDC cell types can also make IFNα depending on the viral stimulus[195]. For example, conventional DCs can produce IFNα in response to influenza ΔNS1 [206]. Recent studies have revealed that while TLR7/9 stimulation is crucial for IFNα production by pDCs, RIG-I regulates IFNα production *in vivo* by conventional DCs in an MyD88 and TRIF-independent manner[233]. Although influenza ΔNS1 is also capable of stimulating pDCs via TLR7 *in vitro*, our results *in vivo* suggest that a non-pDC is predominately responsible for IFNα production (Fig. 4.12B).

In order to evaluate the effects of these distinct IFNα-producing cell populations in regulating adaptive immunity, we directly evaluated the ability of MCMV triggered IFNα *versus* influenza ΔNS1 triggered IFNα to offer priming

signals. Surprisingly, while MCMV triggered pDC IFNα supported Uty-specific T cell priming, influenza ΔNS1 triggered IFNα did not overcome the requirement for CD4+ T cell 'help'(Fig. 4.12C). This result suggests that the type of cell responsible for producing IFNα may be important in determining its effect on immunity (Table 4.1). While pDCs migrate to the spleen following activation, the IFNα producing cell during influenza ΔNS1 infection may not reside at this crucial location for effecting T cell priming. Two chemotactic factors, CXCR3 ligands and SDF-1/CXCL12, play an important role in pDC migration to the T cell areas via high endothelial venules[234] and it will be interesting to evaluate their role in pDC-dependent CD8+ T cell priming.

These findings highlight two important points. First, viruses such as HCV may trigger IFNα production in a manner that does not effectively augment the immune response against it. Second, with respect to therapeutic delivery of IFNα, specific pDC agonists (e.g. TLR7 or TLR9 agonists) may more effectively target IFNα production for promoting antiviral immunity.

Table 4.1. Characteristics of IFNα production and effects by MCMV, CpG, and influenza ΔNS1

	MCMV	CpG	Flu ΔNS1
pDC responsible for serum IFNα	Yes	Yes	No
IFNAR required for serum IFNα	No	Yes	Yes
pDC required for innate immunity	No	N/A	No
pDC required for adaptive immunity	No	N/A	No
Acts as adjuvant for priming	Yes	Yes	No
Overcome CD4 requirement for model antigen	Yes	Yes	No

HCV immunity in vivo

Although our impetus for investigating CD4-dependent CD8+ T cell immunity rested on the role for CD4+ T cell 'help' in HCV-specific immunity, it is clear that the H-Y model system may not be representative of HCV immunity. Ultimately, a small animal model for HCV will be crucial for evaluating cellular requirements in efficient cell-mediated immunity. In lieu of an infectious small animal model, we were able to evaluate CD4-dependent HCV specific T cell responses in a human HLA-A2.1 specific system. A better understanding of HCV immunity in these mice will aid in the advancement of a mouse model for HCV.

Interestingly, our work shows a dominant CD8+ T cell response to the $NS3_{1073\text{-}1081}$ epitope during immunization with full length, cell associated antigen (Fig. 4.13). This epitope cross-reacts with an influenza neuraminidase epitope [228] which may account for the high rate of $NS3_{1073\text{-}1081}$-reactive T cells in HCV-naïve individuals[235]. This epitope may also, however, play an important role in HCV pathogenesis as the strength of the $NS3_{1073\text{-}1081}$ response correlates with associated liver pathology during acute HCV infection [229]. Interestingly, one group reports a high rate of non-synonomous mutations at the C-terminal end of the $NS3_{1073\text{-}1081}$ epitope in patients who progressed to chronic infection from a single source outbreak [236]. This mutation impairs $NS3_{1073\text{-}1081}$ carboxyterminal processing and thus antigen presentation. The selectivity for this epitope offers a target for the virus to avoid detection of priming with cell associated, full length HCV. Although this priming is likely due to cross-priming, the HCV cell line used expresses low levels of human HLA-A2.1 and cross-priming will need to be shown formally in a haplotype mismatched cell line. In any case, mutations in the carboxyterminal end of the $NS3_{1073\text{-}1081}$ epitope may block endogenous

presentation of this epitope by infected hepatocytes and consequently prevent detection by $NS3_{1073\text{-}1081}$-specific T cells[237]. Finally, this epitope is located directly downstream of the protease domain and the contributions of replication fitness, strength of cross-reactivity, and efficiency of antigen presentation in driving escape mutation have not been evaluated. Futher work will be required to see if this is true *in vivo* as well as in other HCV expression systems such as HCVcc.

Activation versus tolerance in HCV-specific models

In light of our *in vivo* findings of no overt DC defects during chronic HCV infection, we proposed that another level of regulation might determine the strength of CD8+ T cell immunity. Similar to our results in the H-Y system, CD4+ T cell 'help' is required for efficient CD8+ T cell priming to $NS3_{1073\text{-}1081}$ (Fig. 4.15). Notably, the role for HCV-specific CD4+ T cells has not been directly evaluated. Specifically, it would be interesting to know if the efficacy of vaccination against HCV antigens by cross-presentation depends on the generation of strong MHCII epitopes[227].

Furthermore, our data suggests that in the absence of CD4+ T cells, functional tolerance of $NS3_{1073\text{-}1081}$ specific T cells ensues (Fig. 4.16). Currently, tolerance is not a heavily favored model in HCV infection because T cell responses can be seen in the liver of chronic patients many years following infection [41]; however, it is possible that not all chronic HCV situations result from a lack of effective CD8+ T cell immunity. Alternatively, although T cells may remain present in patients, the epitope specificity of those T cell responses may be crucial in determining clinical outcome[229]. This epitope selectivity may result from viral evolution[41] or the effects of persistent antigen on T cell anergy[238].

Finally, the role of CD4 'help' in HCV immunity may offer insight into progression and treatment strategy for HCV/HIV co-infection[48].

pDCs and CpG therapy for HCV immunity

In light our finding that pDCs are functionally competent in patients with chronic HCV, the role of pDC IFNα serving in lieu of CD4+ T cell 'help' is extremely attractive. In order to verify this finding in another antigen system besides H-Y and, even better, an HCV specific system, we evaluated the ability of CpG triggered pDCs to overcome CD4 depletion in $NS3_{1073\text{-}1081}$ specific T cell priming. Similar to our findings in the HY system, CpG triggered pDCs enhance HCV-specific CD8+ T cell priming (Fig. 4.17).

Notably, CpG motifs are currently in phase II trials for HCV and have shown good antiviral effect *in vivo*. Although the effects of IFNα on HCV-specific immunity are unclear, it will be important to compare immunologic response in IFNα *versus* CpG treated HCV patients. Moreover, the ability of pDCs to substitute for CD4+ T cell 'help' may make CpG motifs and TLR7/9 therapy suitable for co-infected HIV/HCV patients with compromised CD4+ T cell activity.

Chapter 5: DCs Coordinate HCV Immunity and Pathogenesis

HCV infects nearly 3% of the world's population and over 1 million Americans. In some countries, the prevalence of HCV reactive antibodies is 15-20%. Chronic HCV is a leading cause of cirrhosis and liver cancer. In addition to the morbidity and mortality of these diseases, the disease burden in the 4th and 5th decades of life has significant socio-economic ramifications in some communities. Clearly, more effective therapies and even vaccination would substantially lower disease burden worldwide. But what are the crucial characteristics of effective HCV immunity? Here, I will integrate my findings into the larger literature of HCV immunity to gain insight into this central question.

5.1 Re-examining the role for DCs and CD4+ T cell 'help' in generating effective CD8+ T cell immunity to HCV

Several early studies provided evidence for the role of the CD8+ T cell response in HCV immunity. In humans, the presence of strong HCV specific CD8+ T cell immunity correlates with resolution[40]. Similarly, in chimpanzees, CD8+ T cell immunity, in contrast to humoral responses, correlates with protection against rechallenge[34]. This correlative data was directly tested by CD8+ depletion. As expected, depletion of CD8+ T cells before re-infection rendered the chimpanzees susceptible[239]. Although significant data support the role for CD8+ T cells in HCV immunity, robust CD8+ T cell responses may also

exist in chronic infection[240] and the distinguishing characteristics of successful immunity are not clear.

In order to evaluate the requirements for effective T cell priming during HCV infection, many labs have focused on conventional DCs as the educators of the CD8+ T cell responses. Early studies reported a generalized defect in monocyte derived DC maturation, suggesting that defective maturation prevented robust T cell priming[159-161]. This model, however, is inconsistent with immune competence of chronically infected HCV patients. Furthermore, no mechanism for the modulation of DC function (i.e. virus binding or infection) could be identified to account for this global defect of monocytes. In contrast, we[241,242] and others[243] have concluded that both monocyte derived and circulating BDCA-1 conventional DCs are phenotypically and functionally normal in chronically infected HCV individuals.

These findings offer two significant developments for studies of HCV immunology. First, they suggest that another level of regulation exists to influence the immunologic outcome of HCV-specific T cell priming (Fig. 5.1). CD4+ T cell signals and differential cytokine production during HCV infection are important candidates for this alternate level of regulation. Importantly, our data are consistent with the possibility of HCV-induced modulation of DC cytokine secretion; however, a mechanism for this remains to be identified. Second, our findings lend credence to DC based immunotherapy for HCV. These therapies may prove useful for enhancement of HCV specific immunity.

CD4+ T cell signals are a critical component of a successful CD8+ T cell response[41]. This was directly evaluated in chimpanzees by CD4 depletion

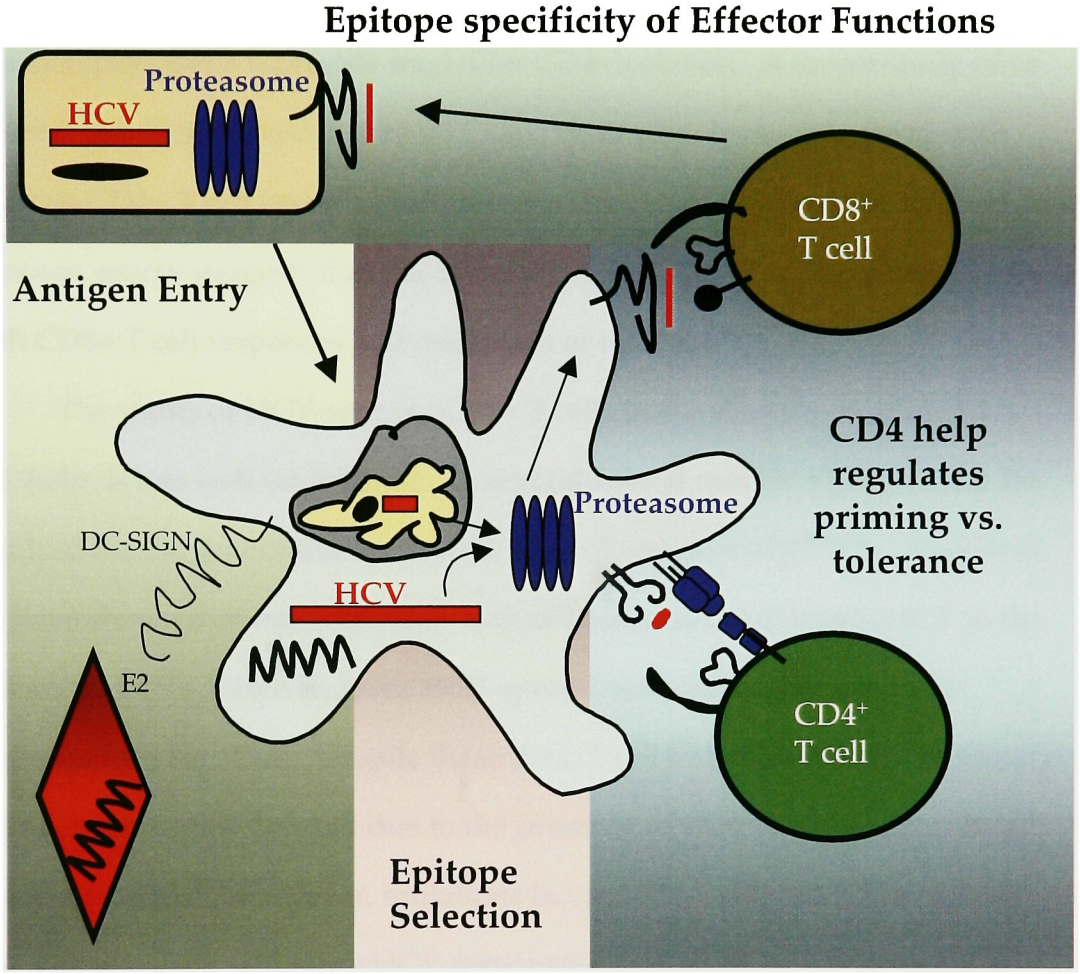

Figure 5.1 Functional DCs regulate CD8+ T cell immunity. In light of normal DC function in chronic HCV, we evaluated several alternate mechanisms of regulating CD8+ T cell priming. Although DC-SIGN binds E2, direct infection of DCs has not been demonstrated. Cross-presentation of both viral particles or cell associated antigen is a mechanism for generating HCV epitopes. Epitope selection may be regulated by primary sequence mutations. CD8+ T cell priming is dependent on CD4 help and plays a critical role in generating effective immunity *in vivo*. Finally, the epitope specificity of CD8+ T cells is an important determinant of their efficacy.

prior to rechallenge[47]. The results showed that CD4+ T cell 'help' was necessary for preventing escape mutations and allowing for resolution of infection. Although these types of experiments cannot be done in humans, HCV/HIV co-infection provides a surrogate model for CD4 depletion. A recent study of co-infected patients showed that HCV CD8+ T cell responses correspond with absolute CD4+ T cell count[48]. Furthermore, data from two patients infected from a known source suggests that the diversity of the CD4+ T cell response correlates with CD8+ T cell responses and resolution of infection[49].

The status of HCV-specific CD8+ T cells in the absence of strong CD4+ T cell 'help' is less well understood. In several animal models, CD4+ T cells are required for the generation of effective CD8+ T cell memory[68,244,245]. Moreover, we have shown that immunization with cell associated full length HCV in the absence of CD4+ T cells induces HCV-specific non-responsiveness to re-challenge (see Fig. 4.16). Despite these data, T cell tolerance in HCV immunity has not been widely favored due to the presence of CD8+ T cells in chronically infected patients[41]. However, mutations in viral RNA makes HCV a rapidly moving target and, although HCV responsive T cells can still be detected in chronic HCV, the epitopes efficiently presented by APCs may be significantly altered[41]. Moreover, the persistence of antigen during chronic infection may induce functional anergy in a subset of HCV-specific T cells[238]. These data suggest that while a crucial decision regarding T cell activation versus tolerance is made during intial exposure, the generation of effective immunity depends on the ability to prevent escape mutations and the adverse effects of persistent antigen. Importantly, therapeutic intervention usually occurs after viral escape and/or the effects of persistent antigen on selective antigen specific T cell

responses. Thus, with better tetramers and larger cohorts of HCV donors, it will be important to measure the relative importance of these processes on T cell immunity to HCV through the course of infection. These data may provide important insight into the important epitopes to target during immunotherapy for chronic infection.

5. 2 Mechanism of antigen presentation in generating CD8+ T cell immunity

Although hepatocytes are generally recognized to be infected during HCV infection, it is not clear if antigen presenting cells are directly infected[141] (Fig. 5.1). Some studies suggest that DCs are infected *in vivo*[246]; however, these results are not reproducible between patients. Furthermore, these findings are not based on negative strand synthesis and therefore indicate the presence, but not necessarily the replication, of HCV. Several studies have shown that HCV E2 glycoprotein binds to DCs via DC-SIGN[94,95], providing a putative mechanism by which HCV RNA is merely bound to surface of DCs. Although one report suggests that DC-SIGN enables HCVpp infection[247], the role for DC-SIGN in HCVcc infection has not been substantiated (see Fig. 2.8). In lieu of mediating direct infection, DC-SIGN binding may play an important role in virus trafficking. This mechanism of "hitching a ride" may account for HIV trafficking from mucosal surfaces to lymph nodes for T cell infection [97]. The importance of this interaction in trafficking HCV from sites of mucosal or intravenous infection to hepatocytes has not been defined.

Although APCs can efficiently generate MHCII epitopes from exogenous antigen, MHCI epitopes for the stimulation of CD8+ T cells are classically generated from cytoplasmically processed antigen. The lack of direct infection of

DCs would prevent classical loading of MHCI; however, it was recently shown that DCs can cross-present antigen from viral particles for stimulation of HCV-specific T cell lines[180]. We have confirmed the ability of full length cell associated HCV antigen to be cross-presented for stimulation of NS protein T cell immunity in mice; however, presentation of HCV epitopes by uninfected DCs in humans or chimpanzees has not yet been shown. This strategy also has the potential to identify new HCV epitopes. Interestingly, cross-presentation of NS3 antigen in the context of cytopathic bovine viral diarrheal virus (BVDV) infection was found to generate robust NS3 T cell immunity, suggesting that cross-presentation may offer unique advantages for vaccination strategies[227].

In light of the alternate methods for antigen presentation, differential antigen processing may be important in epitope generation (Fig. 5.1). In general, peptide epitopes generated by proteasomes are loaded onto MHCI in the endoplasmic reticulum. However, different cells process antigen differently. In the influenza model, direct infection of DCs can generate strong immune responses to both nucleoprotein (NP) and polymerase (PA) epitopes; however, cross-presentation of influenza antigen results primarily in NP-specific priming[248]. This epitope selectivity may have important consequences for the efficacy of the adaptive immune response in mediating viral clearance. There is precedent to suggest that viruses may use epitope preferences to mislead the host. Although cross-presentation generates robust D^b-restricted M45 T cell responses during MCMV infection, immune evasion protein m152/gp40 specifically blocks epitope presentation and subsequent detection in infected tissue[237]. *In vivo* mouse data for presentation of full length, cell associated HCV antigen suggest that $NS3_{1073\text{-}1081}$ is the dominant CD8+ T cell epitope.

Interestingly, a high rate of non-synonomous mutation is seen at the C-terminal end of this epitope which blocks proteasome processing and presentation[236]. In light of the strong T cell immunity to $NS3_{1073\text{-}1081}$, mutation at the C-terminus of this epitope might disrupt T cell stimulation. Alternatively, mutations at this site may allow HCV to evade T cell immunity in the infected tissue, similar to MCMV evasion described above[237]. With the availability of infectious HCVcc, it will be important to determine MHCI epitopes generated by infected hepatocytes *versus* DCs from infected patients by mass spectroscopy[249]. In conjunction with concurrent tetramer data, these experiments will provide a more complete picture of distinct regulation points in effective CD8+ T cell immunity: antigen presentation by APCs, epitope specific T cell responses, and presentation of antigen by infected hepatocyte targets.

5. 3 A role for DCs in the characteristic intrahepatic IFNα/β signature of HCV infection

In contrast to other viral liver infections such as hepatitis B, HCV induces robust expression of IFNα response genes in the liver during acute infection[250]. IFNα is a potent antiviral cytokine that can act by both autocrine and paracrine signaling mechanisms to induce the expression of proteins which protect against viral infection: MxA, PKR, 2'-5' OAS, and ADAR (see Fig. 1.6). These antiviral activities likely account for the potent therapeutic effects of pegylated-IFNα for HCV[101].

Which cells are responsible for IFNα production during HCV infection? Theoretically, all cells are capable of inducing IFNα production in response to

viral infection. The strong data showing the infection of hepatocytes with HCV *in vivo* [141], therefore, suggest that infected hepatocytes may be the most likely producers of IFNα during acute infection. Several reports suggests that hepatocytes are competent to respond to dsRNA via RIG-I and TLR3 signaling pathways[131,137]. On the contrary, although IFNα response genes are significantly upregulated in liver tissue, IFNα transcripts themselves are not found in liver tissue. In response to this finding, it has been argued that IFNα transcripts are present in PBMCs of acutely infected patients[143]. In contrast to hepatocyte infection, HCV infection of PBMCs remains controversial. However, PBMCs express numerous pattern recognition receptors e.g. toll receptors (TLRs), which may make them uniquely suited to detect virus even in the absence of infection. In particular, the natural interferon producing cells in the blood, plasmacytoid DCs (pDCs), express TLR7 (receptor for ssRNA[126]) making them well suited to produce IFNα in response to the HCV RNA. However, a mechanism for HCV induction of IFNα has yet to be defined. In fact, infectious J6/JFH chimeric HCV virus fails to trigger IFNα production in pDCs *in vitro* (Fig. 5.2) suggesting that another PBMC cell type may be involved in the IFNα response to infection; however, the response of liver pDCs *in vivo* also needs to be assessed.

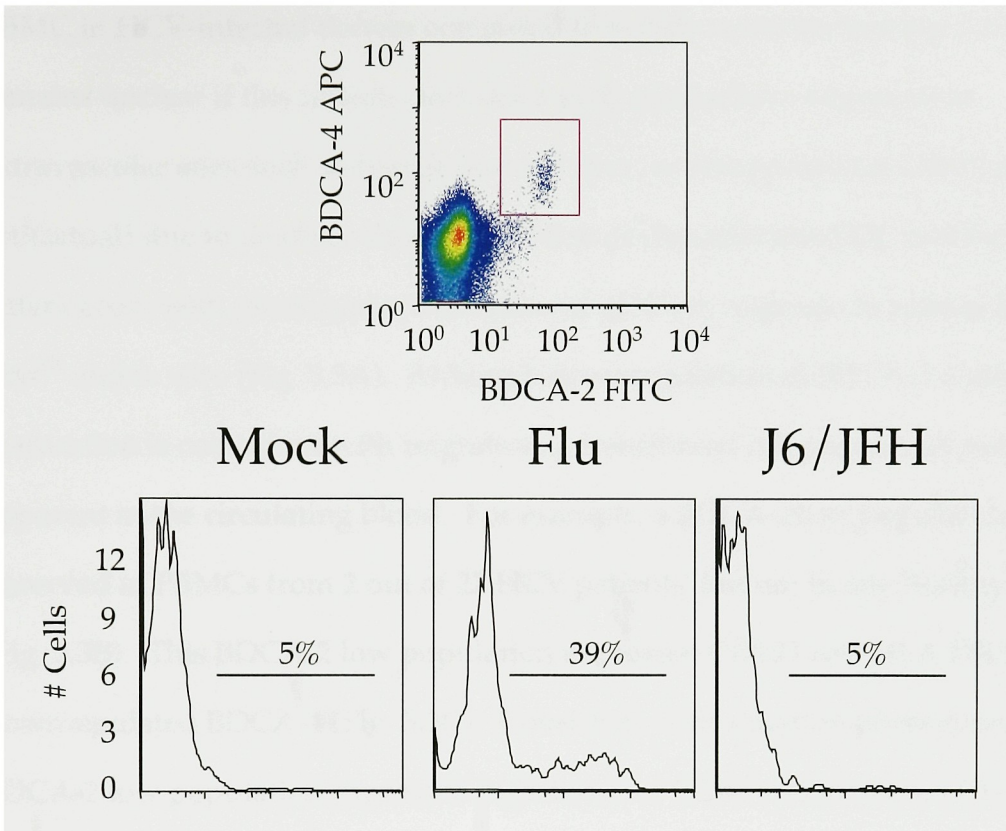

Figure 5.2 J6/JFH chimeric HCV does not induce IFNα production by human pDCs. BDCA-2 and BDCA-4 were used to identify and gate pDCs by FACS. Histograms show IFNα staining in pDC fraction following 6 hr stimulation as indicated.

Despite the lack of evidence for direct pDC activation by HCV, our work[242] as well as that of others[162,174] has revealed lower percentages of pDCs per PBMC in HCV-infected donors compared to healthy controls (see Fig. 2.6). It remains unclear if this reflects decreased pDC production, migration to extravascular sites such as lymph node or liver, or disappearance (albeit real or artifactual) due to death or down regulation of characteristic pDC markers. The latter occurs with phenotypic maturation of pDCs in response to viruses both *in vivo*[251] and *in vitro* (Fig. 5.3A). Although downregulation of BDCA-2 during pDC maturation is coincident with migration, a transitional cell population may be apparent in the circulating blood. For example, a BDCA-2 low population was observed in PBMCs from 2 out of 22 HCV patients, but not in any healthy donors (Fig. 5.3B). This BDCA-2 low population expressed CD123 and HLA-DR but downregulated BDCA-4 (Fig. 5.3C). Consistent with a mature phenotype, this BDCA-2 low population expressed high levels of CD83 and increased CD40. This finding, however, was only present in select HCV+ patients and the defining clinical characteristics could not be identified. Furthermore, exposure to J6/JFH chimeric HCV *in vitro* does not induce phenotypic maturation or enhance survival. *In vivo* studies are required to evaluate the presence of pDCs in infected liver or draining lymph nodes. An important caveat of this data is that these findings are not necessarily HCV-specific (see Fig. 2.6) and may be indicative of chronic infection[172]. Notably, endogenous as well as therapeutic IFNα negatively regulates pDC number and IFNα production[252]; as such, it is important for studies evaluating pDCs in chronic infections to document IFNα serum levels as well as the time elapsed since termination of IFNα therapy.

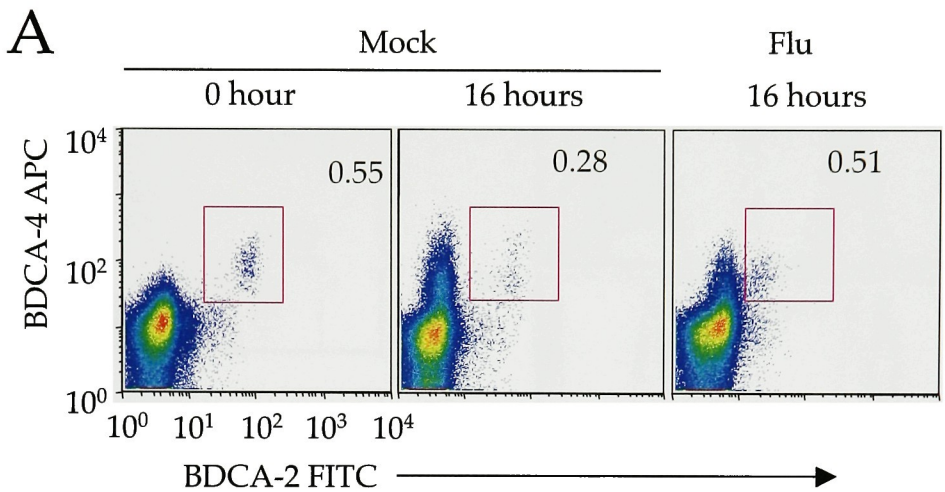

Figure 5.3. BDCA-2 low population in chronic HCV upregulates maturation markers. (Continued on the following page)

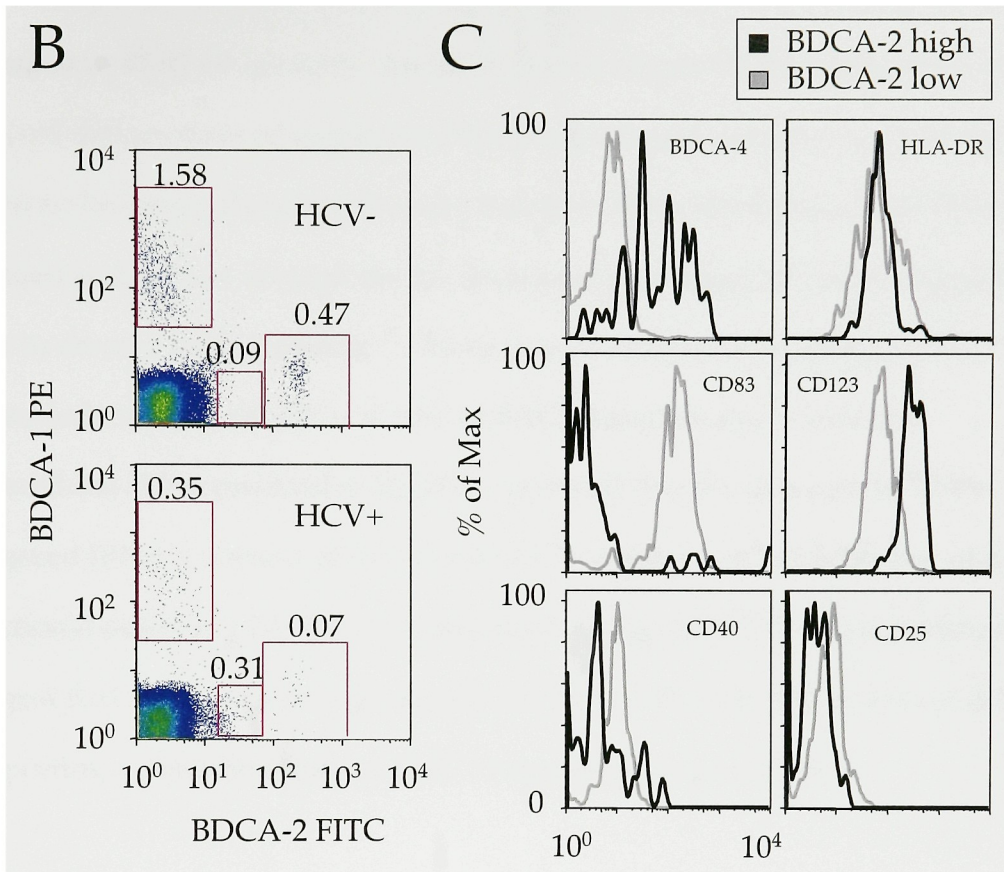

Figure 5.3. BDCA-2 low population in chronic HCV upregulates maturation markers. A. BDCA-2 and BDCA-4 were used to identify pDCs in PBMC cultures by FACS. Numbers indicate pDC percentage per PBMC. Cells were exposed to influenza or not and analyzed at indicated times after exposure. **B.** Enumeration of BDCA-1, BDCA-2, and BDCA-2 low population as described in material methods. FACS plot shows data from HCV- donor 24263 and HCV+ donor 24262. **C.** Surface expression of indicated markers on BDCA-2 low (gray line) compared to BDCA-2 high (black line) cell populations is represented by histogram.

Despite these reported abnormalities in cell numbers, pDCs from chronically infected HCV donors are functionally competent to make IFNα/β in response to TLR7/9 agonists. An initial report suggested that pDCs from HCV infected donors showed impaired IFNα production in response to TLR9 specific agonists; however, these results were based on IFNα levels from bulk PBMC cultures and did not account for the decreased percentage of circulating pDCs seen in chronic HCV infection[162]. More careful analysis accounting for the decreased numbers of pDCs as well as FACS-based assays to monitor intracellular IFNα production by pDCs revealed that the decrease in TLR9 triggered IFNα is a result of decreased pDC numbers and not reflective of a functional defect in pDC IFNα production (see Fig. 2.7)[242,243]. These findings suggest that HCV does not specifically interfere with pDC production of IFNα, supporting a potentital role for TLR7/9 agonist therapy for HCV.

Conventional DCs can also produce significant amounts of IFNα[206]. Using influenza as a stimulus, we showed that iDCs produce IFNα equivalent to pDCs stimulated with CpG (Fig. 5.4A). 2-aminopurine inhibits this IFNα production suggesting the importance PKR or other kinase activity in this response (Fig. 5.4B). Interestingly, DC maturation shuts down this IFNα production (Fig. 5.4B). We hypothesized that this finding reflects an intrinsic inability of mDCs to make IFNα due to their decreased need to detect pathogens (and consequent downregulation of TLRs (Fig. 5.4C)); however, infection with influenza ΔNS1 revealed the capacity of mDCs to produce IFNα (Fig. 5.4D). This finding needs to be evaluated by intracellular cytokine staining to rule out the possibility of contaminating iDCs accounting for the interferon production. This finding suggests that viruses may actively inhibit IFNα production in mDCs. The ability of HCV to trigger conventional DC IFNα production *in vivo* or *in vitro* has not been evaluated.

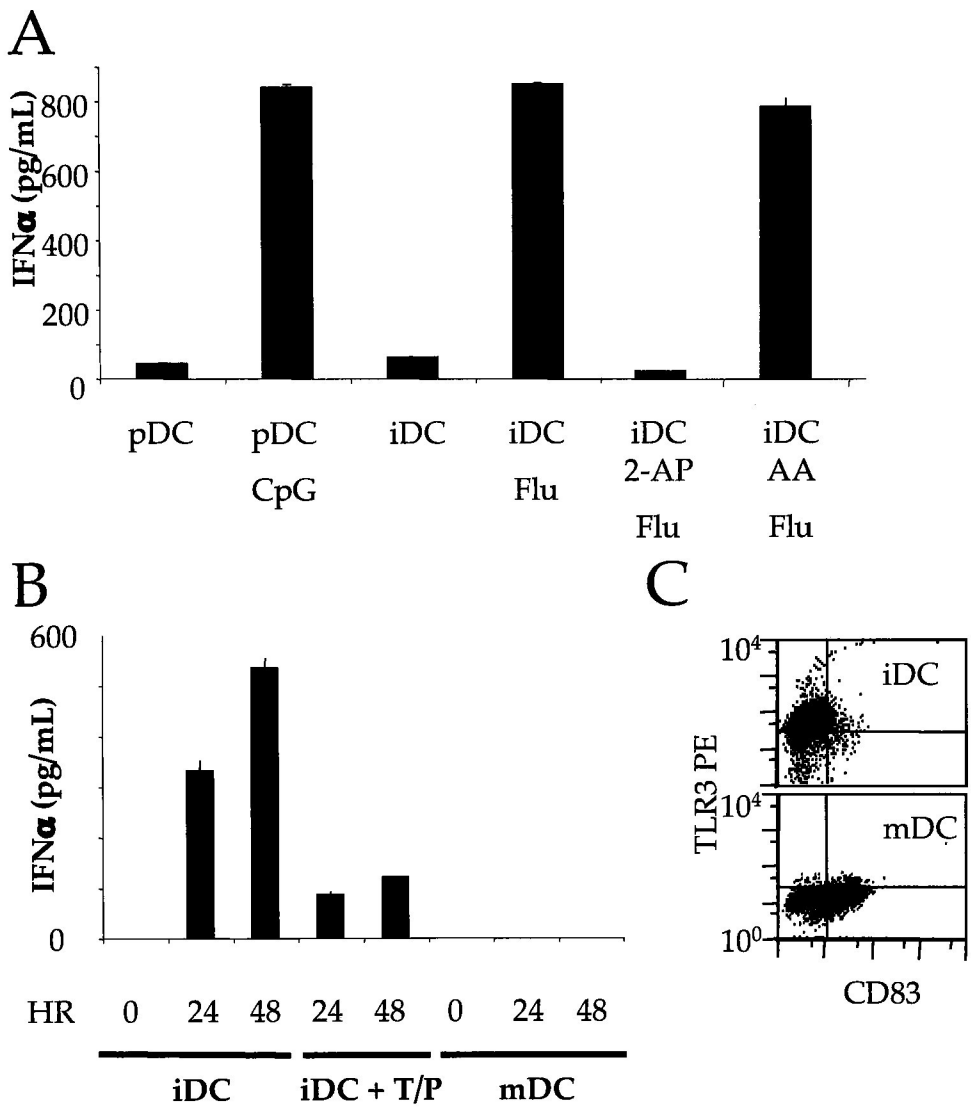

Figure 5.4. Influenza NS1 protein antagonizes mDC IFNα production via non-TLR dependent mechanism. (Continued on the following page)

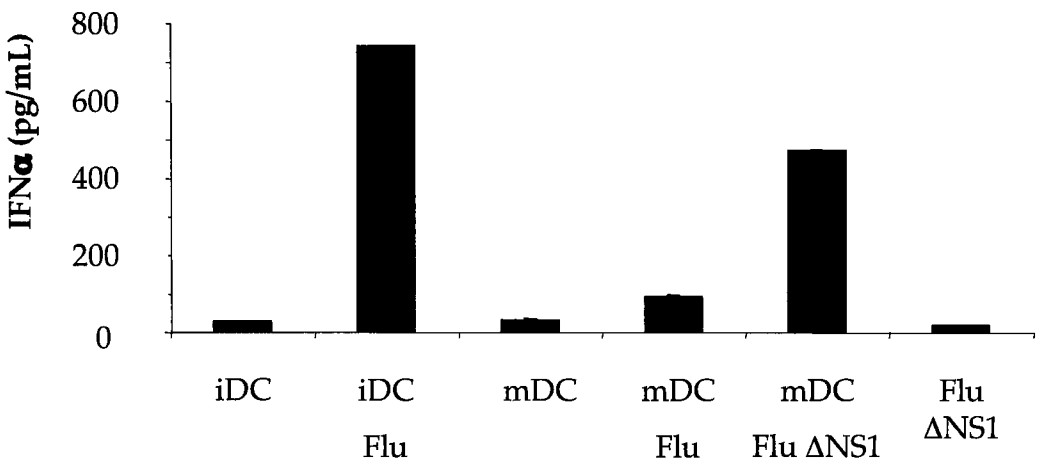

Figure 5.4. Influenza NS1 protein antagonizes mDC IFNα production via non-TLR dependent mechanism. A. pDCs or iDCs at 10^6/mL were stimulated with 3mg/mL of CpG 2216 or 50 HAU influenza (Flu) in the presence of 2-aminopurine (2-AP) or acetic acid solvent (AA). IFNα in 24 hour supernatants was quantified by ELISA. B> iDCs or mDCs at 10^6/mL were stimulated with 50 HAU influenza (Flu) and cultured in media alone or media with maturation stimuli TNFα and PGE-2 (T+P) for the indicated amount of time. IFNα was quantified by ELISA. C. Surface staining of iDCs and mDCs for TLR3 and CD83. D. IFNα in iDC and mDC supernatants containing 106 cells/mL stimulated for 24 hours with media alone, 50 HAU influenza (Flu), or 50 HAU influenza ΔNS1 (Flu ΔNS1) monitored by ELISA.

Despite the clinical efficacy of IFNα and the sensitivity of HCV to IFNα *in vitro*, it is surprising that induction of IFNα response genes does not correlate with resolution of acute HCV infection[139]. Notably, several mechanisms by which HCV proteins antagonize IFNα signaling and production in infected cells have been elucidated[133,134] (Fig. 5.5). The NS3-4A protease plays a key role in disrupting IRF-3 phosphorylation[135] by targeting the RIG-I adaptor, Cardif[136,253] and TLR3 associated TRIF adaptor protein[137]. These antagonistic functions may limit IFNα's anti-viral effect during acute infection. Another study has suggested that the dose of infection may be an important factor in determining the nature of the protective response[37]. In other words, innate immunity and IFNα may play a more important role in low dose exposures. Alternatively, HCV, as well as other pathogens, may take an active role in manipulating the host's IFNα/β response to evade adaptive immunity and promote viral persistence.

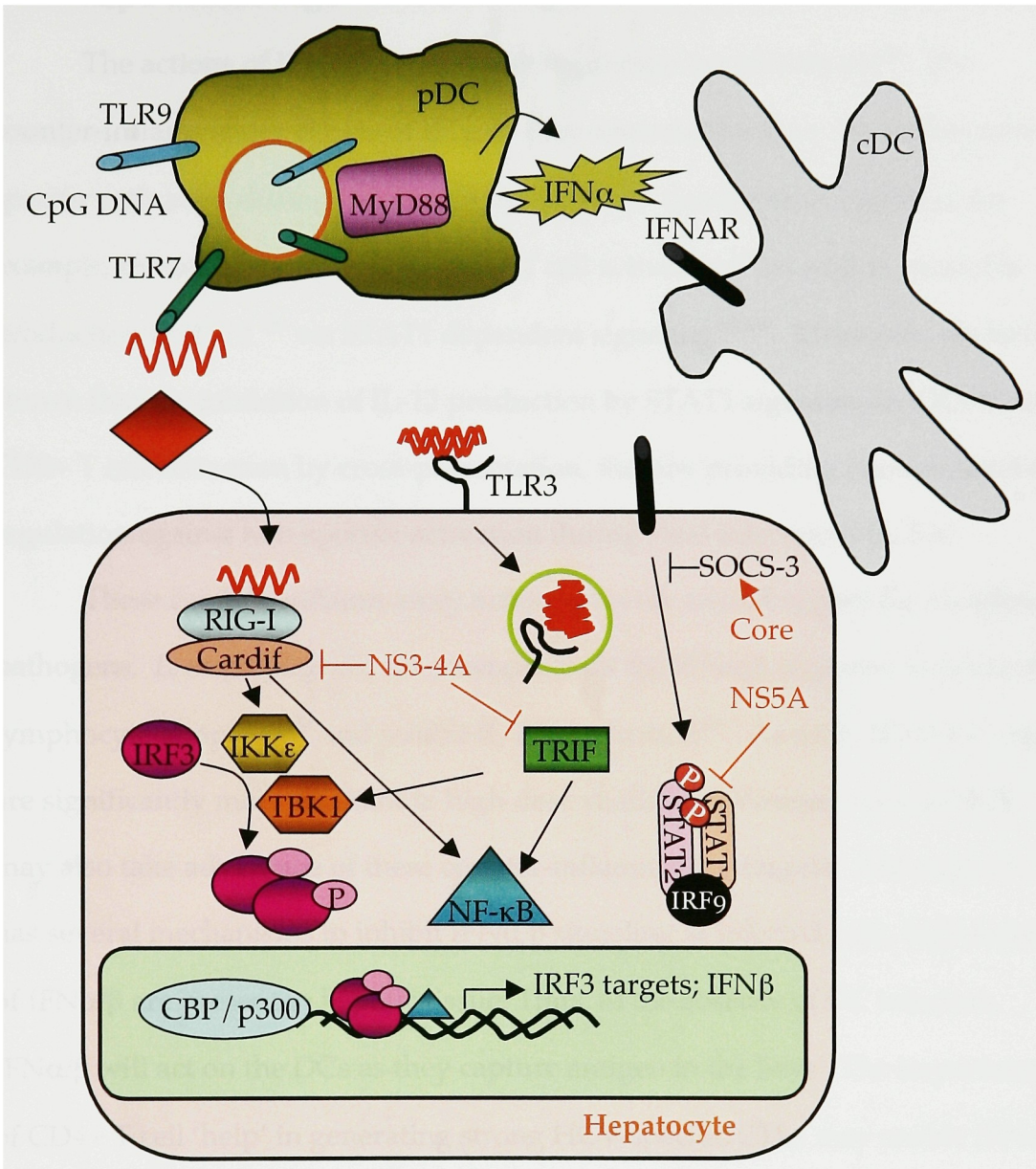

Figure 5.5. Regulation of IFNα production and induction of IFNα response genes during HCV infection. Despite the robust induction of IFNα response genes in liver tissue samples, HCV has numerous mechanisms to inhibit both effector function and production of IFNα. The nature of the IFNα producing cell during acute HCV infection remains unidentified as does the mechanism by which it detects HCV. As proposed in the figure, RIG-I and TLR3 mechanisms may trigger IFNα production directly by hepatocytes. Although a decrease circulating pDC numbers has been documented, it is not clear which PBMC cell type produces IFNα during acute infection.

5.4 IFNα/β's double-edged sword: strategies for immunoevasion and immunity

The actions of IFNα/β on immune regulation are pleiotropic[140]. The counter-inflammatory effects of IFNα/β play important role in controlling non-specific activation during times of hyper-activity. During viral infection, for example, IFNα/β suppresses bystander T cell activation[209] as well as excessive production of IL-12 [186] via STAT1 dependent signaling[140,208]. Moreover, we have shown that the inhibition of IL-12 production by STAT1 signaling in iDCs blocks CD8+ T cell activation by cross-presentation, thereby providing another level of regulation against non-specific activation during viral infection (Fig. 5.6).

These counter-inflammatory actions provide crucial targets for invading pathogens. *L. monocytogenes*, for example, uses the IFNα/β response to promote lymphocyte apoptosis[254] and inhibit IL-12 production[210]. As such, IFNAR-/- mice are significantly more resistant to high dose challenge. Viruses, such as HCV, may also take advantage of these counter-inflammatory targets. Although HCV has several mechanisms to inhibit IFNα/β signaling in infected cells, high levels of IFNα/β are present in hepatic tissue. Thus, in the absence of DC infection, IFNα/β will act on the DCs as they capture antigen in the liver. The importance of CD4+ T cell 'help' in generating strong HCV-specific CTLs may enable IFNα/β in the tissue to play an important role in preventing CD8+ T cell activation by cross-presenting DCs. Polymorphisms in *IL12B* promoters could contribute to the complexity of IFNα/β regulation of IL-12 production, resulting in wide variation in clinical outcomes[255].

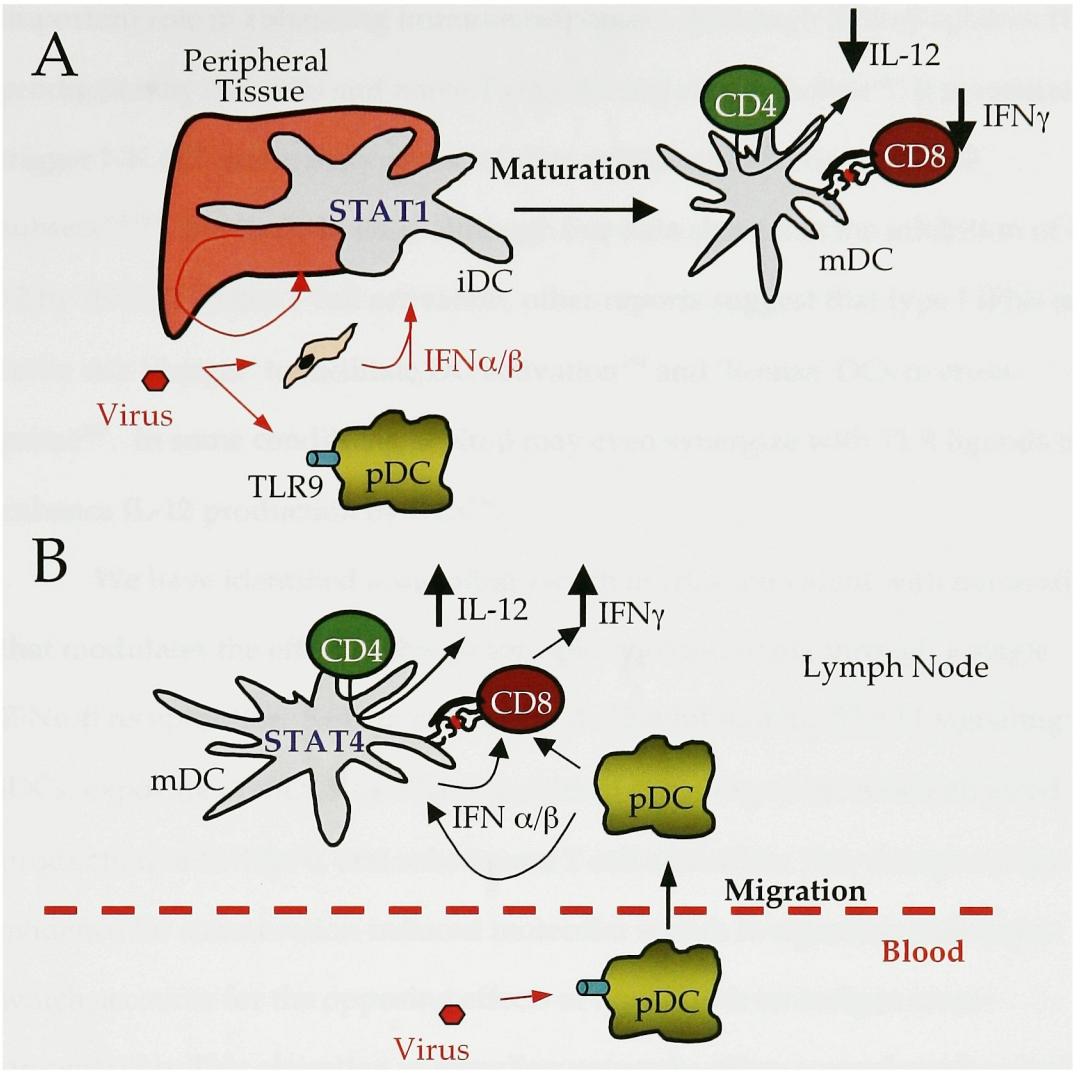

Figure 5.6. Spatial compartmentalization of IFNα reflects both therapeutic and viral strategies of modulating immunologic outcome. A. IFNα in peripheral tissue during an infection or inflammatory response can be produced by infected cells, pDCs, or cDCs. Activation of STAT1 dependent signaling in cDCs decreases consquent IL-12 production and T cell priming. This mechanism may play an important role in viral infections that trigger non-pDC-produced IFNα in tissue such as HCV, inflammatory situations with pDC infiltrates, or therapeutic Betaseron® for multiple sclerosis. **B.** In contrast, IFNα in lymph nodes may facilitate immunity. Activation and migration of pDCs or cDCs to the DC/T cell interface places them in prime location to affect the immunologic outcome. IFNα/β might act on the DC or the CD8+ T cell via STAT4-dependent signaling.

In addition to its counter-inflammatory effects, IFNα/β also plays an important role in enhancing immune responses. Although IFNα/β inhibits IFNγ production by NK cells and naïve T cells during viral infection[181], it is required to trigger NK cell cytotoxicity and stimulate proliferation of certain T cell subsets[182,183]. Similarly in DCs, although our data show that the inhibition of IL-12 by IFNα/β blocks T cell activation, other reports suggest that type I IFNs may serve as a 'danger' to facilitate DC activation[184] and 'license' DCs to cross-prime[185]. In some conditions, IFNα/β may even synergize with TLR ligands to enhance IL-12 production by DCs[198].

We have identified a signaling switch in DCs coincident with maturation that modulates the effect of this pleiotropic cytokine acting through a single IFNα/β receptor (Fig. 5.6). In contrast to IL-12 inhibition by STAT1 signaling in iDCs, exposure of mDCs to IFNα/β results in signaling via STAT4, enhanced production of IL-12p70, and subsequent T cell activation. This data provides evidence for a maturation-induced molecular switch in signaling pathways, which accounts for the opposing effects of type I IFNs on antigen cross-presentation. This alteration in signaling networks offers a novel mechanism by which DCs modulate the integration of signals from the surrounding environment. This switch is reflective of similar intracellular regulation in both NK and T cells[181,199]. Recent data suggest that antigen specific CD8+ T cells regulate total STAT1 levels, thereby reducing their sensitivity to IFNα/β's anti-proliferative effect[208]. In sum, the cell-specific regulation of signaling networks enables multiple, yet specific, effects of IFNα/β in coordinating immunity.

5.5 IFNα/β compartmentalization in disease and immunity

The migration of pDCs to secondary lymph organs upon activation offers a clear mechanism for IFNα/β's immune enhancing effects. IFNα/β production during most viral infections is not restricted to the tissue and may also activate pDCs to produce IFNα. Our experiments suggest that pDC-derived IFNα at the DC / T cell interface can overcome the inhibitory effect of IFNα 'early' (see Fig. 3.13). This finding supports the hypothesis that inflammatory cytokines in the periphery may not be sufficient and that TLR-dependent detection and movement of blood cells is crucial to orchestrating an effective CTL response. In contrast, a recent study suggests that inflammatory cytokines triggered by CpG are insufficient to allow for activation of transgenic CD4+ T cells[256]. These findings may reflect differences in activation of transgenic CD4+ T cells *versus* endogenous CD8+ T cells. Alternatively, the delivery of CpGs in the footpad may not be sufficient to trigger pDC migration and IFNα production in the lymph node at the DC / T cell interface. Another recent study suggests that pDCs are critical for generating herpes simplex virus (HSV)-specific CTLs in response to viral challenge[223]. This finding, however, may be specific for HSV, as pDC depletion does not affect the generation of M45 epitope-specific CD8+ T cell priming in response to MCMV infection(see Fig. 4.8). However, MCMV or CpG activated pDCs can substitute for CD4+ T cell 'help' in priming CD8+ T cells against H-Y model antigens. Thus, in the absence of robust CD4+ T cell responses (e.g. in HIV-AIDS), pDCs may play an important role in T cell priming. This pDC effect is independent of IL-12, but dependent on IFNAR suggesting that IFNα production is required at the DC / T cell interface. These

findings show that pDCs can offer priming signals to CD8+ T cells and provide a functional rationale for their migration to lymph nodes during viral infection.

As previously mentioned, pDCs are not the only cells capable of making IFNα/β. Infected tissue as well as conventional DCs are able to produce IFNα/β. Naturally, the IFNα/β produced by infected tissue is restricted to the local area of infection. Although DCs may migrate to lymph nodes like pDCs, maturation is coincident with decreased IFNα/β production (Fig. 5.4). This may represent active interference by viral proteins to prevent the immune enhancing effects of IFNα/β production at the DC / T cell interface. With regard to vaccine development, viral vectors lacking viral proteins that disrupt IFNα production by mDCs (i.e. influenza ΔNS1) may enhance immunogenicity.

Compartmentalization of IFNα/β production during viral infection may represent a distinct signature of the host-pathogen interaction. Herpesviruses, such as HSV and MCMV, are potent activators of pDC-derived IFNα/β[188]. The activation of pDCs by herpesviruses such as HSV and MCMV may be tightly linked to the importance of cross-priming for robust T cell immunity during infection[257]. In contrast, influenza ΔNS1 triggers IFNα/β production by a non-pDC and can generate an immune response via direct infection of APCs[248]. Notably, the production of IFNα/β during influenza ΔNS1 infection does not substitute for CD4+ T cell 'help' during H-Y priming. LCMV also induces production of IFNα by a non-pDC[195]. Although LCMV-specific CD8+ T cell priming was classically defined as CD4-independent, recent data suggest that CD4+ T cells are important for secondary expansion[68]. It will be interesting to see if pDC IFNα/β affects secondary expansion in this model.

HCV may also induce spatial compartmentalization of IFNα/β. Despite the functional responsiveness to TLR9 agonists[242] *ex vivo*, no direct evidence shows HCV activation of pDC-derived IFNα; nonetheless, IFNα/β response genes are induced in hepatic biopsy tissue[139]. This spatial compartmentalization during HCV pathogenesis may make HCV uniquely suited to take advantage of the inhibitory effects of IFNα/β on CD8+ T cell activation by cross-presenting DCs.

5.6 pDC-based therapy for HCV

Given the correlation between T cell immunity and clinical response, it was hypothesized that IFNα treatment would enhance HCV-specific T cell immunity. A recent report, however, suggests that this is not necessarily the case[213]. Despite the sustained virologic response (SVR), HCV-specific T cell immunity was not enhanced. Several possibilities may account for these findings. First, a simple decrease in antigen load may have led to a decrease in T cell response. Second, as described above, the inhibitory effects of IFNα on DCs may dominate during systemic delivery to decrease T cell activation. Third, treatment may selectivity enhance T cells with an antigen specificity not represented in the functional assays used. As such, it will be important to follow these T cells responses, especially CD8+ T cell response, after SVR to a variety of epitopes expressed by the particular virus that infected the patient.

Targeted activation of pDCs for HCV therapy may offer a more effective strategy for enhancing the immune response. Our results evaluating HCV specific immune responses in mice suggest that pDC-derived IFNα/β enhances T

cell immunity. Although decreases in pDC number have been reported during chronic HCV infection by us[242] and others[162,174], these pDCs still produce significant amounts of IFNα/β upon stimulation. CpG trials by Coley Pharmaceutical Group report good anti-viral activity suggesting that the CpG motifs are effective in triggering IFNα *in vivo*. TLR7/9 agonist therapy may be an important therapeutic strategy to promote CD8+ T cell immunity in patients with compromised CD4+ T cell responses (i.e. HIV/HCV co-infected patients).

In all, host-virus interactions play a key role in dictating the immunological outcome of HCV infection. Although we are still in the early stages of understanding these interactions *in vivo*, the role of IFNα/β in response to HCV infection offers a salient example of the complexity with which HCV modulates the immunologic outcome. Better tools for studying human immunology and the development of a small animal model for HCV will add tremendous insight into the mechanisms involved in the interaction of HCV with its host, as well as novel mechanisms of immune evasion required for viral persistence. A better understanding of the crucial aspects of this interaction will put us on more solid ground for designing therapeutic interventions to prevent and possibly cure HCV infection.

Appendix 1: Learning from our successes—Yellow fever interacts with DCs to generate robust CD8+ T cell immunity

When looking to achieve a specific aim in almost all endeavors, it is helpful to know what worked in the past. Accordingly, HCV research in general and my thesis work in particular takes advantage of both natural resolvers and sustained virologic responders to gain insight into successful immunity, disease pathogenesis, and rational therapeutic design for HCV. Although we frequently learn many things from these situations, these successes are sometimes not widely studied. For example, the vaccine for yellow fever, developed in part here at Rockefeller University, is an extremely effective vaccine that has been in use for over 60 years to immunized over 400 million people; however, the mechanisms associated with the robust immunity it generates are poorly defined. During my thesis work, I had the opportunity to address some of these questions in collaboration with Giovanna Barba-Spaeth in the Rice lab. It is my hope that a better understanding of the effective components of the yellow fever vaccine will bring us closer to developing an HCV vaccine.

A1.1 Abstract

The yellow fever (YF) 17D vaccine is one of the most successful live attenuated vaccines available which confers life-long immunity to pathogenic YF, a flavivirus related to HCV. A single immunization of YF17D induces both YF specific T cell responses and neutralizing antibodies that can be detected for many years after vaccination. Surprisingly, the mechanism for this robust and long-lasting immunity has not been widely studied. Several recent reports have shown flavivirus interaction with dendritic cells (DCs); however, the mechanism of YF17D interaction with DCs and the significance of this interaction in generating T cell immunity have not been explored. Here, we show that YF17D can infect immature and mature human DCs. Viral entry is Ca^{2+}-dependent, but independent of DC-SIGN as well as multiple integrins expressed on the DC surface. Similar to infection of cell lines *in vitro*, YF infection of iDCs is cytopathic. Although infection itself does not induce DC maturation *in vitro*, TNFα-induced maturation protects DCs from YF-induced cytopathogenicity. Furthermore, we show that DCs infected with YF17D or YF17D carrying a recombinant epitope can process and present antigens for $CD8^+$ T cell stimulation. This work offers insight into the immunologic mechanisms associated with the highly effective YF17D vaccine that may guide effective vaccine design.

A1.2 Introduction

"He gave to man control over that dreadful scourge, yellow fever."

President Eliot, Harvard University

Upon conferring master of arts to Dr. Walter Reed

Yellow fever had tremendous public health impact on Central and Northern America from the 1600's until early 1900's infecting over 300,000 people with a mortality of over 40%. It was named "yellow" fever for the characteristic jaundice it produced in infected individuals. In his pioneering work presiding over the Yellow fever Board in Cuba in 1900, Major Walter Reed and his colleagues proved Carlos Juan Finlay's theory, that the mosquito, *Aedes aegypti,* was the vector for this transmissible agent[258]. The following year, Reed's colleague, Dr. James Caroll, showed that yellow fever agent was filterable[259]. YF is classified in the same family as HCV, Flaviviridae, based on sequence and physical similarities.

In 1930's, Max Theiler's seminal work on YF grew out of his observations that propagation of the virus in mice produced an attenuated virus that was less infectious in rheseus macques. After his move to the Rockefeller Foundation (renamed Rockefeller University), Theiler along with Hugh Smith produced an attenuated virus in chicken embryos that conferred protection to pathogenic infection[260]. In 1951, Theiler was awarded the Nobel Prize for his discovery of a live attenuated yellow fever vaccine.

The yellow fever vaccine strain 17D (YF17D) is a live attenuated vaccine that has been used for over 60 years in more than 400 million people for vaccination against yellow fever virus with a remarkable record of safety and

efficacy [261]. The vaccine strain YF17D was generated from the wild-type strain Asibi and differs from Asibi by only 32 amino acids, 12 of which are clustered in the envelope protein [262]. Viscerotropism of the parental Asibi is markedly reduced in YF17D but the basis of the attenuation remains unknown. Furthermore, the YF17D receptor and mechanism of entry are not known.

YF17D vaccination generates both neutralizing antibodies and T cell responses that persist for many years [263,264]. Given its safety and efficacy, YF17D has been engineered as vaccine vector for antigens of other flaviviruses such as dengue, Japanese encephalitis and West Nile viruses. Some of these chimeric viruses are in phase II clinical trials [265]. YF17D can also be engineered to deliver epitopes from unrelated pathogens and it is able to induce protective immunity against heterologous agents[266,267]. The antibody response most likely accounts for the effectiveness of the vaccine; however, a role for cell-mediated immunity in generating an effective immune response has also been suggested [264]. Only recently have YF-specific human $CD8^+$ T cell response been assayed [268], but the immunologic mechanisms associated with YF-specific $CD8^+$ T cell priming are still unknown.

As discussed in the introduction, DCs are potent antigen presenting cells that play a crucial role in regulating the adaptive immune response [52]. DCs may interact with pathogens in peripheral tissue via direct infection or via phagocytosis of either infected cells or viral particles. Upon exposure to inflammatory signals or certain pathogens, DCs undergo a programmed phenotypic maturation program coincident with CCR7 upregulation and migration to the lymph node for engagement and activation of T cells. In addition, DC maturation confers resistance to cytopathogenicity of some viral

infections, facilitating T cell priming [84]. DC-SIGN, a DC specific ICAM-3 grabbing non-integrin that is present most robustly on immature dendritic cell (iDCs), mediates entry of dengue virus into DCs [92,93] and binding of the HCV envelope protein [269].

In this chapter, I explore the interaction of YF17D with human DCs. We investigate the ability of YF17D to infect DCs, the requirements for viral entry, and the cytopathic effect of YF17D in DCs. Furthermore, we evaluate the immunologic determinants of DC infection by monitoring T cell responses to endogenous and model antigens from YF17D vectors. This work provides new insight into the immunologic mechanisms associated with the highly effective YF vaccine and may provide clues relevant to effective vaccine design.

A1.3 Materials and Methods

Isolation and Preparation of cells

PBMCs, DCs, and T cells were prepared as previously described [168]. PBMCs were isolated from whole blood by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ). T cell enriched and T cell depleted fractions were prepared by adherence to plastic in 1% single donor plasma. iDCs were prepared from the T cell depleted fraction by culturing cells in the presence of 1000 U/ml GM-CSF (Berlex, Seattle, WA) and 500-1000 U/ml IL-4 (R&D Systems, Minneapolis, MN) for 6 days [170]. To generate mature DCs, cultures were stimulated on day 6 with 50 ng/ml TNFα (Alexis Biochemicals) and 10mM PGE-2 (Sigma) for 36-48 hr [171]. Patient material was obtained as per protocol approved by the IRB The Rockefeller University Hospital (JKR-0397), and all patients gave written informed consent.

Preparation of virus stocks

Yellow fever 17D (YF17D) viral stocks were derived from pACNR/FLYF plasmid containing the full length infectious YF17D genome under a SP6 promoter [270]. In vitro-generated RNA transcripts were electroporated in SW13 cells as previously described [271]. Virus stocks were harvested at 48 hrs post-transfection with typical yields of 10^7-10^8 PFU/ml as determined by plaque assay on SW13. Single use aliquots were stored frozen at –80°C until use. YF17D/M1 was constructed by inserting the influenza HLA-A2.1 CTL epitope of matrix protein (GILGFVFTL) [272] between yellow fever NS2B and NS3 proteins. Two specific oligonucleotides containing the M1 epitope sequence (forward 5′-aggggagcgcgcagaagtggaattttaggattcgtgttcacgctcggtcaccggagaagt-3′ and reverse 5′-acttctccggtgaccgagcgtgaacacgaatcctaaaattccacttctgcgcgctcccct-3′) and two YF17D-specific primers were used to generate two PCR fragments containing the influenza M1 epitope and a portion of the NS2B or NS3 gene, respectively. One of 50µl of each PCR reaction was mixed together and used as template for PCR with YF17D-specific primers lying in NS2B and in NS3. The final product was digested with BssHII and BstEII and cloned in YF17D2B/3 described elsewhere[267]. The recombinant virus was recovered 48 hrs post-transfection of SW13 cells with infectious RNA and tested for stability by PCR and sequence analysis of the region overlapping the insertion. UV-inactivation was carried out with a GS Gene-Linker UV chamber (Bio-Rad) using the sterilizing program.

Infection of Cells

DCs were washed in RPMI and infected for 1 hr at 37°C using the indicated PFUs. UV-inactivated virus was used as a negative control. The infection was quenched with 5% pooled human serum and washed twice to remove excess virus. For EDTA/EGTA blocking, DCs were washed twice in PBS without Ca^{2+} and Mg^{2+} and 10mM EDTA or EGTA was added before infection. For antibody blocking, DCs were washed in RPMI and resuspended in 1% FBS/PBS. DCs were incubated with 10μg/mL of antibody (DC-SIGN (612) or $\alpha_v\beta_3$ (Chemicon)) or 100μg/mL RGD tripeptides (Sigma) for 15 minutes at room temperature prior to infection. Following 1 hr infection, DCs were resuspended in conditioned media with or without TNFα / PGE-2 maturation stimulus as indicated and incubated 24 hrs before monitoring for infection.

Immunostaining for FACS Analysis

Surface staining was done in serum containing media at 4°C. Anti-CD14, CD25, CD40, CD83, CD86, HLA-DR, and isotype control were obtained from BD Biosciences. Cytoperm / CytoFix Kit from BD Biosciences was used for fixation and permeabilization. MAb 1A5 is a mouse monoclonal antibody against the nonstructural protein NS1 [273] and C12 is a rabbit polyclonal antisera that recognize the nonstructural proteins NS4A and NS4B [274]. Secondary antibodies used were from Jackson (PE) or molecular probes (APC).

Plaque Assay

For plaque titration serial 10-fold dilutions were used to infect monolayers of SW13 for 1 hr at 37°C. After infection cells were overlayed with 0.6% agarose-containing medium and plaques were allowed to develop at 37°C for 4 days. Plaques were fixed in 7% formaldehyde for 1 hr and stained with crystal violet (1.25% in 20% EtOH) [271].

Detection of influenza-specific T cells by ELISPOT

DCs and T cells were plated in 96-well Millipore plates coated with 5μg/ml of anti-IFNγ mAb (Mabtech, Mab-1-D1K). Cultures were incubated for 24-36 hrs at 37°C, washed with mild detergent, and incubated with 1μg/ml biotin-conjugated anti-IFNγ mAb (Mabtech, Mab 7BG-1). Spots were visualized using Vectastain Elite Kit (Vector Laboratories) and AEC substrate. Evaluation was performed in a blinded fashion by an independent service (Zellnet Consulting, Inc., NY) using an automated ELISPOT reader (Carl Zeiss, Inc., NY). Spots represent IFNγ production by single cells and are reported as spot forming cells (SFCs)/10^6 cells.

A1.4 Results

YF17D infects immature and mature DCs

To test the hypothesis that the immune response induced by YF17D vaccination is generated by direct infection of DCs, we exposed both immature and mature human monocyte-derived DCs with characteristic surface phenotype to YF17D virus. FACS analysis for intracellular nonstructural protein NS1 showed robust expression in both immature DCs (iDCs) and mature DCs (mDCs) infected at 2 or 20 PFU/cell (Fig. A1.1A). Detection of nonstructural viral proteins not present within the virion indicates productive infection. Infection was confirmed using an antibody to nonstructural proteins NS4AB (Fig. A1.1B). In addition, virus production in both the iDC and mDC cultures was monitored by plaque assay. Results showed a 3-log increase in infectious particles within 24 hrs that peaks 48 hrs post-infection confirming productive infection (Fig. A1.1C). No significant difference in infectious particle production was seen between iDC and mDC cultures. To exclude antibody-mediated enhancement of YF17D DC infection, DCs were generated in plasma from non-immunized donors and in control experiments, blocking antibodies against the Fc-receptor (FcR) did not block YF17D infection (data not shown). These data demonstrate that YF17D can infect and productively replicate in human DCs, and infection occurs irrespective of their maturation state.

Other cell types were not as susceptible to YF infection. High-level virus production was not seen in T cell, B cell or monocyte enriched PBMC (Fig. A1.1D). In addition, infection of B cell lines (MC116, RAMOS, and Raji) did not result in productive infection (data not shown).

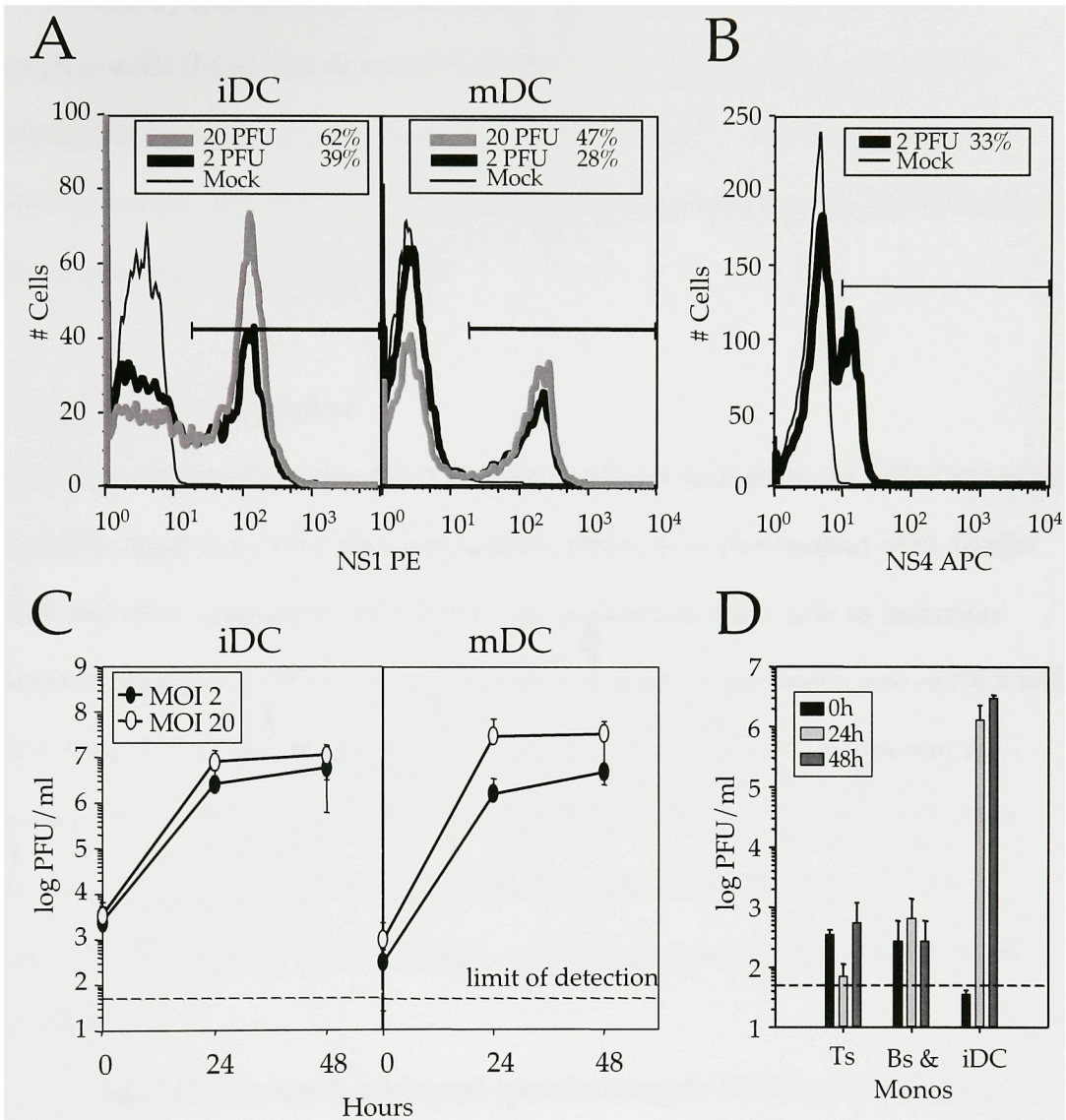

Figure A1.1. Immature and mature DCs are productively infected by YF17D. A. Intracellular FACS analysis of NS1 expression in iDCs and mDCs infected with 2 or 20 PFU/cell 48 hrs post-infection. **B.** Intracellular staining of NS4 expression in iDC infected with 2 PFU/cell. **C.** Virus production by iDC and mDC cultures measured by plaque assay on SW13. **D.** Virus production by T cell enriched and monocyte enriched PBMC fractions compared with iDCs.

In order to test if resident skin DCs exposed to YF during natural transmission by mosquito or subcutaneous vaccination are infected by YF, langerhan cells (LCs) and dermal DCs (DDCs) were generated from CD34+ hematopoetic progenitor cells and exposed to YF17D[275]. FACS analysis for NS4AB revealed that YF17D exhibited a significant preference for CD34+ derived DDCs compared to LCs (Fig. A1.2).

DC infection is Ca^{2+} dependent

The cellular receptor involved in YF17D infection is unknown. In order to assess a possible requirement for divalent cations, iDCs were pre-treated with 10mM EDTA and then exposed to YF17D for 1 hr to evaluate their role in infection. NS4AB expression at 24 hr was completely blocked by pre-treatment of DCs with EDTA (Fig. A1.3A) whereas EDTA pre-treated DCs were still competent for infection with influenza (Fig. A1.3B). EGTA treatment also blocked infection indicating a Ca^{2+}-dependent mechanism (Fig. A1.3A). Consistent with this finding, addition of Ca^{2+}, but not Mg^{2+}, was able to overcome the EDTA block (Fig. A1.3A).

In light of this result, we tested specific integrin blocking antibodies including β_1, β_3, $\alpha_v\beta_3$, β_5, $\alpha_v\beta_5$, $\alpha_v\beta_6$ as well as RGD peptides to evaluate the role of specific integrins in viral entry. Antibodies and peptides were used at concentrations reported to be sufficient for blocking West Nile virus [98]. None of the integrin blocking antibodies significantly inhibited infection as monitored by NS4AB expression 24 hrs post-infection (Fig. A1.3C and data not shown). The lack of inhibition by RGD peptides is consistent with previous reports suggesting YF17D infection is independent from the RGD motif [98,276].

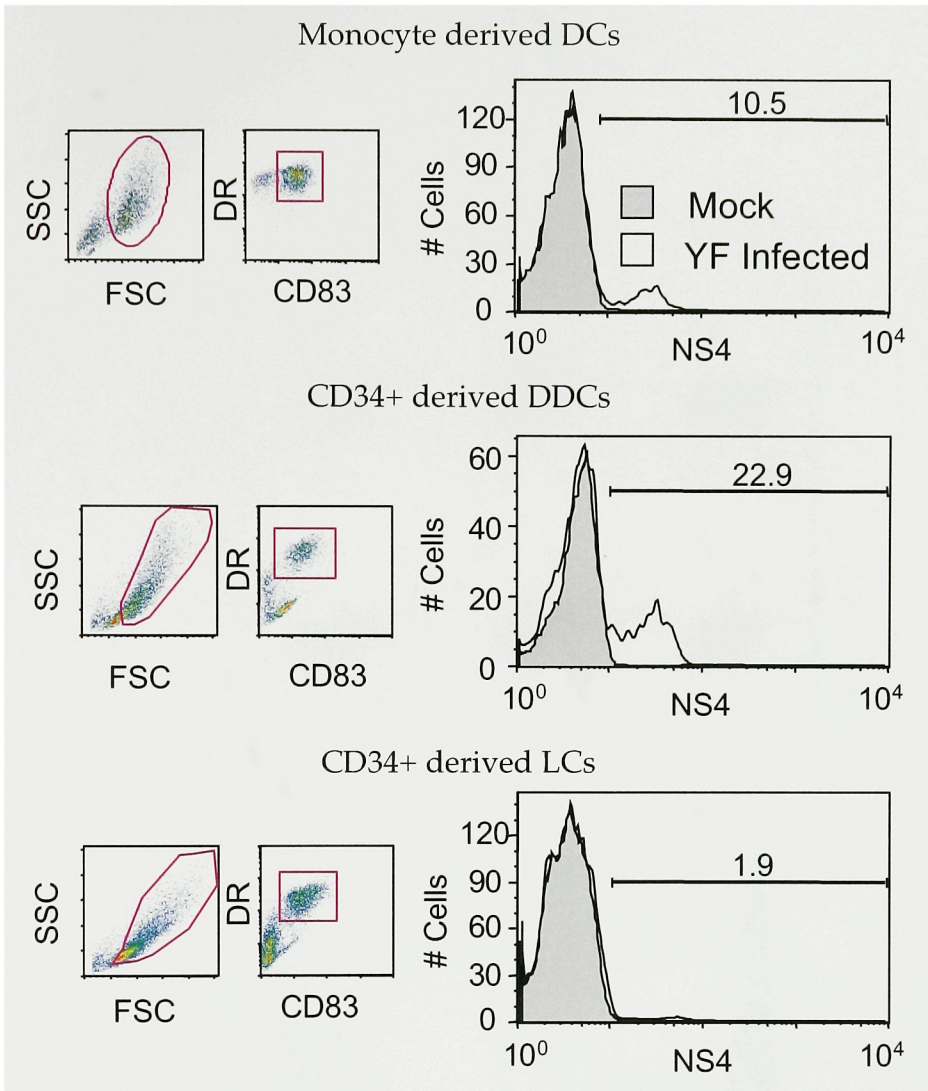

Figure A1.2. Dermal DCs but not LCs are infected by YF17D. Monocyte derived DCs, CD34+ derived DDCs and LCs were exposed to 2 PFU/cell YF17D and stained for FACS analysis after 48 hrs with maturation stimuli. Cells were gated based on side scatter (SSC) and forward scatter (FSC) as well as HLA-DR and CD83 positive staining. NS4A staining in this gated population is indicated by histogram on the right and the number above the gate indicates the percent of cells that stain positive for NS4.

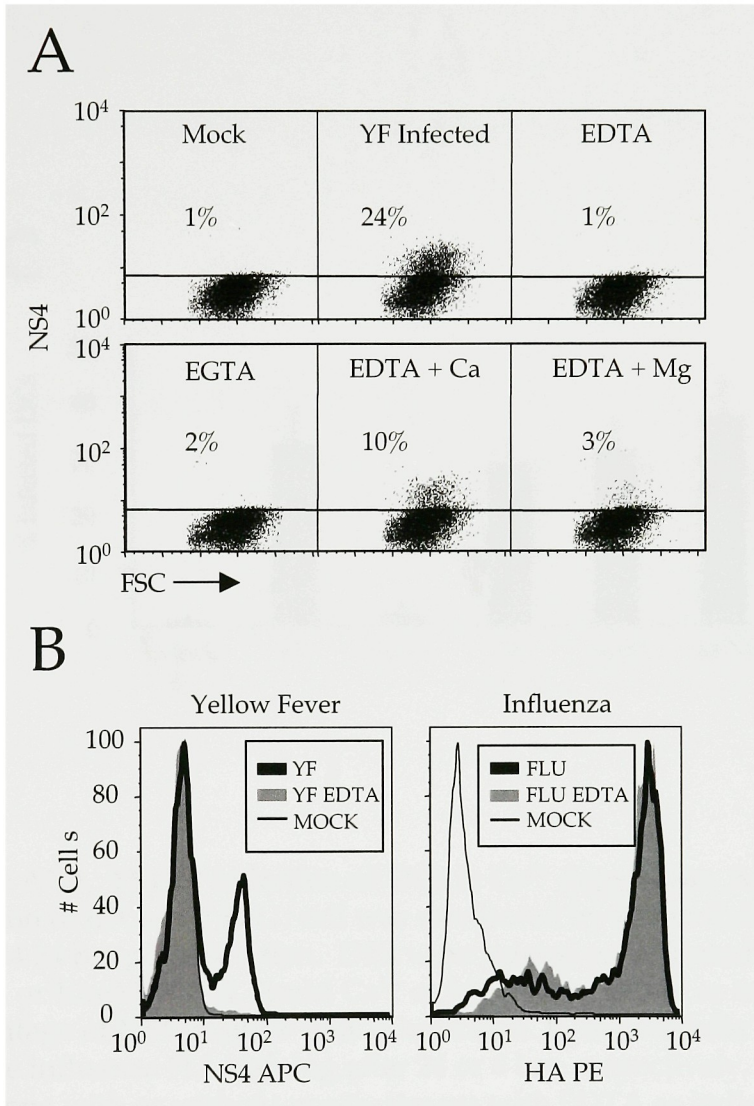

Figure A1.3. YF17D Infection of DCs is Ca^{2+} dependent. (Continued on the following page.)

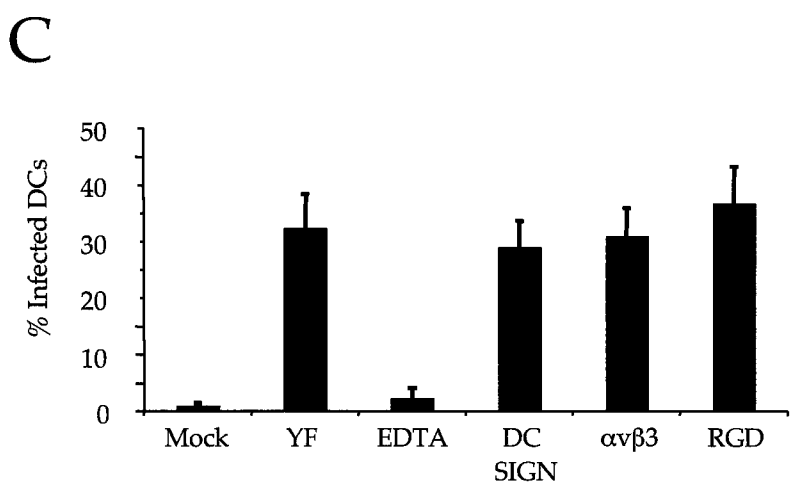

Figure A1.3. YF17D Infection of DCs is Ca^{2+} dependent. A. YF17D infection of iDCs at 2 PFU/cell was monitored by intracellular FACS for YF NS4 24 hrs post-infection. DCs were treated as indicated with 10mM EDTA or 10mM EGTA. 6mM Ca^{2+} or Mg^{2+} were added as indicated. Percentage of NS4 positive cells is indicated. **B.** Intracellular YF NS4 or surface influenza HA staining after 24 or 8 hrs, respectively. Infection was done with or without pre-treatment with EDTA as indicated. **C.** Bar graph represents percent of iDCs staining positive for YF17D NS4 24 hrs post-infection. Samples pre-incubated with 10μg/ml of DC-SIGN or $\alpha_v\beta_3$ blocking antibodies or 100μg/mL of RGD peptides are indicated.

In addition, blocking antibodies against DC-SIGN [93] did not inhibit YF17D infection (Fig. A1.3C). This finding is consistent with our data showing equivalent virus production in iDCs and mDCs which significantly differ in DC-SIGN expression (high in iDC, low in mDC) (Fig. A1.1C) as well as a recent report showing the inability of DC-SIGN to permit infection in THP-1 cells [92]. Although YF17D infection of DCs is Ca^{2+} dependent, the receptor for YF17D remains elusive.

YF17D infection does not alter DC maturation in vitro

Several reports indicate that viral infection of DCs promotes or inhibits maturation [84-86]. In order to evaluate DC maturation as a result of YF17D infection, surface expression levels of CD83, CD86, and MHCII were assayed 36 hrs post-infection. Interestingly, infection alone did not induce robust DC maturation. Although slight upregulation of CD86 and MHCII was observed (data not shown), conventional maturation markers such as CD83 were not expressed (Fig. A1.4A). However, in contrast to inhibition of maturation by several infectious pathogens [85,86], YF17D infection did not inhibit TNFα-mediated DC maturation (Fig. A1.4B).

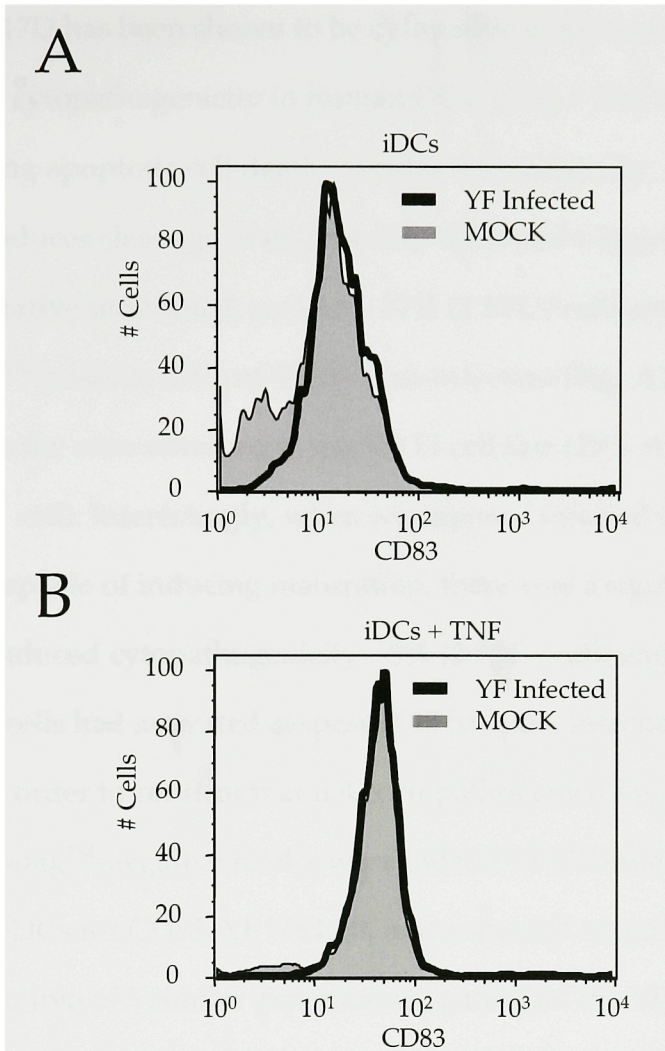

Figure A1.4 YF17D infection does not induce or inhibit DC maturation. A. iDCs infected with 10 PFU/cell were analyzed for CD83 surface expression. CD83 expression of YF17D infected population (compared to mock infected) was analyzed 36 hrs post-infection. **B.** iDCs were infected with 10 PFU/cell and exposed to TNFα / PGE-2 maturation cocktail. CD83 expression of YF-infected population (compared to mock infected) was analyzed after 36 hrs.

Maturation protects DCs from YF17D-induced apoptosis

Since YF17D has been shown to be cytopathic in mammalian cells, we evaluated potential cytopathogenicity in human DCs using a FACS-based assay for monitoring apoptotic cell death. Similar to infection by several other viruses, YF17D induces cleavage of caspase-3 in iDCs and triggers cell death. In a representative individual, we show 37% (2 PFU/cell) and 54% (10 PFU/cell) caspatag™-positive iDCs at 48 hrs post-infection (Fig. A1.5A). These values were similar to the ones obtained in the SW13 cell line (29% at 2 PFU / cell and 69% at 10 PFU / cell). Interestingly, when we exposed infected iDCs to inflammatory stimuli capable of inducing maturation, there was a significant reduction in the YF17D-induced cytopathogenicity—9% (2 PFU/cell) and 13% (10 PFU/ cell) of the total cells had activated caspase-3 48 hrs post-infection (Fig. A1.5A).

In order to confirm that iDC cytopathogenicity was YF17D-specific and that caspatag™-negative mDCs were indeed YF17D-infected, we stained the infected iDCs for CD83, YF NS4AB, and activated caspase-3. In a data set representative of 3 similar experiments, gating on the YF-infected $CD83^-$ cells showed 21% caspase activation compared to 6% in the YF17D-negative population (Fig. A1.5B). In contrast, mature, YF17D-infected DCs ($CD83^+$ / $NS4^+$) showed only 3% caspase activation (Fig. A1.5B). Plaque assays performed on the supernatants from both cultures confirmed comparable levels of virus production (data not shown). Based on this data, we conclude that the resistance to YF17D-induced cytopathogenicity is dependent on DC maturation.

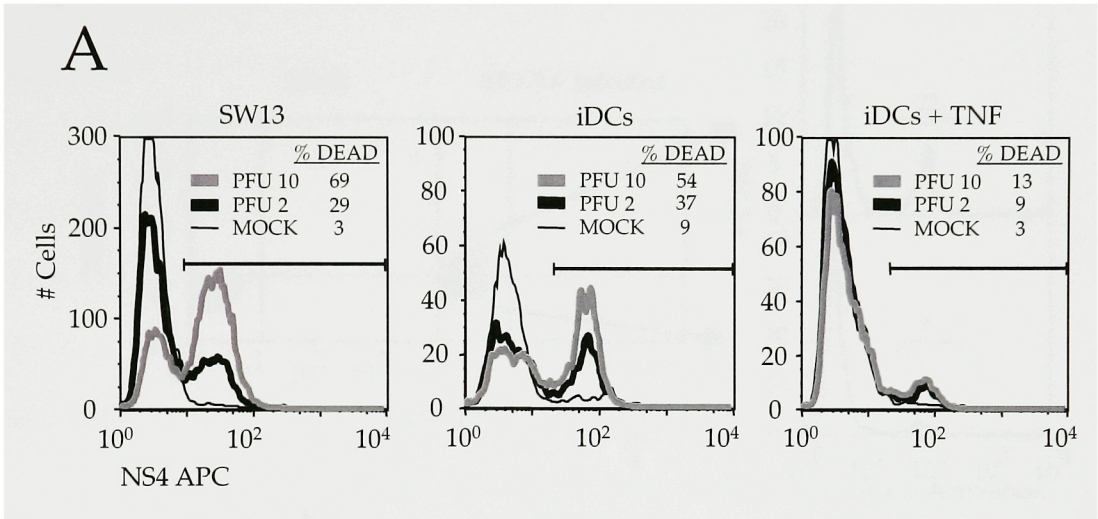

Figure A1.5. Maturation protects iDCs from YF cytopathogenicity.
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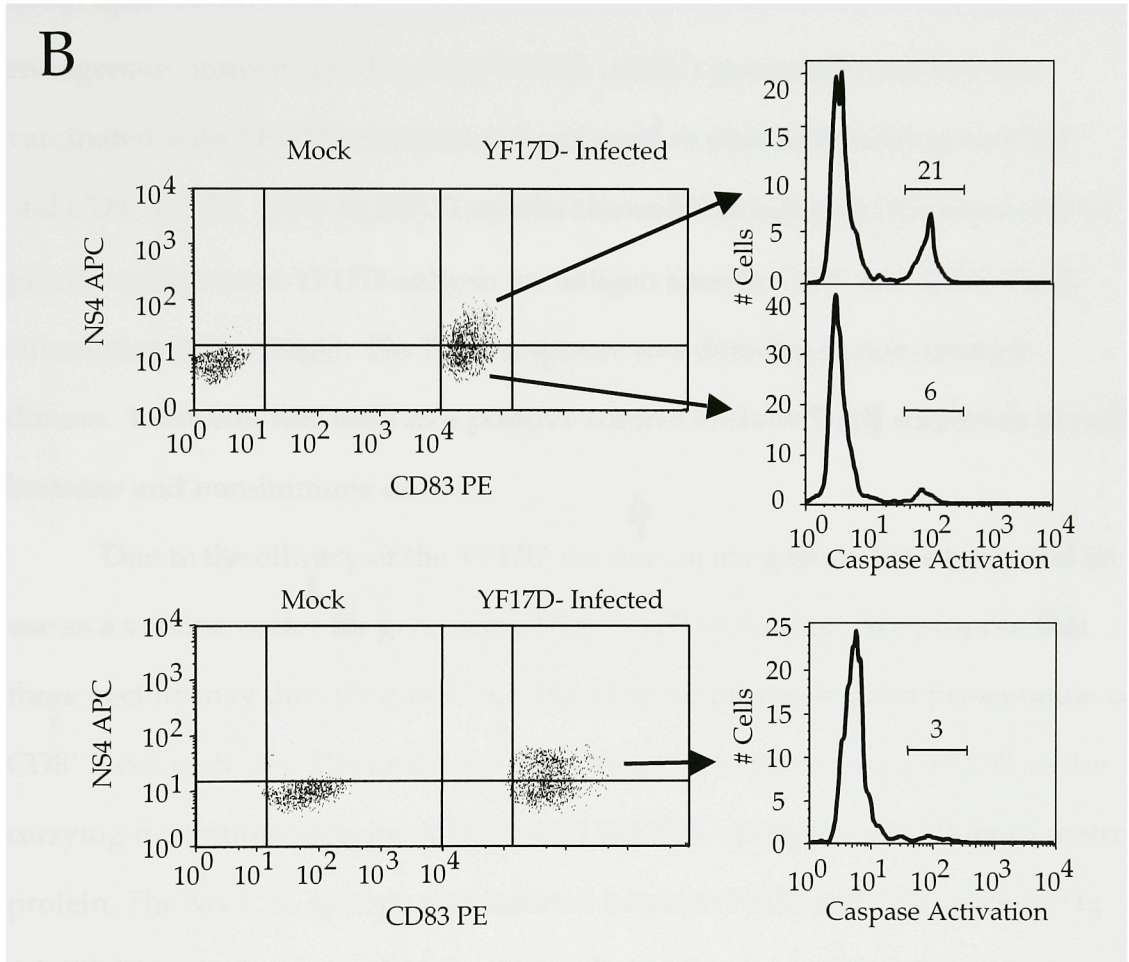


Figure A1.5. Maturation protects iDCs from YF cytopathogenicity. **A.** Caspase activation is measured using Caspatag™-FITC in SW13, iDCs, or iDCs + TNFα, exposed to the indicated PFU of YF17D virus at 48 hrs. Tables indicate the percent of cells that were caspatag™ positive. **B.** YF17D infected iDCs or iDCs + TNFα were identified by intracellular staining with NS4. CD83 staining was used to distinguish iDC vs. mDC. Caspase activation of the indicated cell populations are indicated by histogram and the percent Caspatag™ positive indicated.

Infection of DCs allows for processing and presentation of endogenous and model antigens

In light of the direct infection of DCs and the resistance to YF17D-induced cell death upon maturation, we evaluated the ability of mDCs to process and present endogenous antigens produced by YF17D. mDCs generated from subjects vaccinated with YF17D were infected and used to stimulate autologous $CD8^+$ and $CD4^+$ T cells. IFNγ ELISPOT results showed that infected DCs were able to process and present YF17D antigen for antigen specific $CD8^+$ and $CD4^+$ T cell stimulation (Fig. A1.6A). No T cell response was detected in non-immune donors. Influenza was used as a positive control to show T cell responses in both immune and non-immune donors.

Due to the efficacy of the YF17D vaccine, many groups have proposed its use as a vaccine vector for generating $CD8^+$ T cell immunity. We propose that these vectors may directly infect DCs allowing for processing and presentation of $CD8^+$ T cell epitopes. We tested this hypothesis by constructing a YF17D vector carrying the immunodominant HLA-A2.1 M1 CTL epitope from influenza matrix protein. The M1 CTL epitope was inserted between NS2B and NS3 protein (Fig. A1.6B). This site has been shown previously to tolerate small insertion of foreign sequences [266] mDCs from naive donors were infected with YF17D or chimeric YF17D-M1 virus at 2 PFU/cell as described above and used to stimulate autologous $CD8^+$ T cells. IFNγ ELISPOT results showed that YF17D-M1 infected DCs stimulate robust M1-specific $CD8^+$ T cell responses similar to M1-peptide pulsed or influenza-infected DCs (Fig. A1.6C) while DCs infected with YF17D alone did not stimulate IFNγ secretion in the non-immune donor. Importantly,

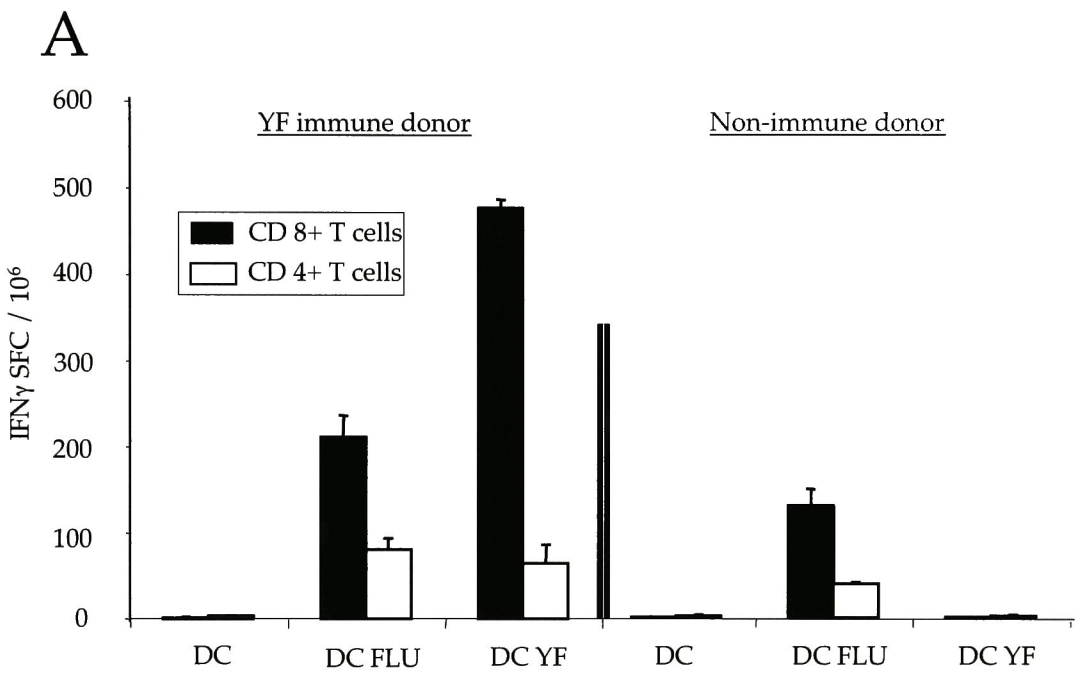


Figure A1.6. YF17D infection of DCs allows for processing and presentation of endogenous and model T cell epitopes. (Continued on the following page.)

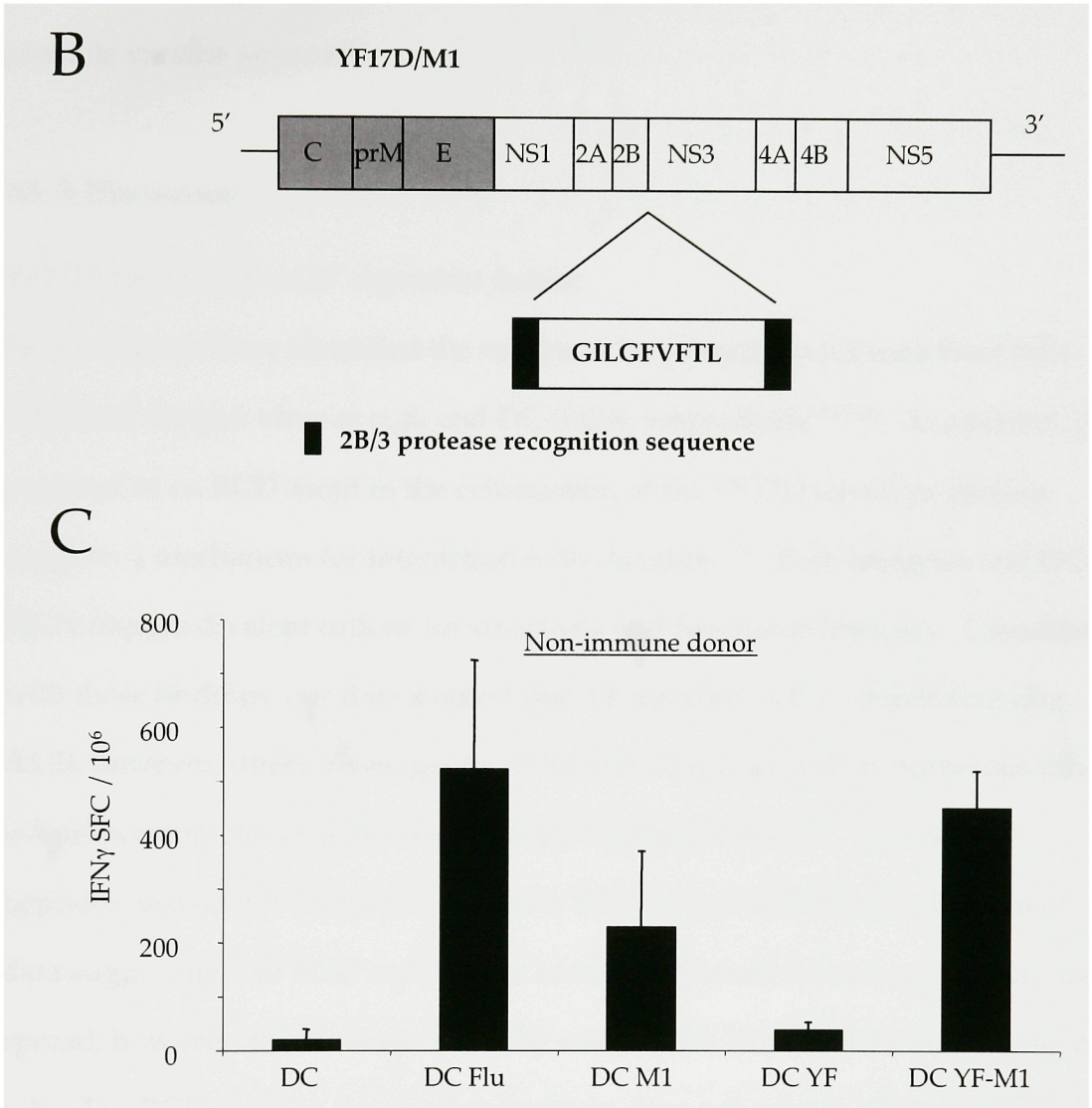


Figure A1.6. YF17D infection of DCs allows for processing and presentation of endogenous and model T cell epitopes. A. DCs infected with influenza or YF17D were used to stimulate purified $CD8^+$ T cells from YF17D immunized donor 1 or a naive donor. Stimulation was measured using IFNγ ELISPOT and data are shown as spot forming cells (SFC)/10^6. **B.** Schematic showing the insertion site of the M1 eptiope at 2B-3 junction. **C.** DCs infected with influenza, YF17D, YF17D-M1, or pulsed with 10μM M1 peptide were used to stimulate $CD8^+$ T cells from a naive donor. T cell stimulation was measured by IFNγ ELISPOT, as mentioned above.

this is the first demonstration of a YF17D 2B/3 chimeric virus presenting an HLA-A2.1 CTL epitope for T cell stimulation, offering proof of principle for a possible vaccine approach.

A1. 5 Discussion

YF17D infects DCs in Ca^{2+} dependent fashion

Recent reports have identified the receptors for related flaviviruses West Nile virus and dengue virus as $\alpha_v\beta_3$ and DC-SIGN, respectively [92,93,98]. In addition, the presence of an RGD motif in the ectodomain of the YF17D envelope protein suggests a mechanism for interaction with integrins [276]. Both integrins and DC-SIGN require divalent cations for structural and functional integrity. Consistent with these findings, our data suggest that YF infection is Ca^{2+}-dependent (Fig. A1.3); however, direct investigation of the role of $\alpha_v\beta_3$ as well as numerous other integrins using blocking antibodies failed to inhibit infection. Cyclo-RGD peptides also do not competitively block YF infection which is consistent with data suggesting that RGD motif in the envelope domain is not required for viral spread; however, this does not formally rule out the RGD motif from playing a role. The RGD motif in the envelope protein does not exist in YF Asibi[262,276]—this motif was acquired as a result of mutation during passage in chicken embryos. The characterization of YF Asibi interaction with DCs may offer insight into the role of this motif and integrins in DC infection.

Furthermore, our results, as well as other's[92], suggest that, in contrast to dengue, YF17D infection of DCs is not mediated by DC-SIGN. One explanation for this difference may be the differential glycosylation of the envelope proteins.

In contrast to dengue virus envelope protein which contains one or two N-linked glycans, the envelope protein of YF17D-204 used in these experiments is not glycosylated [277]. Although other YF17D substrains (YF17DD and YF17D-213) may be glycosylated, our data indicate that DC-SIGN interaction is not necessary for infection and that YF17D interacts with DCs via mechanisms distinct from related flaviviruses.

DC maturation prevents YF-induced cytopathogenicity

Many viruses have been suggested to play an active role in immune evasion via inhibition of DC maturation. Our data, however, suggest that YF17D does not inhibit maturation (Fig. A1.4). These results suggest therefore, that in the context of YF17D vaccination, release of inflammatory cytokines in peripheral tissue may play an important role in triggering DC maturation; this will allow for DC migration to the lymph node and subsequent T cell engagement and priming (Fig. A1.7). Consistent with this model, an increase in TNFα has been detected in subjects vaccinated with YF17D [278].

The ability to undergo functional maturation plays an important role in preventing YF-induced cytopathogenicity. This resistance to cytopathogenicity could play an important role in allowing infected DCs to remain alive for trafficking to lymph node and priming of T cell responses. It will be interesting to evaluate the effect of pathogenic YF Asibi on DC maturation and cytophathogenicity.

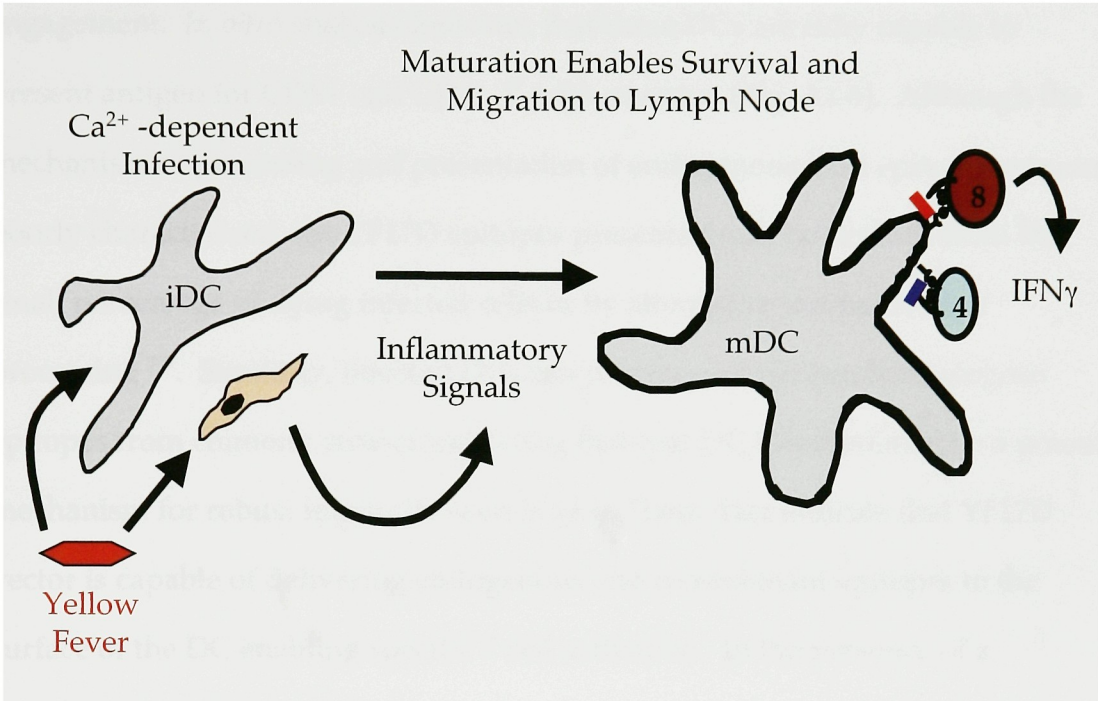


Figure A1.7. Schematic for DC/YF interaction in generating T cell immunity. In the periphery, iDCs may interact with various. Calcium-dependent infection of DCs does not itself mature the DCs; however, infection does not block normal maturation by other inflammatory signals. Maturation prevents the cytopathic effects of YF, enabling migration to the lymph node and priming of T cells.

Infected mature DCs present antigen for T cell activation

The ability of maturation to protect DCs infected in the periphery from apoptosis enables antigen loaded DCs to traffic to lymph nodes for T cell engagement. *In vitro* analysis indicates that these DCs are fully capable to present antigen for CD8+ and CD4+ T cell activation (Fig. A1.6). Although the mechanism of processing and presentation of endogenous CD4 epitopes remains poorly characterized, the YF17D epitopes presented may be derived from the small percentage of dying infected cells or by alternative mechanisms of processing [279]. Similarly, infected DCs can process and present heterologous epitopes from chimeric viruses indicating that that DC infection may be a potent mechanism for robust immunity seen *in vivo*. These data indicate that YF17D vector is capable of delivering endogenous and recombinant epitopes to the surface of the DC enabling specific T cell activation. In the presence of a maturation stimulus produced in infected tissue, we therefore propose that Ca^{2+}-dependent direct infection of human DCs may offer a mechanism for the robust and long-lasting immunity associated with the YF17D vaccine.

More questions remain for YF

It is surprising that even at after 100 years since the discovery of YF, the mechanisms associated with its pathogenesis and immunity are not well understood. In light of the interactions of other flaviviruses with DCs, we designed these experiments to explore the interaction of YF17D with DCs. Although infection of DCs is robust, selective, and Ca^{2+}-dependent, the receptor(s) affording these characteristics remain elusive. A better

understanding of the surface proteins involved in this infection may identify target molecules on DCs for vaccine delivery. Interestingly, the majority of amino acid changes between YF17D and pathogenic YF Asibi exist in the envelope protein[262]. Thus, a better understanding of the molecules involved in infection may offer insight into tropism differences between YF17D and Asibi that result in immunity *versus* disease.

Mouse models are of limited significance since mice are not a true host for YF. As such, it is crucial to evaluate these questions in human models. Of particular note, is the nature of the infection of YF in the skin (during natural transmission by the mosquito) as well as in the subcutaneous tissue (during immunization). While we have made an attempt to evaluate the infectivity of DDCs and LCs using CD34+ derived cells (Fig. A1.2), these experiments need to be performed in skin *ex vivo* to understand YF interaction with resident skin DCs in both immunity and disease.

Finally, the efficacy of YF as a vaccine vector needs further clarification. Although we have shown proof of principle that human DCs can process and present the minimal M1 epitope inserted into the the NS2B/3 site, the components of this process have yet to be defined. The role of the viral protease, host proteasome, length of flanking regions, and site of insertion in mediating efficient presentation by DCs will offer tremendous insight into vaccine vector design.

Appendix 2: The DC database

As mentioned in chapter 3, transcriptional changes associated with maturation and IFNα/β treatment were monitored by affymetrix analysis using human U133A microarrays. The amount data obtained in these experiments is enormous and we are still only beginning to harness this resource. In order to manage such a large amount of data, I adapted a database provide by our collaborator Dr. Nir Hacohen at MIT to facilitate searching and navigation of the data. Our initial work with the database has offered us important insight into DC's regulation of IFNα/β signaling (Chapter 3) as well as novel regulation of indoleamine 2,3-deoxygenase activity in DCs[280].

Preparing the database

Biotinylated cRNA for hybridization was prepared as per Affymetrix Manual (see Chapter 2, Materials and Methods) and loaded onto the U133A microarray. This array analyzes expression of 18,400 transcripts and variants, 14,000 of which are well characterized genes. Each array is chemically loaded with over 22,000 probe sets. Each probe is a 25-mer chosen to allow selectivity for the gene of interest. For each gene probe a 'mismatch probe' exist with a single base pair mismatch in the middle of the 25-mer. This allows for a quantitative index of signal to noise. MAS5.0 analysis software calculates the *p*-value for the intensity signal of the specific probe *versus* that of the mismatch probe. $p<0.05$ is used to determine if the gene expression is present (P) above the noise or absent (A)

below the level of detection. Notably, background depends on the specific probe so the absolute level of signal intensity only indicates relative expression.

The data generated following MAS5.0 analysis was exported to Excel and the comparisons of interest were created. For example, comparison of mDC signal to iDC signal intensity was generated for the whole list of genes. Both forward (mDC / iDC) and reverse (iDC / mDC) ratios were created to facilitate searching. These data were then imported into Filemaker pro according to the specific donor preparation. Data from individual donors were linked for multiple donor comparisons using the Affymetrix ID numbers. A master template was created with search criteria for various comparisons used in our analysis (Fig. A2.1). Searches can be performed based on gene name/symbols, comparison ratios (using >, <, or = functions), absolute value (using >, <, or = functions), and/or absent/present calls (using "a" or "p," respectively). Another template was created to allow for quick scanning of search results (Fig. A2.2). This list displays gene symbol, genename, and absolute signal values. Of note, not all the probes have been associated with defined genes and may therefore appear without a gene name. As the gene names become available, the database can be update with new lists linked to the Affy ID.

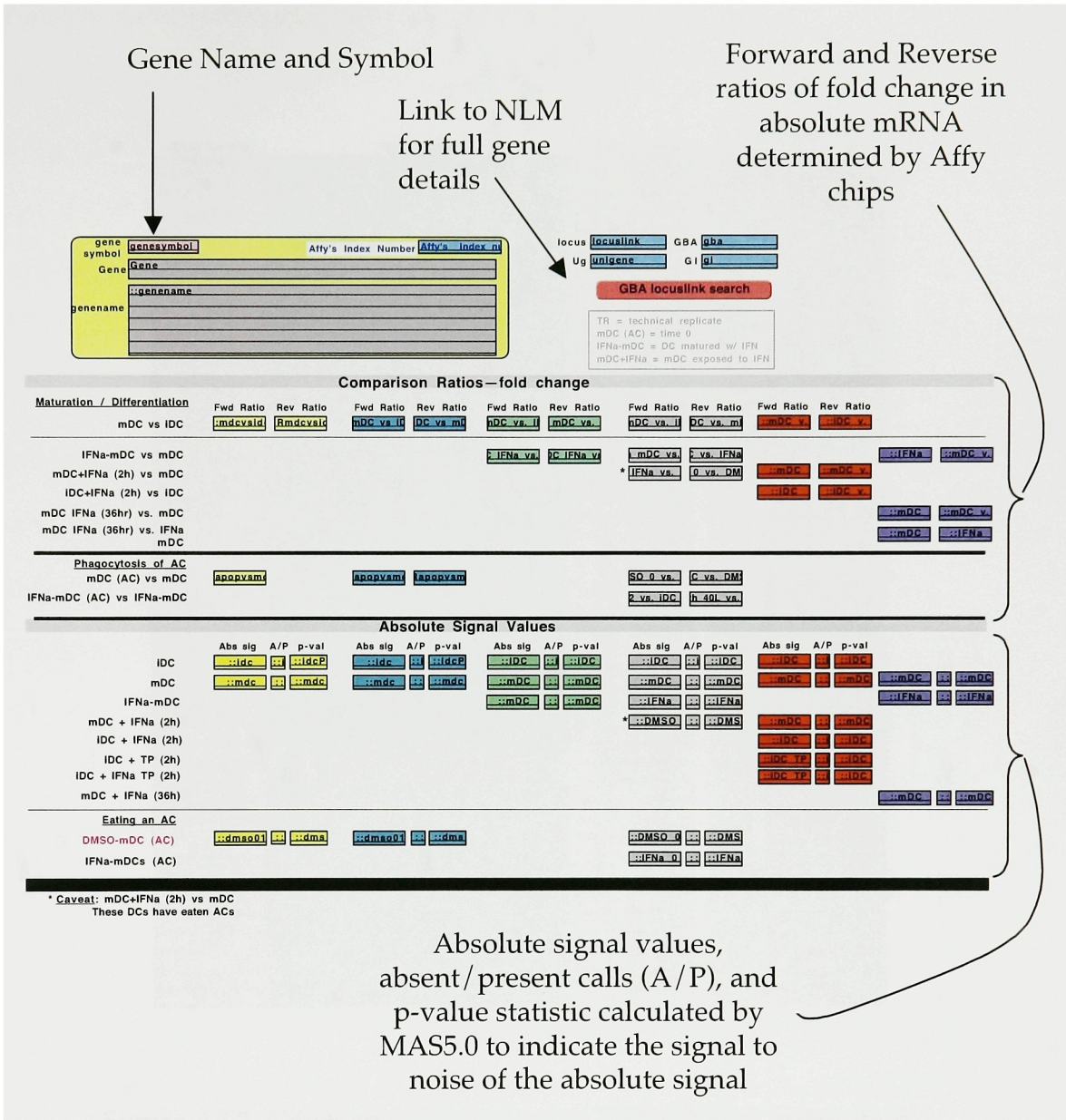
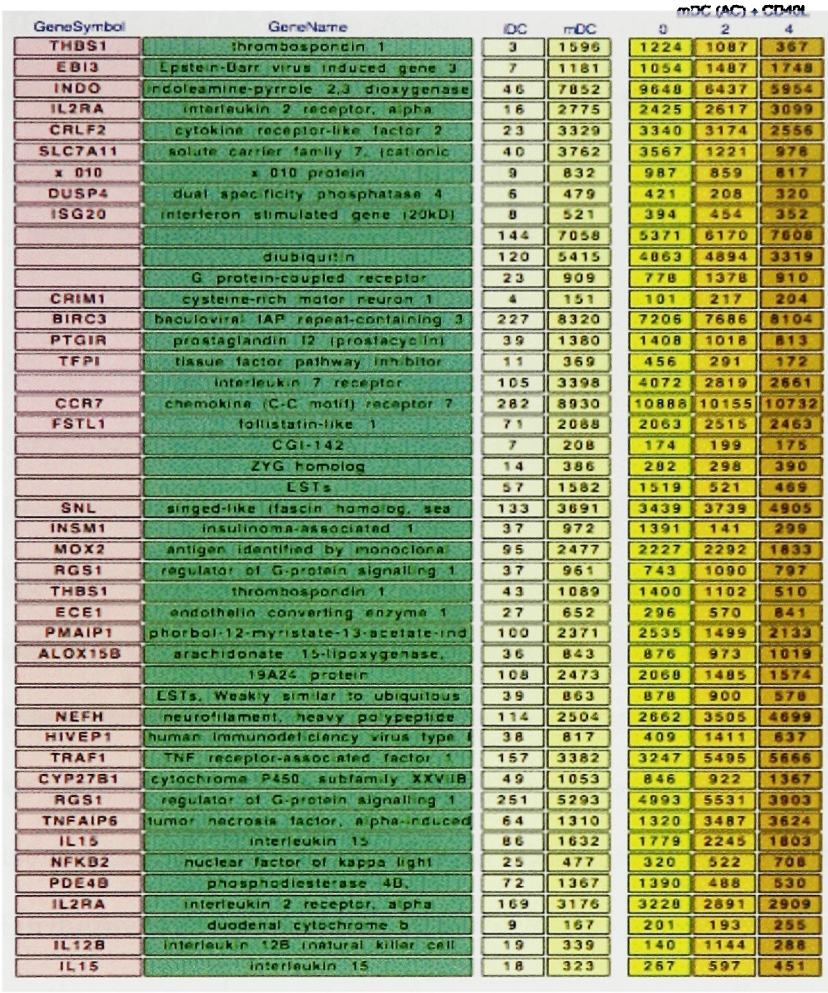


Figure A2.1. DC Database. Affymetrix data from individual donors (indicated by color). The top panel indicates gene symbol, name, and Affy ID. Searches may be performed for any of the fields. The middle panel shows comparison ratios of samples indicated in the left hand column. Both forward and reverse ratios are shown. Searches can be performed using >, <, or = functions in these fields. The bottom panel shows absolute signal value, A/P calls, and p-value calculated by MAS5.0 software of the samples in the left hand column. Absolute signal can be searched with >, < or = functions and A/P call with text A or P.



GeneSymbol	GeneName	iDC	mDC	mDC (AC) + CD40L 0	2	4
THBS1	thrombospondin 1	3	1596	1224	1087	367
EBI3	Epstein-Barr virus induced gene 3	7	1181	1054	1487	1748
INDO	indoleamine-pyrrole 2,3 dioxygenase	46	7852	9648	6437	5954
IL2RA	interleukin 2 receptor, alpha	16	2775	2425	2617	3099
CRLF2	cytokine receptor-like factor 2	23	3329	3340	3174	2556
SLC7A11	solute carrier family 7, (cationic	40	3762	3567	1221	976
x 010	x 010 protein	9	832	987	859	817
DUSP4	dual specificity phosphatase 4	6	479	421	208	320
ISG20	interferon stimulated gene (20kD)	8	521	394	454	352
		144	7058	5371	6170	7608
	diubiquitin	120	5415	4863	4894	3319
	G protein-coupled receptor	23	909	778	1378	910
CRIM1	cysteine-rich motor neuron 1	4	151	101	217	204
BIRC3	baculoviral IAP repeat-containing 3	227	8320	7206	7686	8104
PTGIR	prostaglandin I2 (prostacyclin)	39	1380	1408	1018	813
TFPI	tissue factor pathway inhibitor	11	369	456	291	172
	interleukin 7 receptor	105	3398	4072	2819	2661
CCR7	chemokine (C-C motif) receptor 7	282	8930	10888	10155	10732
FSTL1	follistatin-like 1	71	2088	2063	2515	2463
	CGI-142	7	208	174	199	175
	ZYG homolog	14	386	282	298	390
	ESTs	57	1582	1519	521	469
SNL	singed-like (fascin homolog, sea	133	3691	3439	3739	4905
INSM1	insulinoma-associated 1	37	972	1391	141	299
MOX2	antigen identified by monoclona	95	2477	2227	2292	1833
RGS1	regulator of G-protein signalling 1	37	961	743	1090	797
THBS1	thrombospondin 1	43	1089	1400	1102	510
ECE1	endothelin converting enzyme 1	27	652	296	570	841
PMAIP1	phorbol-12-myristate-13-acetate-ind	100	2371	2535	1499	2133
ALOX15B	arachidonate 15-lipoxygenase,	36	843	876	973	1019
	19A24 protein	108	2473	2068	1485	1574
	ESTs, Weakly similar to ubiquitous	39	863	878	900	578
NEFH	neurofilament, heavy polypeptide	114	2504	2662	3505	4699
HIVEP1	human immunodeficiency virus type I	38	817	409	1411	837
TRAF1	TNF receptor-associated factor 1	157	3382	3247	5495	5666
CYP27B1	cytochrome P450, subfamily XXVIB	49	1053	846	922	1367
RGS1	regulator of G-protein signalling 1	251	5293	4993	5531	3903
TNFAIP6	tumor necrosis factor, alpha-induced	64	1310	1320	3487	3624
IL15	interleukin 15	86	1632	1779	2245	1803
NFKB2	nuclear factor of kappa light	25	477	320	522	708
PDE4B	phosphodiesterase 4B,	72	1367	1390	488	530
IL2RA	interleukin 2 receptor, alpha	169	3176	3228	2891	2909
	duodenal cytochrome b	9	167	201	193	255
IL12B	interleukin 12B (natural killer cell	19	339	140	1144	288
IL15	interleukin 15	18	323	267	597	451

Figure A2.2. Genes upregulated upon TNFα/PGE-2 maturation. The top 45 genes with the highest comparison ratios of mDC / iDC over multiple runs are displayed in a table in filemaker database. This arrangement allow quick scanning of gene names (pink and green), absolute signal for iDC (beige) and mDC (tan), and gene regulation during CD40L engagement (orange).

Transcriptional Profiles of iDCs verus mDCs: the gift that keeps giving

One important analysis performed is the set of genes regulated during DC maturation (Fig. A2.2 and A2.3). The majority of genes do not change by greater than 2-fold during maturation providing an important internal control. However, a significant number of genes are either up or down regulated during maturation. Many of these transcriptional changes are consistent with known phenotypic and functional changes associated with maturation (Fig. A2.3). For example, iDCs express high levels of TLRs and antigen capture receptors to allow them to respond to PAMPs and capture antigen for initiating an immune response. Upon maturation, chemokine receptor (CCR) 7 is upregulated allowing migration to the lymph node. mDCs also upregulate important cytokines such as IL-12 and characteristic maturation marker such as CD83.

The transcriptional profiles associated with maturation have been very important for our analysis. These data were mined extensively to provide a candidate list of genes responsible for the dual effects of IFNα/β described in chapter 3. Furthermore, we have made an effort to characterize the role of several interesting genes whose role in DC maturation is not well understood. Deborah Braun, a post-doc in Matthew Albert's lab, tackled the question of indoleamine 2,3-dioxygenase (IDO) upregulation in mDCs. IDO is an enzyme involved in tryptophan catabolism and is implicated in T cell tolerance[281]. Her work showed that while PGE-2 induces the expression of IDO mRNA, a second signal by TNF-R or TLR engagement is required to induce IDO activity (Fig. A2.4)[280].

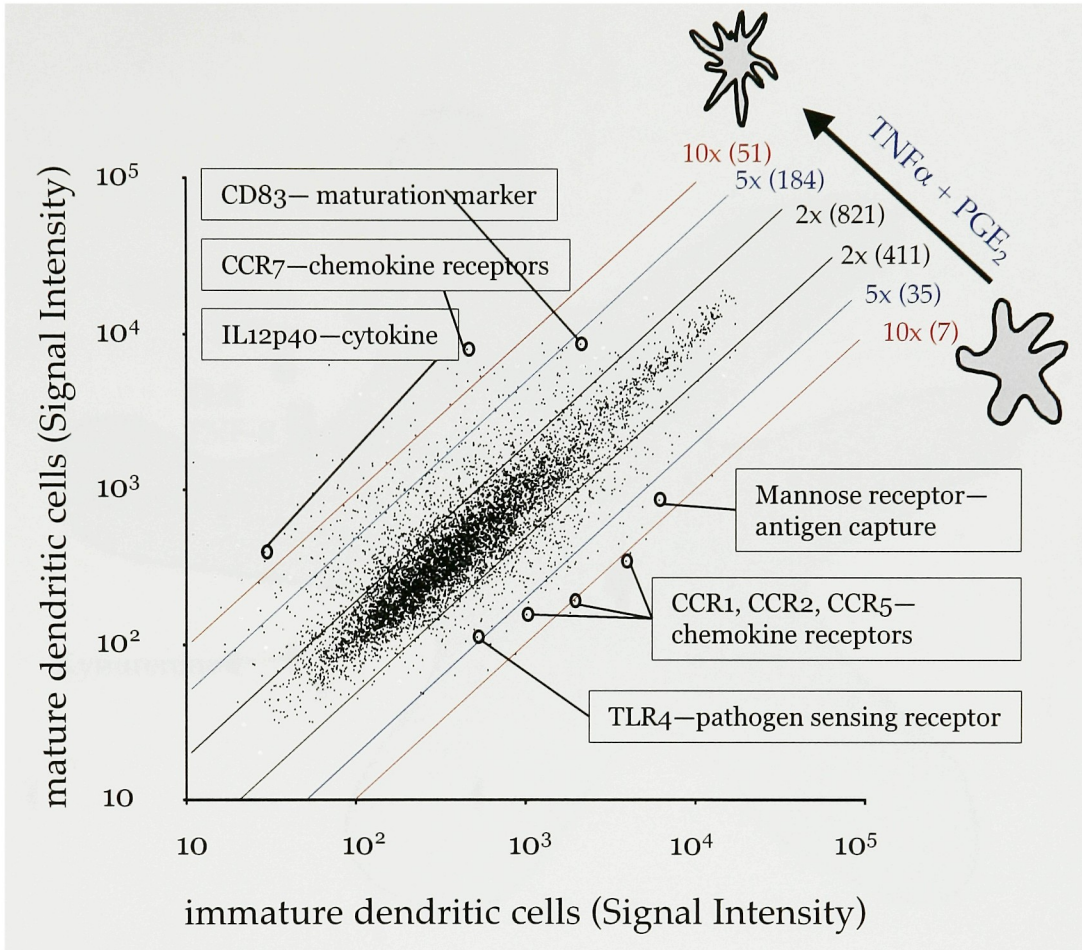


Figure A2.3. Genetic Signature of immature and mature DCs. Absolute signal intensities of iDCs *versus* mDCs are displayed as a dot plot. Red lines indicate a 10x change, blue lines 5x change, and black lines a 2x change in comparison ratios. The numbers in parentheseses indicate the number of genes associated with the fold change. Specific genes are indicated.

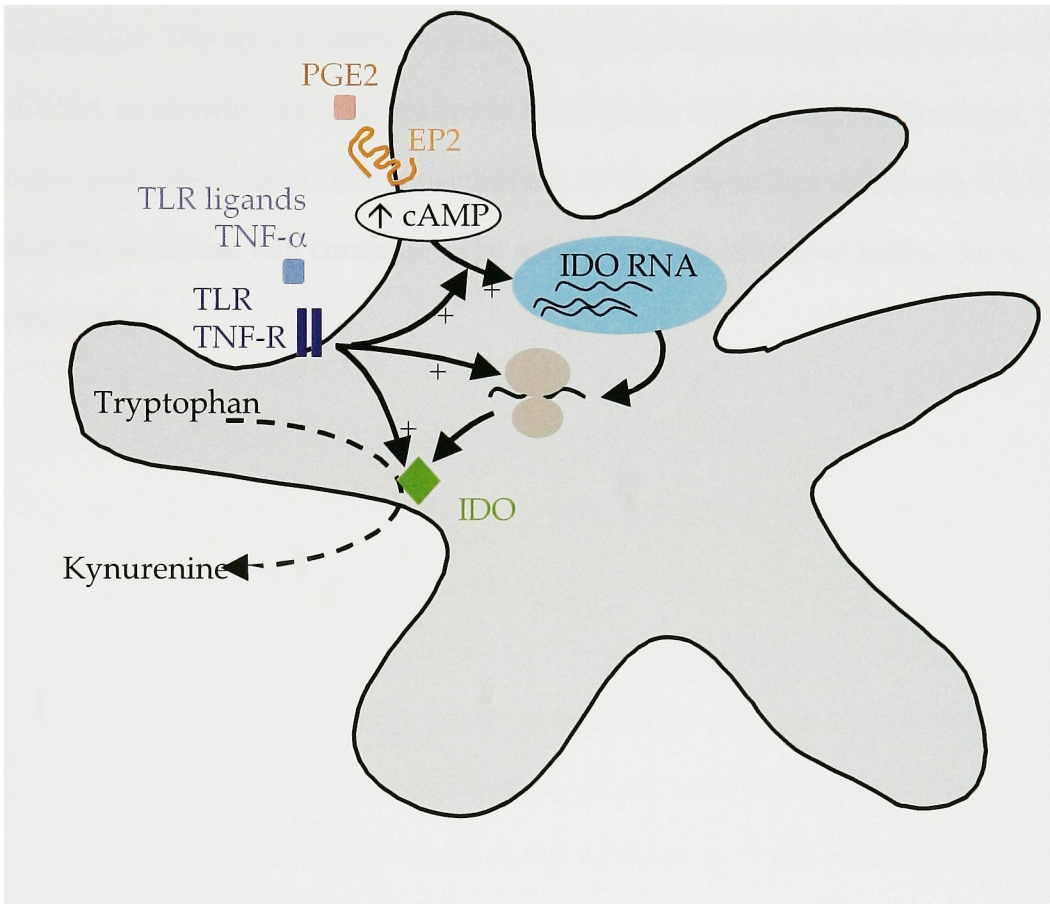


Figure A2.4. Two step regulation of indoleamine 2,3-dioxygenase (IDO) induction during DC maturation. PGE-2 acting through receptor EP-2 activates IDO transcription. However, a second signal from a TLR or TNF-R is required for protein expression and functional IDO. IDO activity was measured by the formation of kynurenine, a metabolic product of tryptophan.

The database allows us to continue to push our functional findings to molecular mechanism in a relatively systematic fashion. For example, several genes have been identified as potential candidates in the regulation of IFNα/β signaling in DCs based on the expression profile and likely interactions with STAT signaling molecules. We are currently evaluating various technologies such as retroviral shRNA to identify the importance of these genes in our functional assays. As we learn more about the interconnectedness of these signaling pathways, I believe that the database will continue to be a very important tool in aiding these discoveries.

Appendix 3: CRI-0505

During my thesis work, I had the unique opportunity to write and participate in a clinical protocol in collaboration with Andy Talal. As I mentioned in chapter 2, our desire to define the function of circulating DCs required larger quantities of PBMCs. As such, we wrote clinical protocol CRI-0505 in order to use leukapheresis to obtain large amounts of PBMCs. This experience was important for me for two reasons.

First, as an MD/PhD student, it gave me my first experience with translational research. This type of research is not easy. The following document shows the preparation that goes into coordinating this type of study. Research on human samples involves numerous concerns and must be extensively reviewed both scientifically and ethically. Once the protocol is approved, technical difficulties exist in physically obtaining samples. Sometimes patients cannot give detailed histories; sometimes you get a smaller sample than you would like; sometimes appropriate controls are not available; and sometimes you get no sample at all. However, for many diseases in which animal models are not available, we must perform the highest quality science with human samples in order to gain insight into human diseases. Despite these difficulties, I was extremely fortunate to have the opportunity to do this work in collaboration with the staff at the Rockefeller University Hospital (RUH). The infrastructure setup at RUH removes a significant amount of administrative and technical problems associated with human research. I am very grateful for the professional and courtesy help of all the staff, nurses, and doctors at RUH.

Secondly, this work served as a constant reminder of why I do this research—to help the people affected by HCV. I was involved in coordinating each leukapheresis and had the opportunity to explain to most of the donors the goals of our research. I am very grateful for their generous contributions.

RESEARCH STUDY SUBMISSION TO THE INSTITUTIONAL REVIEW BOARD OF THE ROCKEFELLER UNIVERSITY

1. **Principal Investigator:** Charles M. Rice, PhD
2. **Investigators Who Will Conduct the Study and Delegation of Authority.**

Charles M. Rice, PhD	**Principal Investigator** 6, 8, 9
Matthew L. Albert, MD, PhD	**co-Principal Investigator** 1, 6, 8, 9
Andrew Talal, MD*	**Assistant Professor, NYH** 1, 2, 3, 4, 5, 6, 7
Stevan Gonzalez, MD*	**Clinical Collaborator, NYH** 1, 6, 7
Ira Jacobson, MD	**Professor of Medicine, NYH** 1, 6
Randy Longman	**Biomedical Fellow** 1, 9

Delegated Tasks Code: 1 = Consent; 2 = Validate Eligibility; 3 = Blood draw at RUH;
4 = Leukapheresis; 5 = Clinical assessment at RUH; 6 = Patient Recruitment; 7 = Clinical Collaboration; 8 = Trial monitoring; 9 = Laboratory investigation.

3. **Title of Study**: HCV Pathogenesis and Dendritic Cell Immunobiology
4. **Grant(s) in Which This Study is Described**: MSTP, NIH
5. **Type of Study**: Ongoing study submitted for continuing review
6. **Date of Study**: April 15 2003
7. **Other Institutions Involved**:

   Investigative aspects of this study to be conducted at Rockefeller University include leukapheresis, and all laboratory study. Patients will be recruited from New York Presbyterian Hospital.

8. **Investigational New Drugs Involved**: None.
9. **Radioactive Isotopes Involved**: None.

Abstract, Rationale of Study, Research Plan and Procedures.

a) Abstract

Hepatitis C virus (HCV) infects approximately 200 million people worldwide. Current estimates indicate that 30% of infected individuals spontaneously resolve the infection. Both resolution and protection correlate with the presence of HCV-specific T cell responses. In the 70% of individuals that progress to chronic HCV infection, pegylated interferon alfa (IFNa) and ribavirin enables ~50% of individuals to sustain virologic responses after treatment is discontinued. The role for HCV-specific T cell responses and IFNa highlight the importance of understanding DC function during HCV infection as myeloid DCs are required for priming T cells *in vivo* and plasmacytoid DCs (pDCs) are the principle cell type responsible for endogenous IFNa production. In this study, we characterize the phenotype and function of these DCs *in vivo*. Furthermore, we evaluate mechanism of HCV antigen presentation by DCs as well as adaptive and innate signals which regulate the immunologic outcome of antigen presentation (T cell activation, tolerance, or ignorance). This study will offer insight into the pathogenesis of HCV and effective immunotherapy strategies.

b) Overview

Hepatitis C virus (HCV) infects more than 100 million people worldwide posing a significant public health risk. Approximately 30% of people infected with HCV resolve infection while 70% progress to chronic infection. Many studies have explored the mechanisms by which the virus is able to circumvent both humoral and cell-mediated immune responses. Although an HCV-specific humoral response is commonly seen in HCV-infected chimpanzees and humans, HCV-specific antibodies are not capable of conferring protection[34]. Instead, protection in chimpanzees has been shown to correspond with expanded populations of HCV-reactive $CD8^+$ T cell responses. Similarly in humans, tetramer analysis of $CD8^+$ T cells from successful resolvers indicates the presence of HCV-specific $CD8^+$ T cells, which are absent in chronically infected patients[40]. To better define these distinct immunologic outcomes, and gain insight into the pathogenesis of chronic HCV infection, we propose to explore the role of dendritic cells (DCs) in HCV infected patients across the spectrum of disease.

b) Hypothesis

i. Circulating populations of dendritic cell in HCV patients are phenotypically and functionally normal.

ii. HCV encoded proteins modulate DC function through direct interaction with surface receptors (e.g. CD81 or DC-SIGN) or through engagement of intracellular signaling proteins (e.g. PKR), thus resulting in the selective inactivation of HCV-reactive $CD8^+$ T cells.

c) Aims

i. To enumerate and characterize circulating populations of DCs in patients with HCV. This will be performed on fresh blood or PBMCs isolated from a leukapheresis sample. We will focus our attention on the rare $CD11c^+$ myeloid DC and the $CD123^+$ plasmacytoidal DC (approximately 0.2 – 0.5% of the PBMC fraction), as we believe the study of such cells will provide better insight into DC function in the native state.

ii. To identify HCV-mediated points of dysregulation whereby dendritic cell / T cell interactions result in the selective tolerization of HCV-reactive T cells.

Aim 1. To investigate phenotypic and functional characteristics of circulating DCs. Using Miltenyi Bead technology, we will isolate CD11c+ myeloid and CD123+ plasmacytoidal DCs. Briefly, using the Miltenyi microbeads, cells can be separated from complex cell mixtures to very high purity within minutes. They are specifically labeled with super-paramagnetic MACS MicroBeads. After magnetic labeling, the cells are passed through a separation column that is placed in a strong permanent magnet. The column matrix serves to create a high-gradient magnetic field. The magnetically labeled cells are retained in the column while non-labeled cells pass through. After removal of the column from the magnetic field, the magnetically retained cells are eluted. Both labeled and non-labeled fractions can be completely recovered. Using this approach it will be possible to isolate the respective DC populations to >95% purity.

We will employ antibodies specific for the BDCA-2 antigen for the isolation of $CD11c^-CD123^+$ plasmacytoid dendritic cells in blood[150,151]. BDCA-2^+ DCs show a plasmacytoid morphology, express the pre-T cell receptor a-chain and are CD45RA+, CD14- and CD2-. They express neither myeloid lineage markers like CD13 and CD33, nor Fc receptors like CD32, CD64 or FceRI, and are negative for BDCA-3 and CD1c (BDCA-1).

Use of the antibody specific for CD1c will be used for isolation of circulating myeloid DCs. CD1c is specifically expressed on dendritic cells which are $CD11c^{high}$ $CD123^{low}$ and they represent the major subset of myeloid dendritic cells in blood[150]. $CD1c^+$ dendritic cells show a monocytoid morphology and express myeloid markers such as CD13 and CD33 as well as Fc receptors such as CD32, CD64 and FceRI. Furthermore, they were determined to be $CD4^+$, Lin, $CD2^+$ and $CD45RO^+$. In blood, in addition to dendritic cells, a subset of B cells also expresses CD1c. For this reason, we will first deplete the PBMC fraction using CD19 for negative selection, followed by isolation of the $CD11c^+$ myeloid DCs.

Cells will be analyzed phenotypically by flow cytometry; their ability to undergo normal maturation will be assessed by placing them in culture for 24-36 hrs; and their ability to prime allogeneic T lymphocytes will be

assessed. Additional studies will include analysis of cells for negative and positive strand HCV RNA; and quantification of IFN-a released in the respective cell populations in response to HCV patient *versus* control plasma.

This study is designed for the collection of large numbers of PBMCs, thus allowing us to study circulating populations of DCs—CD11c+ myeloid DC and CD123+ plasmacytoidal DCs. Note, these populations constitute <0.5% of the PBMCs; therefore to perform functional studies, >250cc of blood are required. This has been difficult to obtain by blood draw and as proposed below, leukapheresis will yield enough material to run replicate studies.

If our hypothesis is incorrect and there is indeed a defect in a subset of dendritic cells that distinguishes patients who resolve *vs.* those that progress with chronic HCV infection, we will re-focus our efforts to characterize this finding.

Aim 2. To monitor the presence of HCV-specific memory T cells with DCs cross-presenting cell associated HCV proteins.

Tetramer analysis has identified HCV-specific T cells in patients who successfully resolve the virus. While useful reagents, this method for T cell characterization is limited to those patients with common MHC alleles and viral epitopes that have been defines as strong binders of the respective MHC molecule. As MHC molecules are highly polymorphic and the HCV genome is itself variable across patient populations, we propose to use antigen cross-presentation assays for the screening of HCV-reactive T cells. In these experiments, we will use an osteosarcoma cell line with tetracycline-repressible HCV protein expression. These cells express full length HCV, genotype 1b. We will monitor the presentation of HCV antigen by patient DCs, whereby antigen is derived from internalized apoptotic osteosarcoma cells. The activation of patient T cells will be monitored using IFN-γ ELISPOT assays. As a positive control antigen in these cross-presentation studies, the osteosarcoma cell line will be infected with influenza virus allowing us to monitor influenza reactive cells present in the patient population.

Cross-presentation of antigen derived from HCV-expressing cell lines. We have previously analyzed the response of HCV-expressing tumor cell lines to apoptotic-inducing stimuli. We have also assessed the phagocytosis of the apoptotic cell lines by DCs and demonstrated their ability to transfer antigen for the loading of MHC I/peptide complexes and the activation of influenza-reactive $CD8^+$ T cells. These assays have been estalished in normal control individuals. With large quantities of PBMCs from HCV patients, we now propose to look at responders *versus* non-responders, comparing the frequency of HCV-specific T lymphocytes. We hypothesize that there will be a qualitative difference in T cell reactivity, present in patients who resolved HCV and absent in those who progress to chronic infection. As previously described[193,282], apoptotic cells will be exposed to

DCs and cultured with patient T cells to evaluate the responsiveness to HCV antigen. Assays for monitoring T cell activity will include ELISPOT and intracellular cytokine stimulation assays.

Regarding the use of the osteosarcoma cell line, our previous results have demonstrated that there is no baseline allo-reactivity in normal individuals. As a result, these cells may be used as a vehicle for the delivery of viral antigen.

In addition to characterizing $CD8^+$ T cells responses, we will also screen for the presence of HCV-reactive $CD4^+$ T cells. Insight into the helper T cell immune repertoire will aid in the testing of our hypotheses regarding cell-mediated HCV-specific T cell tolerance. Positive controls for the $CD4^+$ T cell assays will include reactivity to HCV peptides. We will also employ influenza antigen as a non-modulated antigen control for both the $CD4^+$ and $CD8^+$ T cell immune assays.

d) Primary outcomes

Demonstration of phenotypic and functionally normal DCs in the circulation of HCV patients as detailed above. Briefly, >95% of the isolated circulating DCs will take on a $CD83^+$ $HLA\text{-}DR^{HI}$ mature phenotype after 24-36 hours in culture. These cells will be able to stimulate an allogeneic T cell response with >100,000 counts per minute at a ratio of 100 T cells : 1 DC, as measured in a thymidine incorporation assay.

e) Secondary outcomes

A secondary outcome of this study will be the identification of HCV-reactive T cells in patients who have resolved their HCV infection. A positive T cell response will be defined as >50 SFCs / 10^6 $CD8^+$ T cells.

f) Study design

This is an observational study. Subjects with chronic or resolved HCV infection as defined below are eligible. In this study, we will enroll a total of 10 patients with chronic HCV and 10 patients who resolved infection.

All patients will be leukapheresed for purposes of harvesting white blood cells and plasma for immunologic study. Prior to the leukapheresis, patients will be enrolled by a study investigator or a clinical collaborator; two red-top tubes will be collected at that time for pre-screening of patients for HBV, HIV and syphilis.

We will obtain peripheral blood by leukaperesis, but venipuncture will be offered as an alternative to this procedure.

Initial screening of patients will occur at either The Rockefeller University Hospital or by the collaborating physician. All leukapheresis procedures will take place at The RUH.

i) Identification of Patients

Patients will be identified by collaborating physicians.

ii) Leukapheresis and Blood collections
All patients will be leukapheresed at The Rockefeller University Hospital. Samples will be stored at room temperature.

iii) Schedule of events

Screening. Screening will be completed within 10 days, but no greater than 30 days, and at least 24 hours prior, to study entry.

Medical History: The Principal Investigator's designee will perform a pertinent medical history. A detailed medical history will be provided by the collaborating physician.

Complete Physical Examination: The Principal Investigator's designee will perform a complete physical exam at the indicated times.

If any laboratory result is outside the reference range and is considered to be clinically relevant by the designee of the PI, the test will be repeated at appropriate time intervals until it returns to baseline or becomes a clinically insignificant finding.

Complete Blood Count: including differential white blood cell count, absolute neutrophil count, hemoglobin and platelet count.

Hematologic Tests: PT/PTT.

Blood Chemistries: Calcium, prior to leukapheresis.

Viral screens: Hepatitis B surface antigen, HIV antibodies, Syphilis screen.

HLA haplotype: complete HLA haplotype is obtained as part of the patient database, but is not used as a clinical screening parameter. Results of HLA haplotyping do not need to be available prior to leukapheresis at week 0.

g) Statistical model
The primary variables will be the enumeration of DC subsets and determination of myeloid and plasmacytoid DC phenotype and function. In preliminary data, two tailed non-parametric comparisons (Mann-Whitney test) were used to calculate p values for differences in myeloid and plasmacytoid DC enumeration, as well as total IFNa production by PBMCs, as this method allows for data with a non-Gaussian distribution. p values for data normalized on a per cell basis (e.g. IFNa production per plasmacytoid DC) was evaluated with a two-tailed parametric comparison (unpaired t test, Welch corrected).

Statistical significance will be set using 95% confidence intervals (p value 0.05). Based on preliminary standard deviations for n=17 chronic HCV patients (myeloid DC SD = 0.40 and plasmacytoid DC SD = 0.17), 20 patients will give us 95% power to detect a difference in means of 0.47 for myeloid DCs and 0.20 for plasmacytoid DCs. As with preliminary data from myeloid DCs, it is possible to obtain "statistically significant" results with very low power. Failure to reject the null hypothesis will be complemented with equivalence testing to evaluate the lack of difference between HCV and

normal populations. Briefly, if addtition of one standard deviation statistically increases the HCV population relative to normal controls while subtraction of one standard deviation statistically decreases it, equivalence will be concluded provided the power is significant.

10. **Subjects of Study**:

Study group: Subjects for study will be chosen on the basis of a definitive diagnosis of prior or ongoing HCV infection as based on seropositivity and/or viral load. These patients are expected to fall into three distinct groups based on the natural course of disease: complete resolvers, with no plasma HCV titers; sustained responders, whose HCV titer drops below the level of detection for >6months post-IFN-a and ribavirin therapy; and chronically infected, patients who have persistent high-titer plasma HCV. For purposes of this study we combine groups 1 and 2 as both populations have cleared infection and presumably harbor HCV-specific T lymphocytes[40]. All HCV patients will receive leukapheresis or whole blood donation (360cc).

Inclusion/exclusion criteria

Screening to determine eligibility (with the exception of HLA haplotyping) will be completed within 10 days and not less than 24 hours prior to study entry.

--Disease Characteristics--

1. Confirmed HCV infection based on serologic studies and/or plasma HCV titers

--Prior/Concurrent Therapy--

1. Recovered from toxicity of any prior therapy
2. Biologic therapy:
   No concurrent immunotherapy
   Greater than 6 months post IFN-a and Ribavirin therapy
3. Chemotherapy:
   At least one year post-chemotherapy for other medical condition.
4. Endocrine evaluation/therapy:
   At least 4 weeks since corticosteroid treatment
5. Radiotherapy:
   At least 4 weeks since radiotherapy
   No concurrent radiotherapy
6. Surgery:
   Prior surgery allowed

--Patient Characteristics--

1. Age: 18 and over, able to give written informed consent.
2. Performance status: Karnofsky 70-100%
3. Life expectancy: At least 12 months

4. Hematopoietic:
   WBC greater than 3,800
   Absolute lymphocytes greater than 1,500
   Platelets greater than 120,000
   Hb at least 9.5 g/dl
   INR < 1.5
5. Hepatic:
   No patients with decompensated cirrhosis.
6. Cardiovascular:
   No NYHA class III/IV status
7. Pulmonary:
   No severe debilitating pulmonary disease
8. Other:
   No active infection requiring antibiotics
   Not HIV, syphilis, or hepatitis B; anti-HIV and HbsAg antibody negative obtained within 90 days prior to study entry
   No serious uncontrolled medical illness
   No currently active second malignancy other than non-melanoma skin cancer (note: a patient is NOT considered to have currently active malignancy if they have completed therapy and are now considered by their physician to be at less than 30% risk for relapse)
   No history of vasculitis, including but not limited to systemic necrotizing vasculitides (polyarteritis nodosa group), hypersensitivity vasculitis, Wegener's granulomatosis.
   No receipt of immune modulators or suppressors within 30 days prior to study entry, including but not limited to interferons and thalidomide. No active requirement for corticosteroids; prior use is acceptable.
   No psychiatric illness or social condition that, in the opinion of the investigator, would interfere with adherence to study requirements.
   No alcohol or drug use or dependence that, in the opinion of the investigator, would interfere with adherence to study requirements.

No patient will be excluded who wants to enter this study, except for the reasons detailed above. All women and minority patients will be encouraged to participate. All referrals will be accepted after screening and when appropriate, entered into the study.

Control Group: Normative data will be obtained from individuals without Hepatitis C or other known disease. Initially, buffy coat material obtained from anonymous normal donors will serve as a control population. These samples will be purchased from the The New York Blood Center. Depending on the results for Aim #1, we may wish to include HBV or HIV / HCV co-infected

individuals in this study. At that time, an addendum to the current protocol will be submitted.

11. **Recruitment and Consent Procedures**:

All HCV patients that are not otherwise medically excluded, will be eligible for entry into the study. Recruitment will be performed in an office or hospital setting with patient and family present; recruiters include Drs. Andrew Talal, Ira Jacobson, Matthew Albert and Charles Rice. Consent will be obtained by Drs. Andrew Talal, Ira Jacobson Matthew Albert, Randy Longman or collaborating physicians. Information regarding the current expected prognosis of HCV infection will be offered exclusively by the treating physician. Grounds for exclusion from study will be solely medical.

12. **Risks and Alternative Methods**:

Risks associated with enrollment into this study include the discomfort associated with phlebotomy and leukapheresis. Bruising at the site where the needles are inserted may occur, particularly post-leukapheresis. All of these procedures, in which needles are inserted into the body, have a minor risk of introducing an infection, or causing bleeding in the area if needle insertion.

Leukapheresis is a means of drawing blood meant to save the subject large amounts of blood draws. In leukapheresis, a procedure very much like blood donation for the subject, one needle is placed in each arm, and blood is collected in the blood bank. Unlike blood donation, however, the patient's own red blood cells (one component of the blood) are returned to the patient, in an effort to prevent anemia sometimes associated with blood donation. Only a fraction of the patient's serum and the white blood cells are kept for clinical study; the white blood cells are replaced completely by the patient within a short period of time through trafficking of cells out of the lymph nodes and spleen (minutes). At no time during or after the leukopheresis is the patient immunocompromised[283,284].

The risks involve the use of an invasive needle, which may lead to bruising of the arms where the needle is placed. There is a minor risk of bleeding from anticoagulants given during the procedure; this anticoagulant (citrate) is broken down by the body within one minute, any bleeding will be stopped by terminating the leukapheresis. The use of the citrate anticoagulant may result in hypocalcemia. This is readily detected as the patients lips will begin to tingle. Tums™ will be administered prophylactically and additional Tums™ will be given as per needed. Rarely, patients may faint (suffer hypotension due to a vasovagal reaction) during leukapheresis; for this reason patients lie down during the procedure and are accompanied at all times by medical staff. The leukapheresis will be temporarily stopped and it the patient continues to feel faint, the procedure will be ended prematurely. Patients sometimes feel chilled during leukapheresis; if they do, they will be given blankets to keep warm.

Alternative methods: An alternative to leukapheresis is a standard blood draw. To obtain a sufficient number of cells for study, approximately 360cc of blood would need to be drawn (3/4 unit of blood in a standard blood donation; note however that in some patients, only 30-50cc total blood will be drawn). Leukapheresis is essentially the same for the patient, but has the advantages of providing more cells for study while not depleting the patient of their own red blood cells. Thus wherever possible, leukapheresis will be preferred over standard blood drawing to obtain cells for study when large numbers of cells are required.

13. **Data and Safety Monitoring Plan**:

    a. **Level of Risk:** This project entails moderate risk to subjects. Monitoring will be conducted by the Principal Investigator (PI), a credentialed co-investigator, and the Rockefeller University Institutional Review Board (IRB), through annual reports of progress and by immediate notification of adverse events by the PI to the IRB, the GAC, and any federal regulatory agency (e.g. FDA) when appropriate or required.

    b. **Data and Safety Monitoring Board:** This protocol comprises the collection of patient samples, does not expose participants to substantial risk, does not involve blinding, and does not involve vulnerable populations. It will therefore not need a Data and Safety Monitoring Board. Monitoring will be conducted by the PI and the monitoring group (see below, 14.e.) and the Rockefeller University IRB, through annual reports of progress.

    c. **Safety review plan:** Routine and safety lab tests will be reviewed in real time by the PI and/or co-investigators, as they are returned from the laboratory. Abnormal results will be evaluated for clinical significance and so documented. Clinically significant abnormalities will be followed up by the investigator or referred for care as appropriate. The only "safety data" that will be collected are the CBC, calcium and PT/PTT prior to leukapheresis. These results will be reviewed immediately upon receipt by the physician of record. Patients will not be leukapheresed if any of these tests are outside normal limits. Additionally, the nurse who is performing the leukapheresis will be given study personnel contact information so that any unexpected symptoms may be reported to the study clinicians as they occur. The common toxicity criteria version 2.0 will be used as guidelines to monitor toxicities and adverse events (http://ctep.inof.nih.gov). Adverse events will be reviewed in real time and documented in the medical record of each patient, and then entered into a separate AE log for that patient. On-line adverse event reports generated by the GCRC nursing staff will also be reviewed as they are received and any not already entered in the log will be added. This AE log will be used for tabulation of adverse events in the annual report to the IRB.

d. **Serious Adverse Events (SAE):** A serious adverse event or reaction is any untoward medical occurrence that at any dose results in death, is life-threatening, requires in-patient hopitalization or prolongation of existing hospitalization, results in persistent or significant disability/incapacity, or is a congenital anomaly/birth defect. All serious adverse events will be conveyed to the PI immediately by the GCRC nursing staff or a study team member. Medical intervention will be initiated as appropriate and the patient referred for further care as necessary. All unanticipated AEs of NCI grade 3 or higher occurring during inpatient stay or within 10 days of discharge will be reported to the Rockefeller University IRB and the GCRC Clinical Research Officer within 48 hours. All adverse events tabulated from the AE log (including severity and attribution), as well as total enrollment, demographics of enrollment, reasons for attrition, significant protocol violations, current status of subjects (completed, ongoing, dropout), and any available research data will be reported in the annual progress report to the IRB. The PI will maintain documentation of adverse event (AE) reports, all source documents, research records, and IRB documents.

e. **Data management and Patient Confidentiality:** Data will be collected in a password-protected dedicated computer file and labeled with a patient ID number only. A standard form for the input of patient data will be used for all patient clinical data and to store the results of antibody assays, gene expression studies, and T cell assays. Laboratory data will be collected into bound laboratory notebooks. This data will then be transcribed into an electronic database. This database will electronically date and time stamp entries and will maintain an audit trail. This database will be backed up daily by the Laboratory Administrator. Data records will be reviewed at regular intervals in accordance with the monitoring plan to ensure that data is recorded accurately and properly.

f. **Study monitoring:** The monitoring group will meet every two months to review the QA data. The monitoring group will consist of Dr. Rice, Dr. Albert and Dr. Talal. Medical records of up to three patients will be randomly selected and reviewed for completeness of documentation and to confirm informed consent, eligibility, enrollment note, accurate transcription in to the AE log, and the proper conduct of the protocol. Research laboratory results of up to three patients will be randomly selected and reviewed for SOP compliance. Occurrence of these record and data reviews will be noted in a monitoring log. The monitoring group will also review the AE log for completeness (grading, expected or unexpected, attribution, outcome), and will review for appropriate reporting to the IRB.

g. **CLIA laboratory certification:** Laboratory tests performed as part of this study are not CLIA or CLEP certified. Therefore, no laboratory tests run in the laboratory as part of this research protocol will be communicated to the patient, given to referring physicians, or

influence decisions about the research participants' treatment.

14. **Procedures to Minimize Risks**:

    Leukapheresis or venipuncture will be performed by professional licensed staff at The Rockefeller Hospital or by certified phlebotomy laboratories.

15. **Potential Benefits**:

    There is no direct benefit to participants in this study. The potential benefits are the identification of the immune defect in patients with chronic HCV infection that is in part responsible for the inability to clear the virus. This study might ultimately be useful in identifying surrogate markers for effective anti-viral protocols.

16. **Risk to Benefit Ratio**:

    The balance of the risks versus benefits involved in participation are judged by the investigators to be clearly in favor of the benefits derived from participation. The risks are judged overall to be moderate, but are not anticipated to include serious adverse reactions. This is an observational research study, and the potential benefit of this study is entirely unknown. If the hypothesis proves to be correct, this study will be offer the opportunity to derived new insight into the development of anti-viral protocols aimed at achieving cures in chronic HCV patients.

17. **Compensations**:

Patients will receive compensation for the leukapheresis procedure, $300/donation.

Principal Investigator's Assurance:

As Principal Investigator of this study, I assure the IRB that, as required by federal regulations:

- Proposed changes in approved studies will be presented to the IRB for review and approval prior to initiation except where necessary to eliminate apparent immediate hazards to the subjects.

- The IRB, appropriate institutional officials, the Office for Protection from Research Risks (OPRR) and the FDA, if applicable, will be promptly informed of any unanticipated problems involving risks to subjects or others and research related injuries.

- The informed consent of the subject will be obtained by the investigator in the manner and format approved by the IRB prior to the initiation of the studies.

- The study will be resubmitted to the IRB for continuing review at the interval determined by the IRB to be appropriate to the risk, but not less than once a year.

_____
(Signature of Principal Investigator)

_____
(Date)

Conflict of Interest

Do you, your spouse or dependent children or any Professional Personnel* listed on the application, have any Significant Financial Interest* (as defined by the federal regulations):

(A) that would reasonably appear to be affected by the research?

Yes _____ No _____

(B) in any entities whose financial interest would reasonably appear to be affected by the research?

Yes _____ No _____

(If you answered "Yes" for either (A) or (B), the Principal Investigator and/or appropriate Professional Personnel must provide a confidential statement describing the possible conflict of interest in the sealed envelope marked "Confidential" and addressed to the IRB Chair).

Signed Date

Investigator

*** NOTES:**

Professional Personnel means any other person responsible for the design, conduct, or reporting of this research.

Significant Financial Interest means anything of monetary value, including but not limited to salary or other payment for services (e.g., consulting fees or honoraria); equity interest (e.g., stock, stock options, or other ownership interests); and intellectual property rights (e.g., patents, copyrights, and royalties from such rights). This term does not include:

(i) Salary, royalties, or other remuneration from the institution;
(ii) Any ownership interests in the institution, if the institution is the applicant under the SBIR Program;
(iii) Income from seminars, lectures, or teaching engagements sponsored by public or nonprofit entities;
(iv) Income from service on advisory committees or review panels for public or nonprofit entities;
(v) An equity interest which meets both of the following tests: does not exceed $10,000 in value as determined through references to public prices or other reasonable measures of fair market value when aggregated for the investigator and the investigator's spouse and dependent children; and constitutes more than a 5 percent ownership interest in any single entity when aggregated in the same manner; or
(vi) Salary, royalties or other payments that are not reasonably expected to exceed $10,000 per annum from any single entity when aggregated for the investigator and the investigator's spouse and dependent children.

However, the exclusions in paragraphs (i), (v) and (vi) shall not apply if the compensation or transfer of an equity interest is conditioned upon a particular outcome in the PHS-funded research.

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