PLASMACYTOID DENDRITIC CELLS ARE KEY REGULATORS OF IMMUNE TOLERANCE

by

Benjamin Michael Matta

B.S., The Pennsylvania State University, 2003

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Benjamin Michael Matta

It was defended on

June 27, 2012

and approved by

Lawrence P. Kane, PhD, Associate Professor, Department of Immunology

Adrian E. Morelli, MD, PhD, Associate Professor, Departments of Surgery and Immunology

Anuradha Ray, PhD, Professor, Departments of Medicine and Immunology

Fadi G. Lakkis, MD, Professor, Departments of Surgery and Immunology

Marie C. DeFrances, MD, PhD, Associate Professor, Department of Cellular and Molecular Pathology

Dissertation Advisor: Angus W. Thomson, PhD, DSc, Distinguished Professor, Departments of Surgery and Immunology

Copyright © by Benjamin Michael Matta

2012

PLASMACYTOID DENDRITIC CELLS ARE KEY REGULATORS OF IMMUNE TOLERANCE

Benjamin Michael Matta, PhD

University of Pittsburgh, 2012

Solid organ allograft rejection and many autoimmune disorders remain significant clinical problems despite the efficacy of current immunosuppressive (IS) drug treatments. Moreover, the side effects of global IS drugs that inhibit immune responses in a non-specific manner may leave the host susceptible to development of cancer and chronic infection. The need for antigen (Ag)-specific treatments to reduce or eliminate the need for IS drugs is merited in these cases.

Dendritic cells (DC) are important Ag-presenting cells (APC) that mediate innate and adaptive immune responses, and have been widely investigated as potential cell-based therapeutics to bolster or supplant current IS drug treatments in transplantation and autoimmune diseases. Conventional (c)DC are the most potent APC, however, they can be modified and conditioned to instead modulate immune responses and promote Ag-specific tolerance. Plasmacytoid (p)DC, the primary cells that produce type I IFN and activate anti-viral immunity, are also recognized as holding significant tolerogenic potential. In this dissertation, I present novel data that support the tolerogenic function of pDC and highlight their inability to elicit strong immune responses in the absence of viral infection. pDC exhibit a more immature phenotype compared to cDC that correlates to their weak ability to stimulate T cells. Moreover, liver pDC are less mature compared to pDC in the spleen. They promote regulatory T cell function, suppress delayed-type hypersensitivity responses, and are critical for the spontaneous acceptance of murine liver allografts. My data show that the co-inhibitory molecule B7 homolog-

1 (B7-H1) is important for the immune regulatory capacity of spleen and liver pDC, and that the cytokine IL-27 regulates critical inhibitory functions of liver pDC, including their expression of B7-H1 and their immune suppressive capacity *in vitro* and *in vivo*.

In summary, my data provide evidence that pDC are an attractive target for development of cell-based therapies and identify novel mechanisms utilized by pDC to regulate immune reactivity. I have described for the first time that donor pDC contribute to the spontaneous acceptance of murine liver allografts, a discovery that has important implications for development of future therapeutics to promote immune tolerance, especially in solid organ transplantation.

PREFACE

First and foremost, I would like to thank my family for their support and encouragement in my decision to go back to school and pursue a Ph.D. degree. It has been a difficult journey at times, but having a great family life outside of the lab has made work in the lab more fun, and given me the opportunity to approach challenging questions with renewed enthusiasm.

I would also like to thank my mentor, Dr. Angus Thomson. Your leadership and knowledge in science and in life has been a great inspiration, and has provided me with a strong foundation on which I can continue to build my career as scientist. I appreciate your patience and guidance, and working with you has improved my communication and critical thinking skills, which will serve me well as I continue to develop myself into an independent investigator.

I would like to thank all members of the Thomson lab, past and present, not only for the help and support in my work, but for all of the laughs and the lasting friendships made. I would like to thank our lab administrator Miriam Freeman. There wasn't a question I had that you didn't have the answer to, and you were always happy and smiling, even under the pressure of grant deadlines. Finally, I would like to thank my fellow students, and the faculty and staff in the Departments of Surgery and Immunology, the Thomas E. Starzl Transplantation Institute, and the Graduate Studies office. Everyone has played a role in allowing me to achieve this goal and I appreciate all of your contributions along the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

The immune system encompasses a complex network of tissues and cells that work in concert to protect the host from invading pathogens in addition to regulating unwanted responses against self-antigens (Ags) and harmless or inert Ags. Ag-presenting cells (APC) play a key role in maintaining the balance between immunity and tolerance, and direct immune responses against specific Ags. The most important APC is the dendritic cell (DC), a bone marrow (BM)-derived, professional APC that forms a critical link between the innate and adaptive immune systems. DC are highly specialized for the uptake, processing, and presentation of Ags to initiate adaptive immune responses, and also produce factors that direct innate immune responses, all in an effort to clear invading pathogens and resolve disease pathologies. At the same time, DC also possess distinct regulatory properties that control unwanted and unnecessary inflammatory responses against harmless foreign Ags and maintain tolerance to self. Plasmacytoid (p) DC comprise a highly specialized DC subset that is critical in directing anti-viral responses. In addition, pDC also exhibit potent immunoregulatory functions that make them an attractive therapeutic target for treatment strategies where the regulation of immune responses is warranted.

1.1 DC LINEAGE DEVELOPMENT AND MURINE DC SUBSETS

DC are a heterogenous population of BM-derived cells that arise from common progenitors and comprise multiple subsets [1, 2]. At the transcriptional level, PU.1 expression is required for the

development of all DC subsets [3], with an important function of driving the expression of multiple cytokine receptors that regulate DC development and differentiation (Figure 1.1). The hematopoietic growth factor FMS-like tyrosine kinase 3 (Flt3) ligand (L) is critical for DC differentiation [4]. This is demonstrated by the fact that mice lacking Flt3L have a severe deficiency in lymphoid-related tissue DC [5], and DC can be propagated from BM cultures *in vitro* [6-8] and mobilized and expanded *in vivo* through administration of recombinant Flt3L [9]. Additionally, macrophage (M) colony-stimulating factor (CSF) and granulocyte-macrophage (GM)-CSF can be used *in vitro* to propagate DC from BM [10] and *in vivo* to expand DC [ref], and both play significant roles in DC development and differentiation.

In the steady state in the mouse, there are two major DC subsets arising from the same common DC progenitors [11]. Conventional (c)DC ($CD11c^{+}CD11b^{+}B220^{-}NK1.1^{-}$) are found both in the periphery and resident in lymphoid tissue and are specialized for Ag-uptake, processing, and presentation to $CD4^{+}$ T cells on MHC class II molecules. cDC can be further sub-divided based on their phenotype and tissue localization, and include $CD8\alpha^{+}$ cDC which are reported to be efficient at cross-presentation of exogenous Ags on MHC class I [11]. The other main DC subset in the mouse is the plasmacytoid (p) DC ($CD11c^{low}CD11b^{-}B220^{+}CD8\alpha^{-}$) subset. pDC are the primary cell type responsible for type I IFN production during viral infection [12, 13] and are characterized by unique developmental programming, phenotype, and function. pDC will be characterized further in sections 1.4 and 1.5, and are the focus of the work presented in this dissertation. Commitment to either the cDC or pDC lineages is determined by the balance of transcription factor expression [14] and the timing at which each is predominantly expressed during development. The expression of these transcription factors may be delicately controlled

by the expression of microRNAs (miRNA; short, non-coding RNA sequences) that bind to mRNA and can regulate gene expression by promoting mRNA degradation and translation [15].

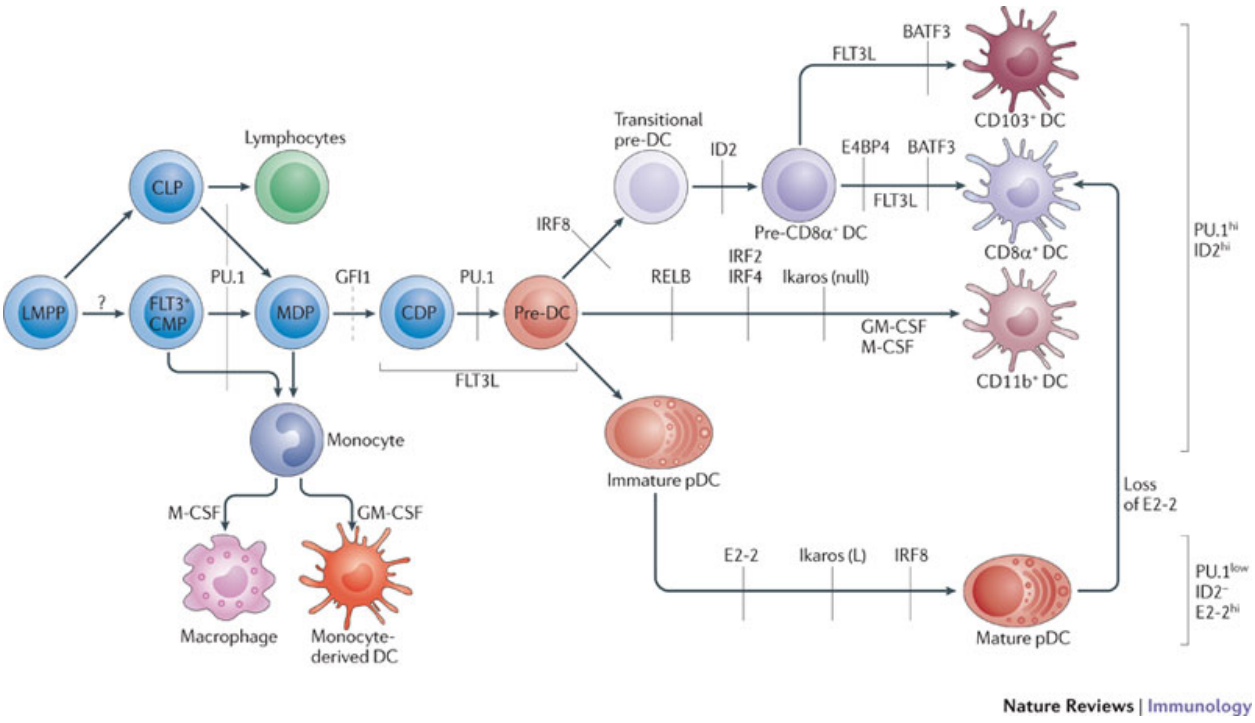


Figure 1.1 Growth factors and transcription factors that regulate DC differentiation.

The developmental pathways from myeloid and lymphoid progenitors to precursor dendritic cells (pre-DCs) in the BM and the peripheral diversification of DC subsets are shown. The approximate points at which key transcription factors are first required for DC development are indicated by vertical lines. Stages at which key growth factors have been determined to be essential are indicated. The development of both DCs and monocytes depends on high concentrations of PU.1, which regulates the expression of the cytokine receptors FMS-related tyrosine kinase 3 (FLT3), macrophage colony-stimulating factor receptor (M-CSFR) and granulocyte–macrophage colony-stimulating factor receptor (GM-CSFR). The development of $CD8\alpha^+$ and $CD103^+$ DCs relies on the stepwise activity of interferon-regulatory factor 8 (IRF8), inhibitor of DNA binding 2 (ID2), E4 promoter-binding protein 4 (E4BP4) and basic leucine zipper transcription factor, ATF-like 3 (BATF3), as well as on FLT3 signaling. $CD11b^+$ DCs depend on a unique set of transcription factors, including RELB, IRF2, IRF4 and Ikaros, and to some extent on the cytokines M-CSF and GM-CSF. The plasmacytoid DC (pDC) lineage requires IRF8, a low level of PU.1 and the absence of ID2. The differentiation of pDC from an immature precursor requires E2-2 and Ikaros, with induced loss of E2-2 converting pDC into cells that closely resemble $CD8\alpha^+$ conventional DCs. CDP, common DC progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; FLT3L, FLT3 ligand; GFI1, growth factor independent 1; LMPP, lymphoid-primed multipotent progenitor; MDP, macrophage and DC progenitor. From Belz GT, and SL Nutt. 2012. *Nat Rev Immunol.* 12:101-113. [1]

1.2 DC LINK INNATE AND ADAPTIVE IMMUNITY

DC serve as a critical link between the innate and adaptive arms of the immune system. In the steady state, DC are highly specialized for internalization of Ags, and following processing, antigenic peptides are loaded onto MHC class II molecules and shuttled to the cell surface where stable Ag-MHC complexes are presented to $CD4^+$ T cells. Inflammatory cytokines, damage associated molecular patterns (DAMPs) or danger signals, and pathogen associated molecular patterns (PAMPs) all serve to stimulate a maturation process that reduces DC capacity for Ag uptake and shifts their function towards Ag presentation and immune activation [16]. During this process, DC upregulate surface Ag-presentation and co-stimulatory molecules and produce cytokines that influence the activity of other cells and direct the immune response towards a resolution.

1.2.1 DC provide 3 signals for optimal T cell activation

Following an orchestrated processing sequence, antigenic peptides of a specific length are generated from acquired Ags, loaded onto MHC class II molecules, and shuttled to the cell surface for presentation to T cells. During this process, DC are being exposed to inflammatory stimuli and initiate signaling cascades that results in maturation into potent stimulators of the immune response. DC form an immunological synapse with T cells [17] and provide three critical signals which results in activation, T cell clonal expansion, and development of Ag-specific memory.

1.2.1.1 Signal 1: Antigen capture, processing, and presentation

Signal 1 provided by DC is presentation of Ag on MHC molecules whereby DC form long-term, stable Ag-MHC complexes on the cell surface to maximize interactions for Ag with the T cell receptor (TCR). DC express a multitude of receptors for recognition of foreign Ags and immune complexes, such as: Fc receptors [18]; Toll-like receptors (TLRs) [19]; Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) [20]; and a class of C-type lectins, including DEC-205/CD205 [21], DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [22], Langerin [23], and Dectin-1 [24]. pDC uniquely express sialic acid binding immunoglobulin-like lectin (Siglec)-H that plays a role in Ag-uptake, and will be discussed later in section 1.4.2.2. Once exogenous Ags are internalized, they enter the endo-lysosomal pathway where they are degraded into short peptides and loaded onto MHC class II molecules, and Ag-MHC complexes are transported to the cell surface for presentation to $CD4^+$ T cells. Small quantities of Ag can escape the endosomal pathway into the cytosol. Degradation of the Ag by the proteasome and trafficking to the endoplasmic reticulum (ER) results in further processing of the peptides for loading onto MHC class I molecules and shuttling to the surface for cross-presentation to $CD8^+$ T cells [25, 26]. In some cases, small peptides may be loaded onto recycled MHC class I molecules within the endosome itself [27].

DC maturation involves down-regulation of the MHCII transactivator (CIITA) [28] which is responsible for regulation of MHC class II expression [29]. Mature cDC also downregulate MARCH1, a ubiquitin ligase that controls MHC class II ubiquitination and degradation, further stabilizing peptide:MHC class II complexes on their surface [30]. Interestingly, pDC differentially regulate MHC class II expression relative to cDC [31], and this may play a

significant role in their T cell stimulatory capacity. The impact of this unique characteristic of pDC and their role in Ag presentation will be discussed in more detail in section 1.4.5.1.

1.2.1.2 Signal 2: Co-stimulation

The upregulation of co-stimulatory molecules on DC provides signal 2 for T cell activation. The B7 family of co-stimulatory molecules is probably the most important functionally for optimal T cell activation [32, 33]. B7-1 (CD80) and B7-2 (CD86) are co-stimulatory molecules with similar functions that bind to CD28 on T cells and drive T cell clonal expansion [34]. CD80/CD86 ligation of CD28 also drives T cell expression of CD40L/CD154, and subsequent interaction with CD40 on DC further increases CD80 and CD86 expression [35]. This is an important consequence of co-stimulation by DC, as it also supports their cytokine production, which provides signal 3 and drives T cell differentiation.

1.2.1.3 Signal 3: Cytokine production and T cell polarization

Production of cytokines by DC provides signal 3 for T cell activation and is important for several reasons. First, production of TNF-α can induce upregulation of integrins and adhesion molecules on T cells to enhance their migration to sites of inflammation. Second, depending on the type of pathogen/Ag that activates the DC, the cytokines being produced will direct polarization of T cells towards different helper T cell (Th) lineages. Recognition of bacterial degradation products, such as the TLR4 ligand bacterial lipopolysaccharide (LPS) induces high levels of IL-12 production, which stimulates IFN-γ production by T cells and promotes T cell polarization towards Th1 responses. During allergic responses though, cytokines such as IL-4 can skew T cells towards a Th2 response, characterized by production of IL-4, IL-5, IL-13, and IL-10. Moreover, Th17 T cells that produce IL-17 can be stimulated by DC-derived TGF-beta, IL-23,

and IL-6 [36]. This subset is highly inflammatory, and induces strong recruitment of neutrophils [37, 38]. IL-6 can further promote the activity of effector T cells by antagonizing the development and function of $Foxp3^+$ regulatory T cells (Treg) [39].

1.2.2 Breakdown of 3 signals can promote immune regulation

The coordination and participation of all three signals from DC is necessary for sufficient and potent T cell activation, clonal expansion, and resolution of pathologic conditions. Deficiencies or alterations of one or more of these signals during T cell activation can lead to negative regulation of T cell function including anergy, activation-induced cell death, and generation of Treg. On the other hand, intentionally disrupting these same mechanisms used by DC during immune activation may allow for manipulation of DC to promote or restore tolerance and prevent or reverse unwanted immune responses, such as would be the case to prevent allograft rejection in transplantation, or reverse the detrimental self-reactivity in autoimmune diseases.

1.3 DC AS REGULATORS OF IMMUNE TOLERANCE

While DC are the most potent APC and are instrumental in driving innate and adaptive immune responses, their Ag-presenting capacity is a vital component in their reciprocal role as regulators of immune tolerance. Thus, DC participate in the maintenance of central and peripheral tolerance to self Ags, commensal bacteria and food Ags, and harmless or inert inhaled Ags, through multiple mechanisms involving negative regulation of T cell function. Significant effort has been put towards harnessing their immune regulatory properties through pharmacologic, biologic, and

genetic manipulation to generate tolerogenic DC to be used as a therapeutic alternative or supplement to current treatment strategies in transplantation and autoimmune diseases.

1.3.1 DC maintain central and peripheral tolerance

Although they are not the only APC involved in thymic selection, DC play an important role in shaping the T cell repertoire [40]. Thymic-resident DC can acquire self Ags (tissue-restricted Ags) that are shed from thymic epithelial cells (TEC) or released from TEC following apoptosis [41], and can acquire blood-borne Ags that enter the thymus through the circulation. DC can also migrate to the thymus carrying Ags obtained in the periphery for presentation and negative selection of $CD4^+$ T cells [42, 43]. In cases where autoreactive T cells do escape thymic selection, steady-state DC in the periphery are capable of targeting these T cells and rendering them inactive [44]. These data suggest that DC have a unique role in negative selection of T cells in the thymus, and in the periphery they provide an additional level of control to prevent the potential deleterious pathology mediated by autoreactive T cells that escape thymic selection.

Due to their enormous surface area and continuous exposure to various Ags, the airway and intestinal mucosal tissues must be carefully regulated to prevent unwanted immune responses against harmless inhaled and ingested Ags while maintaining immune reactivity towards potentially harmful Ags. A population of $CD103^+$ (integrin αE) DC has been described as an important DC subset in maintaining and promoting immune tolerance in mucosal tissues [45-48]. $CD103^+$ DC are believed to be important inducers of Treg that can inhibit unwanted effector T cell responses against inert Ags.

In the steady state, airway DC are continuously sampling inhaled allergens and Ags. They then traffic to the draining lymph nodes (LNs), and in the absence of inflammation, DC remain

immature and induce effector T cell apoptosis and anergy, and can induce Treg [49] due to insufficient T cell co-stimulation and pro-inflammatory cytokine production. DC also play a pivotal role in the phenomenon known as oral tolerance [50], whereby orally administered or ingested Ags induces hyporesponsiveness upon subsequent exposure to that same Ag. Highlighting the role of DC in oral tolerance is the fact that Flt3L-mobilized mice that have significantly elevated numbers of DC exhibit enhanced development or oral tolerance [51]. Some of the mechanisms implicated in mucosal tolerance include, but are not limited to: a shift toward anti-inflammatory (IL-10) versus pro-inflammatory (IL-12) cytokine production [52]; DC production of retinoic acid [53, 54] which can limit effector and memory T cell expansion and promote Treg induction [55, 56]; DC production and activation of TGF-β [47], which is a critical factor for Treg development and function [57]; and deletion of Ag-specific T cells following Ag feeding [58].

These findings indicate that, in the absence of inflammatory stimuli (steady-state), DC can acquire Ag but remain immature, and thus, subsequent interactions with T cells leads to Ag-specific hyporesponsiveness and/or induction of Treg. This critical function of DC serves to maintain tolerance to harmless self Ags, commensal bacteria, and inert inhaled allergens or ingested food Ags. The same properties and mechanisms of DC that maintain and promote tolerance in the steady-state have been the focus of manipulation of DC into what have been termed “tolerogenic DC”.

1.3.2 Conditioning of DC by the local microenvironment: Non-lymphoid tissue DC

DC resident in non-lymphoid tissues are reported to hold greater tolerance-promoting capacity compared to DC found in lymphoid tissues, and therefore, it has been realized that certain factors

in the tissue microenvironment may modulate DC function [59]. For example, the microenvironment of the liver contains factors such as prostaglandins (PGEs), retinoic acid, immunosuppressive cytokines (IL-10 and TGF-β) and enzymes (arginase I) that negatively regulate immune cell function, including DC [60]. Stellate cells [61, 62] and stromal cells [63] in the liver can act indirectly as regulatory cells by producing and secreting many of the above factors that condition DC towards a regulatory phenotype [60]. Maybe of more significance is the fact that the liver lies downstream of the gut and receives blood via the portal vein, which contains detectable levels of gut-derived bacterial degradation products (e.g., LPS; TLR ligands). This blood supply continuously bathes cells in the liver, including DC, and induces changes to their molecular framework that enhances their tolerogenic capacity [64]. Our group and others have worked to characterize hepatic DC [65-68] and dissect their phenotypic and functional differences they exhibit compared to DC in lymphoid tissues [69-73].

Pulmonary stromal cells exhibit an ability similar to hepatic stromal cells to induce regulatory DC that are able to suppress T cell responses [74]. Additionally, TGF-β maintains Langerhans cells (skin-resident DC) in an immature state, as Langerhans cells from TGF-βR-deficient mice show decreased MHC class II and co-stimulatory molecule expression [75]. These effects are not limited to cDC, however, as the regulatory factors (TGF-β, IL-10, and PGE2) found in the gut mucosa can suppress pDC production of type I IFN and IL-12 [76].

As mentioned earlier, $CD103^+$ DC represent a subset of migratory DC found in mucosal tissues (gut, lung, and skin), and may represent an example of conditioning DC in the microenvironment of non-lymphoid tissues that ultimately promotes their regulatory function [77, 78]. $CD103^+$ DC have been linked to the development of oral tolerance and induction of Treg [48, 79, 80], and are an important DC subset involved in regulatory immune responses.

1.3.3 Tolerogenic DC: Working towards antigen-specific immune regulation

Research focused on harnessing the immunoregulatory properties of DC for therapeutic use has grown exponentially in recent years, and for several important reasons. Drug toxicities resulting from prolonged used of pharmacologic agents [81] can have detrimental effects on overall health, and can create new problems in addition to the condition being treated. Additionally, prolonged use of global immunosuppressants (IS) can lead to dysregulation of immune function, resulting in cancer [82, 83] and/or chronic viral infections [84]. Finally, in the case of transplantation, current IS regimens have been successful at preventing acute rejection episodes, but have done little to prevent chronic allograft rejection and associated pathologies, such as chronic allograft vasculopathy (CAV) [85]. The development of Ag- (or patient-) specific therapeutic intervention may reduce the need for or even supplant current pharmacologic treatments, therefore reducing the incidence of drug toxicity. Additionally, an Ag-specific treatment strategy that limits use of global IS would leave the rest of the immune system intact to combat bacterial and viral pathogens and may reduce the development of autoimmune disease and cancer. Finally, targeting DC to regulate Ag-specific immunity may promote long-term allograft survival by reducing the development of CAV and chronic allograft rejection in solid organ transplantation [86].

1.3.3.1 Characteristics of tolerogenic DC

There are several key phenotypic and functional characteristics that have been used to describe tolerogenic DC (Figure 1.2) [87]. Typically, they express low levels of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86), while maintaining relatively high expression of the co-inhibitory molecules B7 homolog 1 (B7-H1; Programmed death ligand-1, PD-L1)

and/or B7-DC (PD-L2). Tolerogenic DC produce high levels of TGF-β and IL-10 and lower levels of IL-12p70, however, certain types of DC that are considered tolerogenic sustain high levels of IL-12p70 production [88].

DC that are propagated or cultured in the presence of IS drugs, regulatory cytokines, or genetically engineered to be tolerogenic are typically resistant to maturation stimuli and maintain an immature phenotype that underlies their ability to negatively regulate effector T cells and promote Treg function [89]. Furthermore, the production of certain factors such as TGF-β or the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) by tolerogenic DC can promote the induction, expansion, and function of Treg [48, 90]. A key feature of tolerogenic DC that is critical for their ability to function *in vivo* is a preserved or even enhanced ability to traffic to secondary lymphoid tissues following adoptive transfer [91]. Overall, tolerogenic DC are: phenotypically immature; resistant to maturation; exhibit poor effector T cell stimulatory capacity; promote effector T cell apoptosis; and are efficient at inducing and/or expanding Treg through multiple mechanisms.

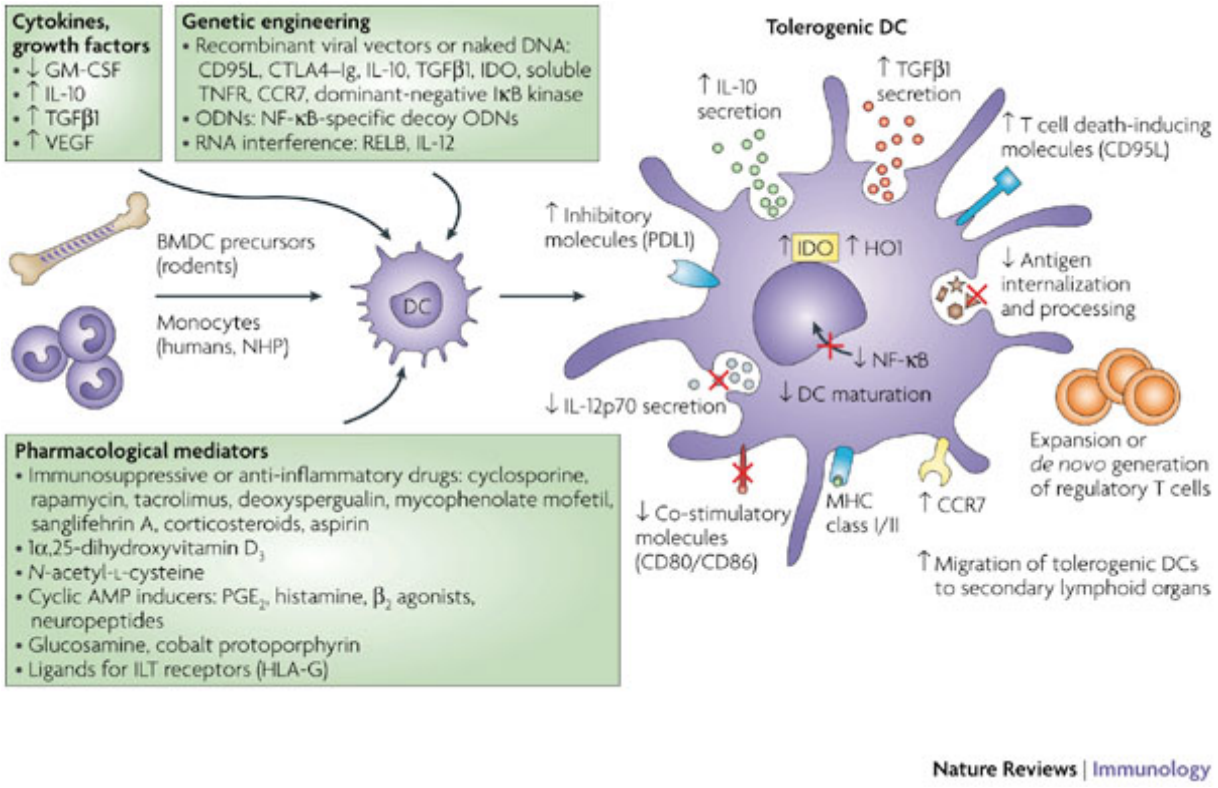


Figure 1.2 Generation of tolerogenic DCs *in vitro*.

Dendritic cells (DCs) that are generated *in vitro* from BM precursors (BMDC precursors) in rodents or blood monocytes in humans and non-human primates (NHP) have been rendered tolerogenic by controlling their culture conditions through exposure to cytokines, growth factors or pharmacological mediators, or by genetic engineering. DCs generated under such conditions can downregulate the outcome of the T-cell response by a single predominant function or, more frequently, by a combination of complementary mechanisms. CCR7, CC-chemokine receptor 7; CTLA4–Ig, cytotoxic T-lymphocyte antigen 4–immunoglobulin fusion protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; HO1, haem oxygenase-1; IDO, indoleamine 2,3-dioxygenase; IB, inhibitor of NF-κB; IL, interleukin; ILT, immunoglobulin-like transcript; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; PDL1, programmed cell death ligand 1; PGE2, prostaglandin E2; TGF1, transforming growth factor-1; TNFR, tumour-necrosis factor receptor; VEGF, vascular endothelial growth factor. From Morelli, AE and AW Thomson. 2007. *Nat Rev Immunol*. 7:610-621. [87]

1.3.3.2 Generation of tolerogenic DC

The natural ability of DC to promote and maintain tolerance to self Ags and the conditioning of DC within certain tissue microenvironments have provided cues for the generation of tolerogenic DC *in vitro* for therapeutic purposes, especially in the fields of autoimmunity and transplantation. The over-arching goal of these efforts is to generate DC that have many of the characteristics described above in section 1.3.2.1 and shown in Figure 1.2.

Cytokine-conditioned tolerogenic DC

Early studies focused on the regulatory properties of IL-10 and its ability to downregulate the stimulatory capacity of APC (macrophages and monocytes). This included negative regulation of MHC class II [92] and co-stimulatory molecule expression [93], as well as inhibition of pro-inflammatory cytokine secretion [94, 95], leading to regulation of T cell responses [96]. Eventually this work shifted to DC, as IL-10-conditioned DC were identified as tolerance-promoting cells [97]. IL-10-conditioned DC have potent immune regulatory functions that includes an enhanced ability to negatively regulate effector T cells and induction and promotion of Treg function [98-100]. TGF-β is another cytokine known to modulate DC function [101, 102]. Interestingly, when IL-10 and TGF-β are combined to generate what has been termed "alternatively-activated" dendritic cells, they acquire potent immunosuppressive function [103-105].

Exposure to other cytokines has been shown to enhance the tolerogenic properties of DC. Human monocyte-derived DC conditioned in IL-27 upregulate the expression of B7-H1 and fail to effectively stimulate T cell proliferation and cytokine production [106]. Moreover, in a model of insulin-dependent autoimmune diabetes mellitus, mice that were given DC exposed to IFN-γ were protected from disease onset [107].

Pharmacologic manipulation of DC to promote tolerance

The use of IS to inhibit immune responses in transplantation and autoimmunity led to investigation into the effects of these drugs on individual components of the immune system to determine their mechanisms of action. Although its use in the generation of tolerogenic DC began more recently than many other pharmacologic agents, rapamycin (RAPA)/sirolimus has garnered significant attention for its ability to condition DC to favor their tolerance-promoting capacity [108, 109]. Early work by our lab uncovered important features of RAPA-DC: they exhibit reduced expression of co-stimulatory molecules; are resistant to common maturation stimuli; and exhibit a reduced T cell allostimulatory capacity compared to control DC [10]. RAPA-DC also show a profound ability to spare Treg over effector T cell function, and upon adoptive transfer, can prolong experimental cardiac [110, 111], vascularized skin [112], and composite tissue allograft [113] survival.

In addition to RAPA, there has been extensive study of the influence of other IS drugs and pharmacologic agents on DC phenotype and function, and overall the effects are similar, despite different mechanisms of action. Again, as described above, these pharmacologically-modified DC are characterized by low expression of co-stimulatory molecules, resistance to maturation stimuli (including reduced upregulation of co-stimulatory molecules and decreased IL-12 production), preserved or upregulation of IL-10 production, decreased T cell stimulatory capacity, increased induction of T cell production of IL-10, and induction or expansion of Treg. Pharmacologic agents implicated in these findings include, but are not limited to: cyclosporine A (CsA) [114]; FK506/tacrolimus [115]; dexamethasone [116]; vitamin D3 [117, 118]; aspirin [119]; PGE2 [120]; mycophenolate mofetil (MMF) [121, 122]; and Sanglifehrin A (SFA) [123, 124].

There are many characteristics of cDC that make them an ideal candidate for therapeutic use in promoting tolerance, including: ease of propagating these cells *in vitro*; their propensity for acquisition, processing, and presentation of target Ags; the ability to influence their phenotype and function through pharmacologic, biologic, and genetic manipulation; the capacity to migrate to lymphoid tissues upon adoptive transfer; and their ability to regulate T cell responses and promote Treg function. Many of the studies on cDC and tolerance, however, have relied on *in vitro* or *ex vivo* manipulation to maintain cDC in an immature state and skew their functional properties in favor of immune regulation. To this end, there has been increasing investigation into whether pDC, based on their unique immunoregulatory properties which favor immune regulation and tolerance, even in the steady state and in the absence of manipulation, may have similar tolerance-promoting capacity.

1.4 PLASMACYTOID DC: A SPECIALIZED DC SUBSET

As mentioned earlier, pDC are one of the major DC subsets in the mouse. Their main function in the immune system is as the primary producers of type I IFNs during viral infection, and they effectively link innate and adaptive anti-viral responses. They display many unique characteristics that define their functional role in the immune system and distinguish them from other DC subsets.

1.4.1 pDC lineage development

The properties that define pDC may be partially explained by their unique developmental programming compared with other DC subsets (Figure 1.1). pDC arise from the same common DC progenitors as cDC [125, 126], yet they express a genetic profile that more closely resembles lymphoid (T and B) cell development [127]. Murine pDC can be readily propagated from BM cultures using Flt3L [6, 128], whereas GM-CSF and IL-4 that are used to generate cDC in traditional BMDC cultures inhibit pDC development [7]. One study has also identified M-CSF as a growth factor able to drive pDC development from BM cultures *in vitro* and *in vivo* in Flt3L-deficient mice [129].

pDC lineage commitment is controlled by expression of the transcription factor E2-2 [130], a member of the E protein family, which plays a crucial role in lymphoid cell development [131]. E2-2 regulates expression of several critical proteins expressed at high levels by pDC relative to cDC, including IFN-regulatory factor (IRF) 7 and 8 [131, 132], and the transcription factor Spi-B in human pDC [133], and may be the most important genetic determinant of pDC development. The balance and strength of E2-2 expression versus PU.1, which is critical for development of all DC subsets, has been reported to be critical for directing pDC versus cDC development [1, 14].

1.4.2 Phenotypic characterization of pDC

pDC are phenotypically distinct from cDC [134] and are characterized by expression of unique cell surface proteins. Human pDC are characterized by expression of blood DC Ag (BDCA)-2/CD303 [135], immunoglobulin (Ig)-like transcript (ILT)7 [136], and leukocyte-associated Ig-like receptor-1 (LAIR-1) [137]. They were initially described as a unique DC subset from human

monocyte-derived precursors that expressed high levels of the IL-3R and drove T cell differentiation towards a Th2 response, and thus, were termed DC2 [138, 139]. Around the same time, another group described a population of "plasmacytoid monocytes" [140] that was linked to a previously described population of high type I IFN-producing cells [141, 142], but had the ability to stimulate Th1 responses and promote anti-viral immunity.

Shortly thereafter, a murine equivalent of these cells was identified as a $CD11c^+$ DC subset capable of producing high quantities of type I IFN [143]. pDC in the mouse express lower levels of CD11c than cDC and are $B220^+Gr\text{-}1^+(Ly\text{-}6C/^+Ly\text{-}6G^+)$ and $CD123/IL\text{-}3R^-CD11b^-CD19^-$ [143, 144]. They are typically referred to as a $B220^+CD11c^{low/int}$ population of cells, however, this classification does not exclusively define mouse pDC [145]. Thus, within the bulk $CD11c^+$ DC pool, several pDC-specific markers have been identified recently and are described in detail below.

1.4.2.1 PDCA-1/BST-2/CD317/Tetherin

Plasmacytoid DC Ag (PDCA)-1 is probably the most widely recognized and commonly used marker for pDC. The overwhelming majority of studies on mouse pDC target this molecule for isolation of pDC by immunomagnetic bead isolation and for cell sorting [refs]. Anti-PDCA-1, and the previously described monoclonal Ab 120G8 [146], both recognize BM stromal cell Ag (BST)-2 [147]. In the naïve mouse, BST-2 is expressed exclusively on pDC, but is upregulated on multiple cells types following immune activation involving type I IFN [147]. BST-2 is also known as CD317/Tetherin [148], and may function to inhibit the spread of enveloped viruses by binding to them and retaining them on the surface of the cell as they replicate and are released, slowing the spread of infection. BST-2 may also be an important molecule for direct targeting of pDC *in vivo* for therapeutic enhancement of anti-viral and anti-tumor immunity [149].

1.4.2.2 Siglec-H

Sialic acid-binding Ig-like lectin (Siglec)-H, is a pDC-specific marker that plays a key role in regulating production of type I IFN [150]. Siglec-H functions as an endocytic receptor, and targeting Ags for delivery to pDC via Siglec-H offers therapeutic potential in stimulating [151] or regulating [152] immune responses. Siglec-H lacks a cytoplasmic tail, and as a result, associates with the intracellular adaptor protein, DNAX-activating protein (DAP) 12 [150]. Ab stimulation of Siglec-H blunts TLR9-mediated production of type I IFN, and DAP12-deficient mice exhibit elevated levels of type I IFN production, highlighting Siglec-H as an important molecule that regulates critical functions of pDC.

1.4.2.3 LAG-3

Lymphocyte activation gene (LAG)-3 is a CD4-like molecule [153] that is expressed by activated T and NK cells and is only exclusive for pDC within the DC compartment. Like CD4, it binds to MHC class II with high affinity and can down-regulate Ag-specific T cell stimulation [154]. LAG-3 is also expressed on $Foxp3^+$ Treg [155, 156], and has been associated with their regulatory function. It can be cleaved from the cell surface, and soluble LAG-3 (sLAG-3) can be detected in normal mouse serum [157].

Interestingly, LAG-3 mRNA was detected in tissues where T and NK cells were not normally distributed [158] and sLAG-3 was detected in $Rag1^{-/-}\gamma c^{-/-}$ mice [157] which lack T, B, and NK cells, suggesting a significant level of LAG-3 expression by another cell type. Further analysis identified LAG-3 expression within the DC compartment, and specifically identified LAG-3 on $B220^+CD11c^{int}$ cells that were $PDCA\text{-}1^+$ [159]. An extensive study on the role of LAG-3 in the functional immunobiology of pDC has not been performed. However, the fact that pDC have the capacity to suppress cDC function [160] and pDC can secrete up to five times

more sLAG-3 than activated T cells [159], suggests this could possibly be an important, previously unreported mechanism through which pDC may be able to regulate MHC class II^+ cell function.

1.4.3 pDC localization and trafficking

In the steady state, pDC can be found in the T cell areas of the spleen, LNs, and Peyer's patches [146]. cDC, on the other hand, are found in greater numbers in the marginal zone surrounding the T and B cell areas. pDC localization following activation may be dependent upon the specific TLR that is activated [161], most likely due to the resultant chemokines that are produced. Activated pDC form clusters and co-localize in the T cell areas, and the kinetics of pDC migration and localization within lymphoid tissues appear to differ from those of cDC.

Although CCR7 mediates pDC migration to LN [162], they also have a unique ability to directly enter LN from the circulation via high-endothelial venules (HEVs) [163, 164]. Several receptor-ligand interactions have been reported to contribute to pDC transmigration across HEVs [165], including multiple integrins, selectins and intracellular adhesion molecules (ICAMs) [164]. Steady-state pDC express CXCR4, a receptor for CXCL12 which is expressed on the surface of HEVs and can promote transmigration into LNs [166]. Following activation, pDC become sensitive to additional ligands that direct migration to inflamed LNs, including the CCR7 ligand CCL21, and CXCR3 ligands [167], the latter of which further enhance the response to CXCL12 [168]. These same interactions are thought to control the kinetics and localization of pDC migration in the spleen during inflammation [161].

CCR9 has been identified as the receptor for thymus-expressed chemokine (TECK/CCL25) and directs T cell trafficking during thymic development and to the epithelium

of the small intestine [169-171]. Recently, a subpopulation of $CCR9^+$ DC in the small intestine has been shown to co-express classical pDC markers (B220, PDCA1, Ly6C), secrete IFN-α, migrate to CCL25, and home to the small intestine upon adoptive transfer [172]. This same subpopulation of cells was also reported to transport peripheral Ags to the thymus, where they deleted Ag-specific thymocytes [43]. Expression of CCR9 may be a key marker for pDC that possess significant tolerogenic function, and will be discussed further in section 1.5.2.1 relating to central tolerance, and section 1.5.3.4 regarding pDC in the setting of transplantation.

1.4.4 pDC, type I IFN, and anti-viral immunity

pDC are the primary type I IFN-producing cells in humans and mice [141-143]. They express high levels of endosomal TLR 7, 8 and 9 that sense single-stranded viral RNA (ssRNA; TLR7 and 8) and unmethylated, CpG-rich viral and bacterial ssDNA (TLR9) [173]. Upon recognition of these TLR ligands, pDC rapidly induce transcription of anti-viral genes leading to the secretion of large amounts of type I IFN (up to 10 pg/cell, which is nearly 1,000 times more than any other cell type) [142], as well as the pro-inflammatory cytokines IL-6 and TNF-α [174] and up-regulation of co-stimulatory molecules such as CD80 and CD86 (Figure 1.4) [175]. pDC production of type I IFN serves as a critical link between the innate and adaptive responses to viruses through inhibition of viral replication and activation of neutrophils [176], macrophages [177], NK cells [178, 179], T cells [180], B cells [181], and cDC [182, 183]. Although their unique ability to induce such potent anti-viral immune responses through type I IFN production suggests pDC might not be suited to promote tolerance, in the steady state or in the absence of type I IFN production, pDC are well-equipped to regulate immunity.

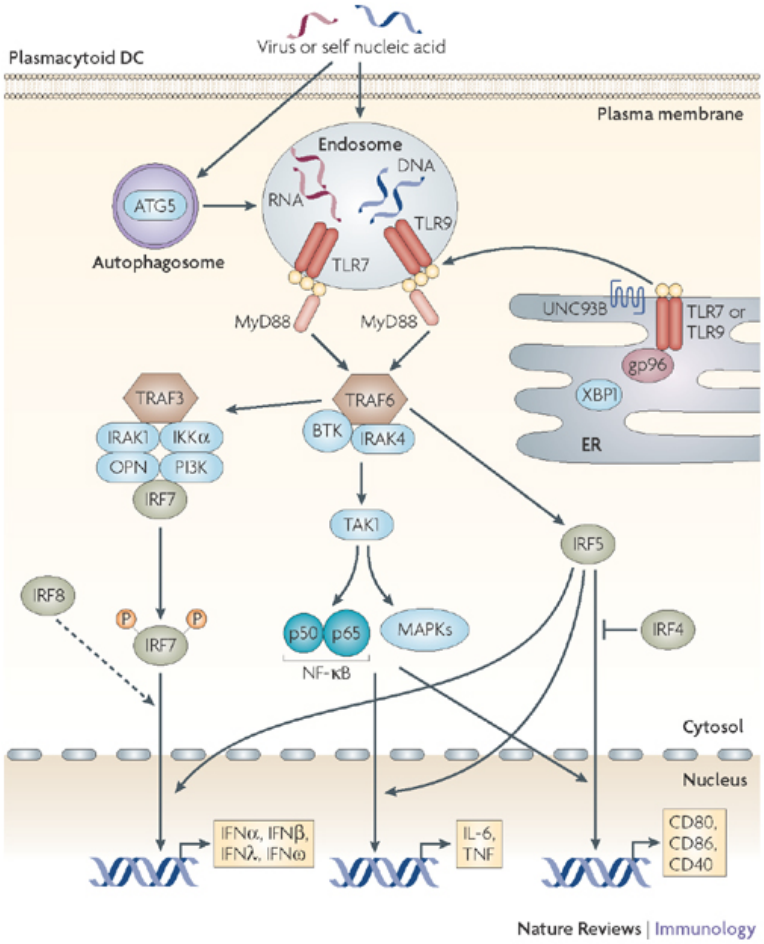


Figure 1.3 Activation pathway in plasmacytoid dendritic cells responding to nucleic acids.

Resting plasmacytoid dendritic cells (pDCs) predominantly express Toll-like receptor 7 (TLR7) and TLR9, which reside in the endoplasmic reticulum (ER) in association with UNC93B and gp96. Following exposure to virus or nucleic acids, TLR7 and TLR9 relocate from the ER to the endosomes to engage with their RNA or DNA agonists. Conformational changes in the TLRs lead to the activation of MyD88 (myeloid differentiation primary-response gene 88) and its further association with TRAF6 (tumour-necrosis factor (TNF) receptor-associated factor 6), BTK (Bruton's tyrosine kinase) and IRAK4 (interleukin-1-receptor-associated kinase 4). The MyD88–TRAF6–IRAK4 complex then activates IRF7 (interferon-regulatory factor 7), TAK1 (transforming-growth-factor--activated kinase 1), nuclear factor-kB (NF-kB) and IRF5 to propagate the downstream signals. Most importantly, IRF7 is activated through TRAF3, IRAK1, IKK (inhibitor of NF-B kinase), osteopontin (OPN) and phosphoinositide 3-kinase (PI3K). Following ubiquitylation and phosphorylation, IRF7 translocates to the nucleus and initiates the transcription of type I interferons (such as IFN, IFN, IFN and IFN). TAK1 triggers the activation of NF-B and MAPKs (mitogen-activated protein kinases) and, together with IRF5, leads to the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules. IRF8, although not involved in the initial induction of IFN, magnifies IFN production through a feedback mechanism. By contrast, IRF4 inhibits the function of IRF5 through direct competition. Autophagosomes, which are constitutively formed in pDCs via ATG5 (autophagy-related gene 5), are probably involved in transferring the nucleic-acid agonists to endosomal TLRs for the production of IFNs. IL-6, interleukin-6; XBP1, X-box-binding protein 1. From Gilliet M, Cao W, and YJ Liu. 2008 *Nat Rev Immunol*. 8:594-606. [173]

1.5 TOLEROGENIC pDC: MEDIATORS OF IMMUNE REGULATION

There are many properties of pDC that clearly define them as the primary type I IFN-producing cells during viral infection. Within hours of activation by a virus, nearly two-thirds of the genes being transcribed in pDC are related to type I IFN production and are IFN response genes [173]. They produce up to 1,000-fold more type I IFN than any other cell type which both activates and protects other cells from being infected. There are other characteristics of pDC, however, that limit their immunostimulatory capacity and make them an attractive therapeutic target to promote immune tolerance.

1.5.1 Immune regulation by pDC via the 3 signals of T cell activation

During initiation of the adaptive immune response, DC provide three critical signals to T cells that drives their expansion, differentiation, and function (section 1.2.1). Several unique characteristics of pDC regarding these three signals results in their ability to promote T cell hyporesponsiveness and regulatory function due to sub-optimal activation.

1.5.1.1 Signal 1: Regulation of MHC class II expression

Following activation and maturation, cDC are the most potent stimulators of Ag-specific T cell-mediated immune responses. This is due in part, to downregulation of the MHC II ubiquitin E3 ligase membrane-associated RING-CH1 (MARCH1) [30], resulting in formation of long-term, stable Ag:MHC II complexes on their surfaces following activation. pDC, in comparison, exhibit weaker T cell stimulatory capacity [73, 128, 184], which may be due to their failure to downregulate MARCH1 [31] following activation. Thus, Ag:MHC II complexes on pDC are

continuously ubiquitinated, internalized, and in a constant state of turnover. We tested this property of pDC by utilizing B6 $CD4^+$ transgenic (tg) T cells that have a TCR specific for BALB/c MHC class II-derived peptide (Eα52-68) presented in the context of B6 MHC class II ($I\text{-}A^b$). $B220^+$ pDC propagated from BM culture using Flt3L and pulsed with Eα52-68 peptide showed a poor ability to stimulate Ag-specific T cells, even if they were stimulated with CpG B (Figure 1.5). When peptide was instead added to pDC-T cell cultures, the extent of pDC-induced T cell proliferation was much greater when compared to pulsed pDC. These data are highlighted by the fact that unstimulated pDC in the presence of continuous Ag induced similar levels of T cell proliferation compared to peptide-pulsed pDC that were stimulated with CpG B. This unique feature of pDC most likely arose so that pDC could continuously sample viral Ags allowing them to constantly update the T cell repertoire against viruses. In a setting such as transplantation where graft-derived Ags are continuously present due to cell death and cell turnover, this characteristic of pDC could contribute to their ability to promote Ag-specific tolerance.

Several studies have shown that low levels of Ag presentation to T cells can promote Treg development [185, 186]. It is reasonable to assume then, that rapid Ag:MHC II turnover by pDC could translate into presentation of low levels of Ag (i.e. weak TCR stimulation), consequently enhancing their ability to promote Treg induction and function. Induction and support of Treg function by pDC has been reported in several models [187-189], thus, this unique characteristic of pDC suggests a previously unexplored mechanism through which they may promote tolerance via Treg. In support of this, it has been shown that silencing of the critical DC developmental transcription factor PU.1 leads to reduced MHC class II expression [190]. During development (Figure 1.1), DC precursors downregulate PU.1 as they differentiate into pDC [1] (compared to cDC which maintain high PU.1 expression), suggesting that low expression of MHC class II is

regulated transcriptionally during pDC development and differentiation, and thus, the ability to regulate effector T cell activation and promote Treg function is an inherent property of pDC.

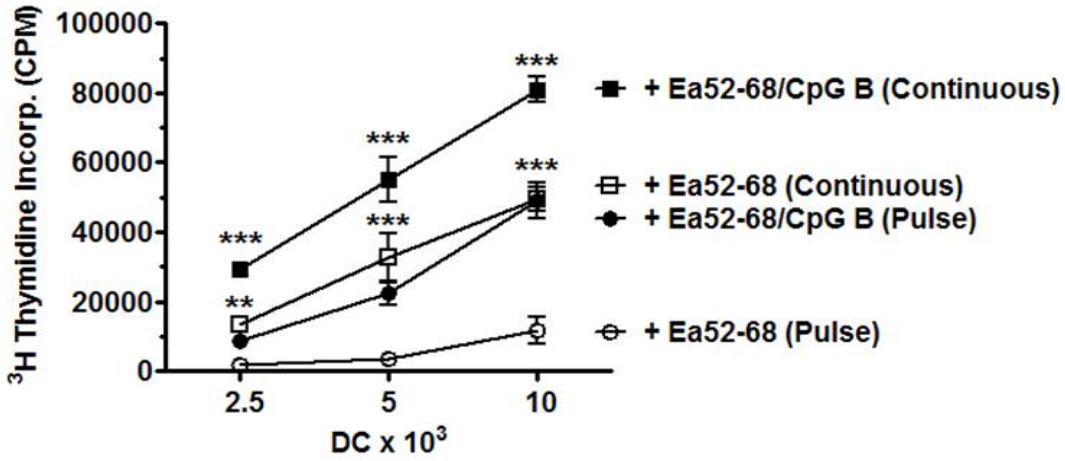


Figure 1.4 T cell stimulatory capacity of pDC is increased upon continuous exposure to Ag.

pDC were propagated from B6 BM using Flt3L in 10 d culture. pDC were isolated by B220 positive selection using immunomagnetic bead isolation. Cells were cultured for 18 h in the absence or presence of BALB/c MHC class II-derived peptide $E\alpha_{52\text{-}68}$ and 100 ng/ml CpG B. Cells were collected, washed, counted, and cultured with $CD4^+$ TEa tg T cells for 3 d. Unstimulated and CpG B-stimulated pDC that were not pulsed overnight received peptide in culture with T cells for the duration of the assay. Proliferation was quantified by radioisotope incorporation using a liquid scintillation counter. Statistical significance was calculated for the difference between Ea52-68 (Pulse; open circles) versus Ea52-68 (Continuous; open squares), and Ea52-68/CpG B (Pulse; filled circles) versus Ea52-68/CpG B (Continuous; filled squares). ** $p \leq 0.01$, *** $p \leq 0.001$.

1.5.1.2 Signal 2: CD86 versus B7-H1: Tipping the balance towards immune regulation

Despite their ability to regulate immune responses, DC remain the most potent inducers of T cell-mediated immunity. An important component of their function is the expression of co-stimulatory molecules that support T cell activation by providing signal 2. It is recognized, however, that the outcome of T cell responses is ultimately determined by the balance of co-

stimulatory versus co-inhibitory molecules expressed by DC [191, 192]. To this end, there are two key factors that underscore the regulatory nature of pDC: first, they exhibit a low expression of co-stimulatory molecules; and second, they display relatively high expression of the co-inhibitory molecule B7-H1 [128]. Further evaluation of the B7-H1:CD80 and B7-H1:CD86 ratios shows that pDC express comparatively high ratios relative to cDC [69, 128]. To highlight the significance of this, we have noted that the B7-H1:CD86 ratio on pDC is significantly higher in tolerant pediatric liver transplant patients that were successfully weaned from IS compared to those on maintenance IS, and this high B7-H1:CD86 ratio correlates with an increased frequency of $CD4^{+}CD25^{hi}$ Treg [193]. B7-H1 is a well-known negative regulator of T cell responses that has been implicated in an immune regulatory role [194] in many disease states and experimental models, and will be discussed in greater detail in section 3.2.3.

1.5.1.3 Signal 3: Pro- vs. anti-inflammatory cytokine production

In addition to production of type I IFNs, pDC also produce other cytokines that drive T cell differentiation and regulation. Upon recognition of various pathogens, pDC secrete IL-6 and TNF-α [195], IL-12 [196] and IL-10 [72]. Contributing to their weak T cell stimulatory capacity relative to cDC, pDC have been shown to produce less bioactive IL-12p70 compared to cDC [69]. Moreover, certain tissues such as the liver, may condition pDC to acquire a more tolerogenic phenotype relative to pDC in lymphoid tissues, and thus their cytokine production may be skewed in favor of immune regulation (e.g., liver pDC produce greater levels of IL-10 and less IL-12 compared to pDC from the spleen) [72].

1.5.2 Regulating immune responses: pDC in the steady state and pathologic conditions

The unique properties and multitude of regulatory mechanisms inherent in pDC suggest they may be important in preventing unwanted immune responses in the maintenance of central and peripheral tolerance as described above in section 1.3.1. Moreover, their potent immunoregulatory function may be targeted for promotion of tolerance in the setting of transplantation and autoimmunity. In contrast, immunoregulation by pDC can have a negative impact on health and disease by suppressing desired immune responses, such as in the setting of cancer.

1.5.2.1 Regulation of central tolerance: pDC impact T cell selection in the thymus

Two recent reports suggest that, like thymic cDC, human thymic pDC can drive natural (n) Treg development. Thus, Martin-Gayo et al [197] have shown that, following activation with CD40L and IL-3, mature thymic pDC efficiently promote the generation of $CD4^+CD25^+Foxp3^+$ nTreg from autologous, positively-selected $CD4^+CD8^+$ thymocytes. The nTreg induced by pDC are better IL-10 producers and inferior TGF-β producers compared with those induced by thymic cDC, reflecting the ability of the two major DC subsets to induce distinct Treg repertoires, based on their distinct Ag-presenting capacities [31]. Studies by Hanabuchi et al [198] have further shown that activated human thymic medullary pDC express the thymic stromal lymphopoietin (TSLP) receptor and become responsive to TSLP, inducing the generation of $Foxp3^+$ Treg from $CD4^+CD8^-CD25^-$ thymocytes. As in the former study, these Treg expressed more IL-10 but less TGF-β than those induced by TSLP-stimulated cDC.

Although it was shown previously for cDC [42], it is now recognized that pDC can acquire Ag in the periphery and migrate to the thymus to participate in T cell selection [43].

Their ability to traffic to the thymus was dependent on expression of the chemokine receptor CCR9, consistent with $CCR9^+$ pDC being a tolerogenic population (see section 1.5.2.4). Taken together, these novel data suggest that pDC play a critical role in maintenance of central tolerance, through elimination of autoreactive (Ag-specific) T cells and selection of Treg that can preferentially secrete IL-10 in response to self Ag in the periphery.

1.5.2.2 Autoimmunity: Regulation of unwanted immune reactivity against self-antigens

Type-I IFN production by pDC has been implicated in the pathogenesis of autoimmune diseases, such as psoriasis, systemic lupus erythematosus (SLE), arthritis and type-1 diabetes [199]. Under normal, healthy conditions, the intracellular localization of TLR9, high concentration of extracellular DNases and low levels of unmethylated CpG motifs within mammalian DNA prevent pDC from sensing self DNA and secreting type-I IFN. However, several host factors can break down innate tolerance to self DNA, resulting in autoimmunity. For example, the anti-microbial peptide LL37 and nuclear protein HMGB1, released by damaged and/or dying cells, bind self DNA fragments also released by dying cells to form aggregates that are protected from extracellular DNase degradation and delivered into early endosomes of pDC [200, 201]. Moreover, DNA-specific Abs bind self-DNA-LL37-HMGB1 complexes, increasing their translocation into TLR9-containing endosomes. The outcome of these responses is increased IFN-α production, activation of cDC, and promotion of autoreactive T cell responses.

Although recent observations show that pDC are involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), by promoting the induction of myelin oligodendrocyte glycoprotein (MOG)-specific Th17 cells [202], several reports suggest that tolerogenic pDC may regulate the severity of autoimmune disease (Table 1.1). Thus, mature pDC from rheumatoid arthritis patients with low disease activity express high levels of the

tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) and promote the differentiation of naïve $CD4^+CD25^-$ T cells into IL-10-secreting Treg or T regulatory type-1 (Tr1) cells [203]. Moreover, *in vivo* depletion of pDC exacerbates joint pathology and augments cell-mediated and humoral immunity to type II collagen in experimental arthritis [204], and enhances disease pathology in a model of EAE [205]. The regulatory role of pDC in EAE was dependent on suppression of IL-17- and IFN-γ-producing T cells through production of IDO. In an independent study, mice lacking MHC class II on pDC also show enhanced EAE severity due to lack of Ag presentation by pDC in LN and failure to induce myelin-Ag-specific Treg [206]. In support of these observations, targeting myelin Ags to pDC via Siglec-H led to inhibition of T cell-mediated development of EAE [152].

In a murine model of lupus nephritis, low dose peptide therapy induces TGF-β production by pDC, expansion of autoAg-specific Treg and reduction of kidney-infiltrating inflammatory Th17 cells [185]. Moreover, a protective effect of pDC against TCR transgenic T cell-induced type-1 diabetes (T1D) in NOD mice has been demonstrated [207]. It has also been shown in both viral-induced and spontaneous models of T1D that pDC in the pancreatic LN can be induced to produce TGF-β, which contributes to the conversion of islet-specific effector $CD4^+$ T cells into $Foxp3^+$ Treg that can prevent the development of T1D [208]. Thus, pDC appear to play important roles in regulation of several experimental autoimmune disease states and may serve as potential targets for restoration of tolerance to self Ags.

Table 1.1 pDC and the regulation of autoimmunity.

Description of pDC	Disease/Model	Mechanism(s)	Reference
BDCA-4$^+$ (CD304) Isolated from PBMC	Rheumatoid Arthritis (Human)	IDO-dependent induction of IL-10-producing Treg	[203]
120G8 mAb depletion of pDC	Experimental Arthritis (Mouse)	Systemic depletion of pDC enhanced cellular (T cell) and humoral (B cell) autoimmune responses;	[204]
mPDCA-1$^+$Ly-6C$^+$ Isolation by negative selection using Plasmacytoid Dendritic Cell isolation kit	Experimental Lupus (Mouse)	Adoptive transfer of pDC reduced autoantibodies and T cell production of IFNγ and IL-17; T cells produced greater levels of TGFβ and pDC produced TGFβ but less IL-6; pDC enhanced function of CD4$^+$CD25$^+$ and CD8$^+$ Treg	[185]
mPDCA-1 mAb depletion of pDC	NOD model of type I diabetes (T1D) (Mouse)	Systemic depletion of pDC enhances disease progression and severity; IDO contributes to disease regulation	[207]
120G8$^+$CD11c$^+$ From pancreatic LN cells	Viral-induced and spontaneous T1D (Mouse)	Induction of TGF-β production in pancreatic LN pDC by NKT cells results in conversion of anti-islet effector CD4$^+$ T cells into Foxp3$^+$ Treg in pancreatic LN which prevents T1D development	[208]
Siglec-H$^+$CD11c^{lo} or BST2$^+$CD11c^{lo} Spleen and LN	Experimental Autoimmune Encephalomyelitis (EAE) (Mouse)	Siglec-H-mediated targeting of MOG peptide to pDC *in vivo*; induction of CD4$^+$ T cell hyporesponsiveness and decreased Th1 and Th17; reduced severity of EAE	[149]
B220$^+$CD11c^{int}CD19$^-$ Flow sorted from pooled LN cells	EAE (Mouse)	Lack of MHC class II on pDC increases disease severity due to loss of interaction with Ag-specific CD4$^+$ T cells in LN	[206]

1.5.2.3 Mucosal tolerance: pDC regulate immunity in gut and airway mucosa

DC play an important role in maintaining tolerance to harmless inhaled or ingested Ags in mucosal tissues. Thus, several reports have recently implicated pDC in the induction and regulation of oral tolerance. The liver is a site of presentation of ingested Ags, and liver pDC appear to rapidly induce anergy or deletion of Ag-specific T cells via a CD4$^+$ T cell-independent

mechanism. Goubier et al [209] have shown that pDC prevent fed Ag-induced T cell priming and are responsible for systemic tolerance to $CD4^+$ and $CD8^+$ T cell-mediated delayed-type hypersensitivity (DTH) responses induced by Ag feeding. Depletion of pDC *in vivo* prevents the induction of tolerance by Ag feeding, and transfer of oral Ag-loaded liver pDC to naive recipient mice induces Ag-specific suppression of $CD4^+$ and $CD8^+$ T cell responses. The anatomical and immunological location of the induction of oral tolerance likely occurs in the mesenteric LN, Peyer's Patches, and liver, however, the presence of significant numbers of pDC in the tonsils that can induce and co-localize with $Foxp3^+$ Treg [210] suggests they may act very early on during induction of oral tolerance. These data demonstrate that oral tolerance relies on Ag presentation by pDC to T cells, and suggest that pDC could represent a key therapeutic target for intestinal and systemic inflammatory diseases.

It has also been shown that systemic depletion of pDC during inhalation of inert Ag leads to classic features of asthma: IgE sensitization, airway eosinophilia, goblet cell hyperplasia, and Th2 cytokine production [211]. Adoptive transfer of pDC prior to sensitization abrogates this effect, likely by inhibiting the generation of effector T cells induced by Ag-presenting cDC. In a model of *Chlamydia pneumonia* infection, pDC depletion during airway challenge allowed Ag sensitization to occur leading to asthma [212], suggesting that pDC play an important role to suppress asthma development as a result of pulmonary infection. In another model of airway tolerance induction, DC recovered from tolerized mice were dominated by $B220^+CD11c^+Gr\text{-}1^+CD11b^-$MHC class II^{low} cells that were poor T cell stimulators in MLR [213]. Together, the foregoing observations show that pDC can protect against unwanted inflammatory responses to harmless Ags and play a significant role in the development of tolerance at mucosal tissues.

1.5.2.4 Transplantation tolerance

Although the concept that pDC have potential to promote transplant tolerance has emerged in recent years [188, 214-216], the role of pDC in experimental transplant tolerance remains poorly characterized. Reports of the influence of pDC on transplant outcome are summarized in Table 1.2. Two of the first reports to highlight the importance of pDC in alloimmune tolerance identified these cells as a critical and necessary component of a population of facilitating cells (FC) in BM that could promote hematopoietic stem cell engraftment [217] and induce Ag-specific Treg *in vivo* [218]. More recently, this population of FC was shown to facilitate human kidney allograft tolerance with no incidence of GVH disease [219]. Adoptive transfer of donor-derived pre-pDC suppresses GVH disease through IDO-dependent promotion of Treg function [220]. This may be an important mechanism through which pDC are able to promote allograft tolerance that is not dependent on donor Ag specificity. Highlighting this point, pDC propagated from donor strain, recipient strain, and even third-party BM using Flt3L could prolong murine cardiac allograft survival [215]. Another potential mechanism for this phenomenon is the induction of IL-10-producing T cells by pDC via ICOS:ICOSL (B7RP-1) interaction [221, 222].

Flt3L-mobilized splenic pDC of donor origin significantly prolong experimental cardiac allograft survival in the absence of IS or in combination with anti-CD154 (CD40L) mAb therapy [223]. Studies suggest, however, that recipient DC/APC may be more important in chronic rejection (and thus, more appropriate targets for therapeutic intervention), since donor DC have a finite life span in the recipient and are not replenished. In an experimental model of cardiac allograft tolerance induced by donor-specific transfusion and anti-CD154 mAb, recipient PDCA-1^+ pDC acquired donor MHC II-derived alloAg from the graft and migrated to LN (but not spleen), where they induced alloAg-specific $CD4^+Foxp3^+$ Treg necessary for tolerance induction

[188]. Depletion of pDC or prevention of pDC homing to LN inhibited Treg development and tolerance induction. Moreover, in CCR7-deficient mice that almost completely lack pDC in the LN, adoptive transfer of syngeneic pDC (but not cDC) restores cardiac allograft tolerance induced by DST and anti-CD40L/MR1 [224]. Interestingly, in a rat model of cardiac allograft tolerance induced by CD40Ig, pDC accumulated in the graft and the spleen, but not LN, and induced $CD8^+$ Treg [225]. Regulation of alloreactive $CD4^+$ T cells by pDC occurred either directly, through an IDO-dependent mechanism, or indirectly through $CD8^+$ Treg, in a contact-dependent manner. Similar results were observed for human pDC, as they induced a population of $CD8^+$ Treg in an IDO-dependent manner that could suppress alloreactive T cells [226].

We have reported that analysis of peripheral blood DC, especially pDC, in liver transplant patients can be a helpful immune monitoring tool. Consistent with the more tolerogenic properties of pDC, higher incidences of circulating pDC relative to cDC are found in operationally tolerant pediatric liver allograft recipients, and in patients on low dose IS therapy undergoing prospective drug weaning, compared with patients on maintenance IS [227]. In addition, high PD-L1:CD86 ratios on pDC correlate with elevated $CD4^+CD25^{hi}Foxp3^+$ Treg in tolerant patients [193], consistent with evidence that the balance between expression of inhibitory PD-L1 and co-stimulatory B7-1 (CD80)/B7-2 (CD86) ligands regulates the outcome of their interaction with T cells [191, 192]. Further emphasizing their importance, a lower ratio of pDC to cDC was associated with rejection in pediatric small bowel transplant recipients, suggesting that greater frequencies of pDC following transplant are critical for graft survival [228].

Table 1.2 pDC and transplantation tolerance.

Tolerogenic pDC	Model	Mechanism(s)	Reference
$B220^+CD11c^{lo}CD11b^-$ ($CD8^+TCR^-$) Bone marrow or peripheral blood-derived, expanded using Flt3L, flow sorted	Hematopoietic Stem Cell (HSC) Transplantation (Mouse); Combined kidney and HSC transplantation (Human)	Not reported; pDC may require interaction with effort from other cells within $CD8^+TCR^-$ population - pDC alone are not sufficient to promote engraftment; may contribute to ability of FC to promote induction of Ag-specific $CD4^+Foxp3^+$ Treg	[217-219]
$B220^+Lin^-CD11c^+PDCA1^+$ Flow-sorted from bone marrow	HSC Transplantation (Mouse)	T cell-derived IFN-γ induces IDO production by donor pDC which reduces GVH disease by promoting Treg and inhibiting effector T cell functions	[220]
$CCR9^+B220^+CD11c^{int}$ ($CD3^-CD19^-$) Mobilized using Flt3L-secreting B16 melanoma, flow sorted from CD11c bead-purified LN cells	Graft-Versus-Host Disease (Mouse)	Expansion of $Foxp3^+$ Treg; suppression of IL-17-producing Th17 pro-inflammatory cells; low T cell stimulatory capacity/T cell hyporesponsiveness	[229]
$B220^+CD11c^+CD11b^-$ Flow-sorted from Flt3L expanded BM cells (Donor)	Cardiac Allograft (Mouse)	Induction of T cell hyporesonsiveness; regulation of T cell responses by expression of B7-H1	[215]
$B220^+CD11c^+CD11b^-$ Flow sorted from Flt3L-mobilized, splenic CD11c purified cells (Donor)	Cardiac Allograft (Mouse)	Infusion of donor pDC combined with anti-CD40L prolongs cardiac allograft survival; mechanism not reported	[223]
$PDCA\text{-}1^+B220^+CD11c^+Gr\text{-}1^+$ ($CD19^-$) Host pDC within the allograft, host lymph node and spleen	Cardiac Allograft (Mouse)	pDC acquire donor antigen from the graft and induce donor Ag-specific $Foxp3^+$ Treg in the host lymph node	[188]
$PDCA1^+B220^{int}CD11c^{int}$ Mobilized using Flt3L-secreting B16 melanoma, magnetic bead isolation using pDC isolation kit from spleen and LNs (Syngeneic with recipient)	Cardiac Allograft (Mouse)	Recipient pDC are required in LN for anti-CD40L/DST induced cardiac allograft tolerance; may be important for induction of $Foxp3^+$ Treg	[224]
$CD45R^+/B220^+CD4^+$ ($TCR^-CD45RA^-$) Flow sorted from gradient-enriched splenocytes	Cardiac Allograft (Rat)	Accumulation of pDC in allograft and spleen; suppression of $CD4^+$ T cells through induction of $CD8^+$ Treg and IDO	[221]
$CD123^+HLA^-DR^{hi}Lin^-$ Flow cytometric analysis of patient whole blood samples	Liver Allograft (Human)	Elevated pDC:cDC ratio and elevated B7-H1 expression on pDC correlates with increased frequency of circulating $CD4^+Foxp3^+$ Treg, and is associated with successful withdrawal from IS compared to patients on maintenance IS	[193, 227, 228]

As mentioned earlier, CCR9 is a chemokine receptor expressed by pDC that directs their migration to the thymus and small intestine [172]. $CCR9^+$ pDC express very low levels of Ag-presenting (MHC II) and co-stimulatory molecules, a phenotype associated with DC immaturity or tolerogenicity [229]. When LN $B220^+CD11c^+$ DC were sorted based on CCR9 expression, pulsed with OVA and cultured with $CD4^+$ T cells, the $CCR9^+$ pDC failed to induce $CD4^+$ T cell proliferation *in vitro* and failed to prime T cells *in vivo*, suggesting that these OVA-loaded pDC could promote Ag-specific T cell tolerance [229]. The potent tolerogenic potential of $CCR9^+$ pDC is evidenced by their capacity to suppress acute graft-versus-host (GVH) disease when co-transferred into lethally-irradiated recipients with allogeneic $CD4^+CD25^-$ effector T cells [229]. One mechanism to account for these observations is an enhanced ability of $CCR9^+$ pDC to induce highly suppressive $Foxp3^+$ ($CD4^+CD25^+$) Treg and to inhibit the outgrowth of inflammatory IL-17-producing T cells [229]. Further investigation has revealed that $B220^+CCR9^-$ cells are precursors of cDC, suggesting that, in conjunction with other classical pDC markers, CCR9 may be a key marker for terminally-differentiated pDC and does not necessarily indicate a pDC subpopulation [230]. Collectively, the evidence from experimental and clinical studies suggests that pDC have potent regulatory capacity in transplantation, including multiple mechanisms that are independent of donor Ag, and clearly support a role for pDC in promoting transplantation tolerance.

1.5.2.5 Immune regulation in cancer

Due to their ability to secrete high levels of type-I IFN and TNF-α, pDC would appear to have the potential to promote anti-tumor immunity through activation of NK cells and $CD8^+$ T cells with potent cytolytic function. On the contrary however, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape (Figure 1.6). Tumor-infiltrating pDC are

associated with poor prognosis [231], and are recruited into the tumor microenvironment (Figure 1.6A) via several receptor:ligand interactions, including CCL20:CCR6, stromal cell-derived factor (SDF)-1/CXCL12:CXCR4, and very late Ag (VLA)-5:vascular cell adhesion molecule (VCAM)-1 interactions [232, 233]. pDC exposed to the tumor microenvironment are protected from tumor macrophage IL-10-induced apoptosis; they exhibit reduced IFN-α production upon TLR9 stimulation and can induce IL-10-producing $CD4^+$ and $CD8^+$ Treg [234-236]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the tumor, and by limiting anti-tumor cytolytic activity through induction of regulatory $CD4^+$ and $CD8^+$ T cells.

In addition to intratumoral immune regulation, pDC also exhibit immunoregulatory properties in tumor-draining LN (TDLN) (Figure 1.6B). A subpopulation of $CD19^+$ pDC in TDLNs produces high levels of IDO [237]. Production of IDO by pDC has been linked directly to activation of naturally-occurring $Foxp3^+$ Treg through modulation of the general control non-depressive kinase 2 (GCN2) pathway, which leads to inhibition of protein synthesis and Treg activation [187]. IDO-activated Treg can suppress effector T cell responses directly, or indirectly, via the PD-L1:PD-1 pathway [187]. IDO plays a dual regulatory role by preventing conversion of these Treg into pro-inflammatory Th17 cells through autocrine inhibition of IL-6 production via upregulation of GCN2 in pDC [238]. IDO has also been reported to inhibit production of IFN-α, which may limit their ability to activate innate and adaptive anti-tumor immunity [239]. In relation to other tolerogenic pDC populations already described, it would be of interest to determine if the reported $CD19^+$ IDO-producing subpopulation of pDC in TDLN corresponds to the tolerogenic $CCR9^+$ pDC reported to induce $Foxp3^+$ Treg, inhibit Th17 development and suppress GVH disease.

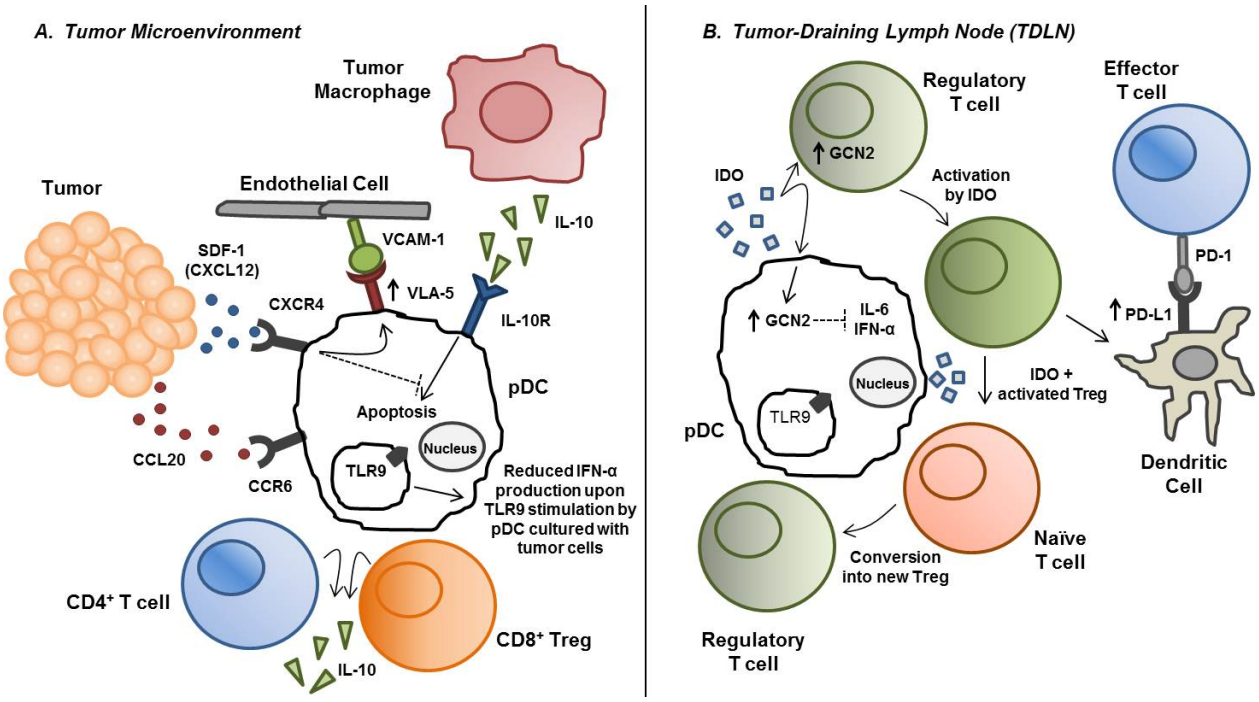


Figure 1.5 pDC regulate anti-tumor immunity and can promote tumor escape.

A, pDC are recruited into the tumor microenvironment via multiple receptor–ligand interactions. Interaction of stromal cell-derived factor-1/ CXCL12 with CXCR4 on pDC can upregulate very late Ag-5 for transendothelial migration via vascular cell adhesion molecule-1 and can protect pDC from IL-10-induced apoptosis. CCL20 binding to CCR6 on pDC can recruit pDC to the tumor site. Factors produced within the tumor microenvironment can modulate IFN-α production via TLR9 stimulation. pDC cultured with tumor cells can induce IL-10 production by CD41 T cells and CD81 Treg. **B**, CD191 pDC in TDLN produce high levels of IDO, which can activate mature Treg via activation of the GCN2 pathway of protein synthesis inhibition. Activated Treg can then activate other DC, leading to upregulation of PD-L1, which suppresses anti-tumor T-cell responses. pDC-produced IDO and activated Treg can also convert naïve T cells into new Treg. IDO acts in an autocrine manner to suppress pDC production of IL-6, which prevents the conversion of Treg into IL-17-producing Th17 pro-inflammatory cells. IDO also downregulates IFN-α production by pDC. From Matta BM, Castellaneta AC, and AW Thomson. 2009. *Eur J Immunol*. 40:2667-2676. [240]

1.5.2.6 Conditioning pDC for tolerance

Although steady-state pDC exhibit many features of classical tolerogenic DC (Figure 1.2 and section 1.3.2.1), studies have investigated the effects of *in vitro* and *ex vivo* manipulation of pDC using pharmacologic and biologic agents. Human pDC treated with tacrolimus/FK506 prior to

TLR9 stimulation failed to upregulate co-stimulatory molecules and HLA-DR to the same extent as untreated controls, and showed impaired production of TNF-α [241]. The PGE2 analog iloprost showed similar effects on pDC through suppression of TNF-α and IFN-α production following stimulation with CpG [242], while enhancing their regulatory potential through upregulation of IL-10 production. PGE2-treated human pDC secreted less IFN-α and skewed T cell differentiation towards Th2 cytokine production [243], highlighting the potential for PGE2 in targeting pDC to regulate Th1-mediated pathologies. In a recent study, cDC that were cultured with T cells that constitutively secreted high amounts of TGF-β acquired a "plasmacytoid-like" phenotype, and upregulated secretion of the immunoregulatory cytokine IL-27 [244]. In correlation with these findings, pDC exposed to TGF-β exhibited enhanced regulatory function, including elevated expression of IL-27, as well as a greater capacity for induction of $Foxp3^+$ Treg [245]. Together, these data suggest that the inflammatory and regulatory functions of pDC may be manipulated for therapeutic purposes.

1.6 SPECIFIC AIMS

1.6.1 Specific Aim I (Chapter 3): To evaluate the contribution of B7-H1 to the immune regulatory capacity of splenic pDC

The development of cell-based therapies for translation to clinical use has been studied extensively, and due to their ability to deliver Ag-specific signals, DC have garnered much of this attention. Recent data have highlighted the immune regulatory capacity of pDC (section 1.5) in the steady state and various disease pathologies, and suggest several mechanisms contributing

to their tolerogenic capacity. We demonstrate that splenic pDC are immature APC that exhibit a distinctly different cell-surface phenotype compared to splenic cDC, characterized by lower expression of Ag-presenting and co-stimulatory molecules which correlates with their weak T cell stimulatory capacity. We have also identified B7-H1 as a critical molecule that regulates the allostimulatory capacity of splenic pDC. The high expression of B7-H1 relative to co-stimulatory molecule expression (CD40, CD80, and CD86) limits the ability of splenic pDC to induce potent allogeneic T cell activation and proliferation, both *in vitro* and *in vivo*.

1.6.2 Specific Aim II (Chapter 4): To investigate the mechanisms that contribute to the inherent tolerogenicity of liver pDC

Liver pDC have been described as an important regulatory cell population able to suppress Ag-specific DTH responses and that is critical for the development of oral tolerance. The mechanisms underlying these phenomena have not yet been reported. We show that the immunoregulatory cytokine IL-27 plays a critical regulatory role in the functional biology of liver pDC by promoting expression of B7-H1 which subsequently increases the incidence of $CD4^+$ T cells expressing Foxp3 in MLR. The effects of IL-27 on liver compared to spleen pDC suggest the potential for unique signaling pathways in these cells which may be critical in regulating their tolerogenic capacity. Our data also show that IL-27 produced by liver pDC also restrains their ability to efficiently stimulate immune responses *in vitro* and *in vivo*.

1.6.3 Specific Aim III (Chapter 5): To determine the necessity for donor liver pDC in the spontaneous acceptance of fully MHC-mismatched murine liver allografts

Highlighting the inherent tolerogenic nature of the liver, liver allografts are accepted spontaneously in the absence of IS treatment in fully MHC-mismatched strain combinations in rodents. Moreover, induction of tolerance in human liver allograft recipients typically requires less IS drug therapy compared to other solid organ allografts. We demonstrate for the first time that donor liver pDC are required for spontaneous and indefinite survival of mouse liver allografts. The weak ability of liver pDC to prime immune responses to alloAgs and high expression of important adhesion molecules suggests that as liver pDC migrate from the donor liver into recipient lymphoid tissues, they may be a critical component for the development of tolerance to liver allografts.

2.0 MATERIALS AND METHODS

The following is a comprehensive description of all mice, reagents, experimental protocols, and statistical methods used to generate all of the data contained in this dissertation and publications resulting from work presented herein.

2.1 MICE

Six-week old C57BL/6 (B6; $H2^b$), C3H/HeJ (C3H; $H2^k$), and BALB/cByJ (BALB/c; $H2^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh School of Medicine. $Ebi3^{-/-}$ mice (Ebi3tm1Rsb/J) purchased from Jackson and B7-$H1^{-/-}$ mice (breeding pairs kindly provided by Dr. Lieping Chen, Yale University, New Haven, CT), both on a B6 background, were bred and maintained at the University of Pittsburgh. Experiments were conducted under an Institutional Animal Care and Use Committee (IACUC)-approved protocol and in accordance with National Institutes of Health guidelines. The animals were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received tap water *ad libitum*.

2.2 MEDIA AND REAGENTS

The endogenous DC poietin recombinant human Flt3L (CHO cell-derived) was obtained from Amgen (Seattle, WA). Complete medium was composed of RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 10% v/v fetal calf serum (Nalgene, Miami, FL), non-essential amino acids, L-glutamine, sodium pyruvate, penicillin–streptomycin, and 2-mercaptoethanol (all from Life Technologies, Gaithersburg, MD). The TLR9 ligand CpG type B oligodinucleotide (ODN) 1826, certified endotoxin free, was obtained from Invivogen (San Diego, CA). Low-endotoxin, azide free (LEAF) anti-B7-H1 neutralizing and LEAF Rat IgG2b isotype control Abs were purchased from BioLegend (San Diego, CA). Recombinant murine IL-27 was purchased from eBioscience (San Diego, CA) and STAT3 Inhibitor VII and the STAT3 inhibitor JSI-124/Cucurbitacin were purchased from Calbiochem/EMD Chemicals (Gibbstown, NJ). 120G8.04 mAb for *in vivo* depletion of pDC was purchased from IMGENEX (San Diego, CA).

2.3 FLT3L-MOBILIZATION AND PURIFICATION OF DC SUBSETS

pDC were purified as described [69]. Livers and spleens were harvested from mice administered Flt3L (10 μg/mouse/day; 10 days) and digested in collagenase (Sigma, St. Louis, MO). DC were enriched from total liver non-parenchymal cells or splenocytes by density gradient centrifugation using Histodenz (30% for liver, 16% for spleen) (Sigma). Liver and spleen cells were depleted of $NK1.1^+$ cells to remove NK cells and NKDC [145] by incubation with biotin-conjugated NK1.1 (BD Pharmingen, San Diego, CA) and anti-biotin magnetic microbeads (Miltenyi Biotec,

Auburn, CA), followed by depletion on an LS column (Miltenyi Biotec). pDC were positively selected using PDCA-1 magnetic microbeads (Miltenyi Biotec). Purity was consistently between 93 and 97%, where the majority of contaminating cells were $B220^{-}CD11c^{+}$ cDC, with negligible contamination by $B220^{+}CD11c^{-}$ B cells, as reported previously [69]. In some experiments, cDC were isolated from $NK1.1^{-}PDCA\text{-}1^{-}$ cells by CD11c positive selection and stimulated with the TLR4 ligand LPS at 1 μg/ml.

2.4 FLOW CYTOMETRIC ANALYSIS OF DC PHENOTYPE

Liver and spleen DC were incubated in 5% v/v normal goat serum and FcγR-blocking Ab (anti-CD16/32, BD Pharmingen) to block non-specific binding. For cell surface staining, cells were analyzed by 5-color analysis using FITC-, PE-, APC-/AlexaFluor647-, Pacific Blue-, and PE/Cy7-conjugated Abs. For phenotypic analysis of DC, surface expression of MHC class I (K^{b}) and II ($I\text{-}A^{b}$), CD40, CD80, CD86, B7-H1 (PD-L1), B7-DC (PD-L2), ICOSL (B7RP), Siglec-H, CCR9, LAG-3, and WSX-1/IL-27Rα was quantified on a population of $B220^{+}CD11c^{low}$ (PDCA-1-purified pDC) or $B220^{-}CD11c^{+}$ (CD11c-puified cDC) gated cells. . The gating strategy and expression of B220/CD45R, CD11c, and Siglec-H on purified $PDCA\text{-}1^{+}$ pDC is shown in Figure 2.1.

2.5 INTRACELLULAR CYTOKINE AND FOXP3 STAINING

Purified PDCA-1$^+$ pDC were cultured for 18 h with 1 μg/ml CpG B ODN or 10-25 ng/ml IL-27 and treated with Golgi Plug Protein Transport Inhibitor (1 μl/ml; BD PharMingen) for the final 5 h. The cells were labeled for surface proteins as described above. For analysis of cytokine production, cells were fixed with 4% v/v paraformaldehyde and permeabilized using 0.1% saponin. pDC were stained for intracellular expression of IL-27p28 (BioLegend, San Diego, CA) and IL-12p35 (R&D Systems, Minneapolis, MN) and T cells stained for expression of Foxp3. For analysis of Foxp3 expression following cell surface staining, cells were incubated overnight using the Foxp3 Fix/Perm kit (eBioscience).

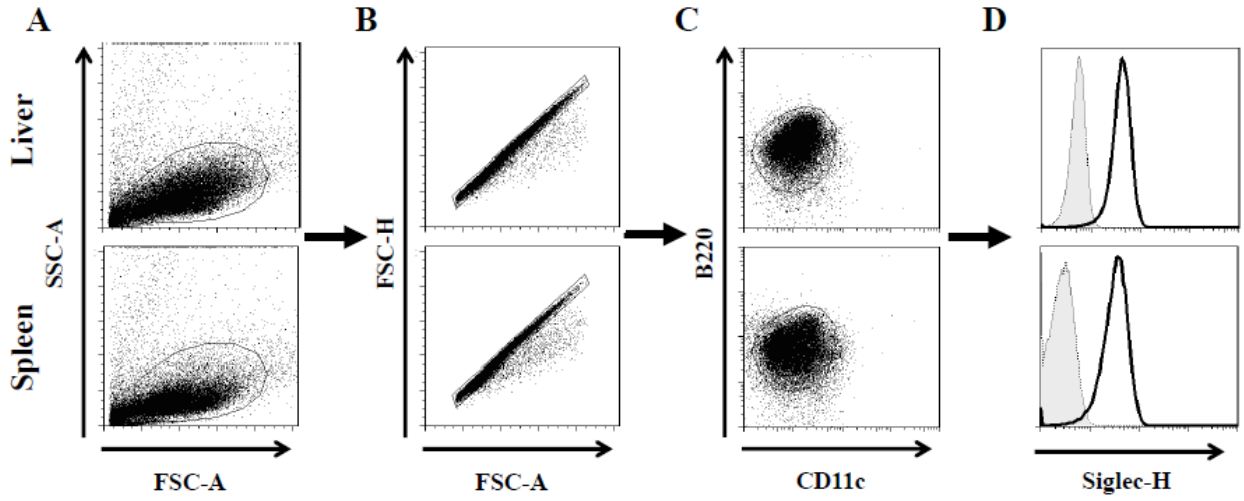


Figure 2.1 Flow cytometry gating strategy for PDCA-1$^+$ liver and spleen pDC.

For identification of pDC, cells were stained using antibodies against B220 and CD11c. A, First, a live cell gate was made using forward (FSC) versus side (SSC) scatter plot. Cell doublets were excluded using (B) FSC-Height (H) versus FSC-Area (A). Analysis of surface molecule expression was analyzed on B220$^+$CD11c^{low} cells (C), including the pDC-specific marker, Siglec-H (D).

2.6 ELISA

Cytokine levels in culture supernatants were determined by ELISA using kits from BioLegend (IL-2, IL-6, IL-10, IL-12p40, and IFN-γ), and R&D Systems (IL-27p28), respectively, following the manufacturers' instructions.

2.7 T CELL ISOLATION

Bulk T cells were purified from spleens and LNs of normal BALB/c mice. Single cell suspensions were incubated with an Ab cocktail consisting of anti-CD45R/B220 (RA3-6B2), anti-CD16/CD32 (2.4G2), anti-TER-119, anti-I-A/I-E, anti-CD11b, and anti-Ly6G (RB6-8C5) obtained from BD Pharmingen. Non-T cells were eliminated from the cell suspension by negative selection using Dynabeads (Invitrogen) following the manufacturer's instructions.

2.8 MLR

Unstimulated, CpG B- or IL-27-conditioned pDC ($2x10^4$/well) were used as stimulators of normal allogeneic BALB/c T cells ($2x10^5$/well) in a 3 d MLR using 96-well, round-bottom plates at a 1:10, DC:T cell ratio. T cells were labeled with the cell tracer CFSE (Invitrogen) for identification and proliferation analysis. At the end of the culture period, supernatants were harvested for cytokine quantitative analysis by ELISA, and cells were harvested for flow cytometric analysis of T cell proliferation and Foxp3 expression.

In separate experiments, freshly-isolated, irradiated (2000 rad) or non-irradiated liver or spleen pDC or cDC ($5x10^3$ up to $2x10^4$) were used as stimulators of normal, allogeneic C3H T cells ($2x10^5$) with or without 10 ng/ml CpG B (pDC) or LPS (cDC). On day 4, 1 μCi ^{3}H-thymidine was added to each well for the final 18 hours of culture. Plates were harvested on day 5 and radioisotope incorporation was quantified using a scintillation counter. Results are presented as mean counts per minute (cpm) from 6 wells per group.

2.9 RNA ISOLATION AND SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) and then reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad). IL-27p28, Ebi3, IL-12p35, WSX-1, and β-actin DNA products were amplified with Fast SYBR Green PCR Master Mix (Applied Biosystems; Carlsbad, CA) using an ABI PRISM 7000 Fast Sequence Detection System (Applied Biosystems). Relative gene expression was determined by comparing to a standard curve for each gene and then normalized to β-actin. Primers used for IL-27p28 were forward: 5′-ATCTCGATTGCCAGGAGTGA-3′ and reverse: 5′-GTGGTAGCGAGGAAGCAGAGT-3′; Ebi3, forward: 5′- ATTGCCACTTACAGGCTCGG-3′ and reverse: 5′-AAGCAGGGGGATGCCAGA-3′; IL-12p35, forward: 5′- CTGTGCCTTGGTAGCATCTATG-3′ and reverse: 5′- GCAGAGTCTCGCCATTATGATTC-3′; WSX-1, forward: 5′-CAAGAAGAGGTCCCGTGCTG-3′ and reverse: 5′- TTGAGCCCAGTCCACCACAT-3′; and β-actin, forward: 5′-AGAGGGAAATCGTGCGTGAC-3′ and reverse: 5′-CAATAGTGATGACCTGGCCGT-3′.

2.10 WESTERN IMMUNOBLOTTING

Freshly-isolated liver or spleen PDCA-1^{+} pDC were washed in PBS and cell pellets lysed using Cell Lytic M (Sigma) supplemented with complete Mini EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Cellular debris was removed by centrifugation at 13,000 x *g*. Protein was quantified and 20 µg loaded onto 10% Tris-Glycine gels and separated using the XCell SureLock Blot Module (Invitrogen). Gels were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blotted using primary Abs: Ebi3 (2.5 µg/ml; eBioscience) or IL-12/IL-35 p35 (1 µg/ml; R&D Systems) and GAPDH (Novus Biologicals, Littleton, CO). Primary Abs were followed by HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein was visualized using Super Signal West Pico ECL kit (Pierce – Thermo Fisher Scientific, Rockford, IL).

2.11 ORAL TOLERANCE AND DELAYED-TYPE HYPERSENSITIVITY

The protocols for oral tolerance and delayed-type hypersensitivity (DTH) were adapted from Goubier et al [209]. For oral tolerance experiments, 8 d Flt3L-mobilized, B6 wild-type (WT) or Ebi3$^{-/-}$ mice were fed 25 mg OVA (Grade II, Sigma) in 200 µl PBS by gavage using 3" 20G straight, 2.25mm ball animal feeding needles (Fisher Scientific). Flt3L was administered for 2 d after OVA feeding. On day 7 after gavage, mice were immunized with 50 µg OVA in 50 µl PBS:Complete Freund's Adjuvant (CFA) emulsion by s.c. injection at the base of the tail. Mice were challenged 7 d after immunization s.c. in the hind footpad with 12.5 mg/ml heat-aggregated (HA) OVA (Grade VII, Sigma) in 20 µl PBS. HA-OVA was prepared by incubating OVA in

PBS at 80°C for 60 min followed by centrifugation at 1900 g for 10 min [246]. The supernatant was removed and HA-OVA resuspended in PBS for injection. PBS alone was injected into the opposite hind footpad as a control. Footpad thickness was measured (Figure 2.2) at time 0 prior to injection and daily thereafter through 96 h post-challenge using Quick Mini Series 700 digital calipers (Mitutoyo). Footpad swelling was calculated as the difference in footpad thickness after challenge compared to baseline footpad thickness.

For DTH experiments, DC were mobilized in B6 WT, Ebi3$^{-/-}$ and B7-H1$^{-/-}$ mice, as described above. On day 9 of Flt3L administration, the mice were fed 25 mg OVA (Grade II, Sigma) in 200 μl PBS by gavage. After 18 h, the animals were euthanized and liver pDC purified as described above. For immunization, pDC (10^5) were co-injected s.c at the base of the tail with day 7, GM-CSF/IL-4-propagated CD11c-purified BMDC [247] at a 1:1 ratio in 50 μl PBS. The BMDC were pulsed with 1 mg/ml OVA (Grade VII, Sigma) (BMDC-OVA) for 3-4 h prior to injection. Un-pulsed BMDC or BMDC-OVA were injected alone as controls. Mice were challenged in the footpad with HA-OVA as described above, 7 d after immunization. Footpad swelling was quantified and spleens from recipient mice were harvested after 96 h. Total splenocytes ($2.5x10^6$/ml) were stimulated with 1 mg/ml OVA (Sigma) for 48 h and culture supernatants were collected for quantitative analysis of cytokines by ELISA.

2.12 ALLO-DTH

Protocol for Allo-DTH was modified from section 2.11. Liver or spleen pDC or cDC ($5x10^6$) from B6 mice were administered by s.c. injection at the base of the tail of C3H recipient mice (C3H used as recipients to mimic strain combination in liver transplant experiments). After 7

days, C3H mice were challenged in the footpad with $1x10^7$ B6 splenocytes. Footpad thickness was measured every 24 hours for 3 consecutive days. Spleens were harvested from C3H mice on day 4 post-challenge and total splenocytes were restimulated *ex vivo* with irradiated (2000 rad) splenocytes from normal B6 (donor), BALB/c (third party), and C3H (syngeneic) mice at a 1:1 ratio for 4 days. 1 µCi ^{3}H-Thymidine was added to each well for the final 18 hours of culture. Radioisotope incorporation was quantified as described above from 6 wells per group. Supernatants were harvested from cultures for determination of cytokine production (IL-2 and IFN-γ) by ELISA.

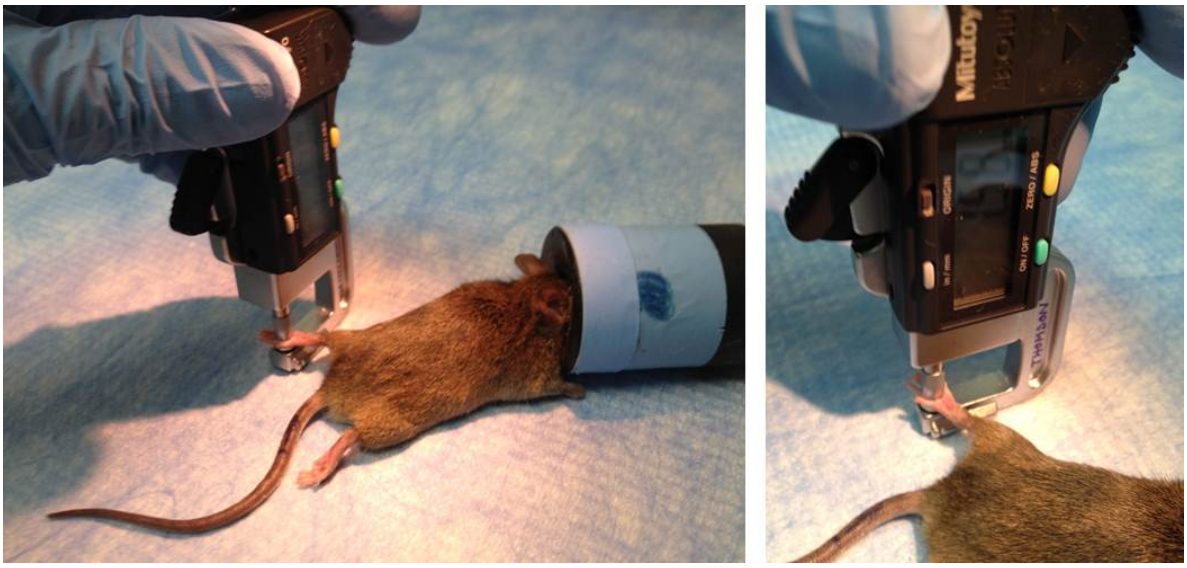


Figure 2.2 Quantification of footpad swelling using spring-loaded digital calipers.

Twenty-four hours after mice were challenged in the footpad with heat-aggregated OVA, swelling was quantified using Mitutoyo Series 700 digital calipers. Mice were maintained under inhaled anesthesia (isoflurane) and three measurements were taken and then averaged for each foot at each time point. Baseline footpad thickness was subtracted from footpad thickness at each time point, and this value was used as footpad swelling, and reported in µm.

2.13 IN VIVO DEPLETION OF pDC

For pDC depletion *in vivo*, mice were administered 250 µg of 120G8 mAb by intraperitoneal (i.p.) injection on days -2 and -1 prior to liver transplant. We have shown this to be an acceptable and effective method for depleting pDC *in vivo* (Figure 5.11).

2.14 LIVER TRANSPLANTATION

The basic techniques of liver harvesting and orthotopic liver transplantation without hepatic artery reconstruction were based on the method described [248] with minor modifications [249]. B6 liver grafts were perfused with 1 ml of University of Wisconsin (UW) solution via the portal vein, stored in UW solution for 24 hours at 4°C, and were implanted orthotopically into C3H recipients by the anastomosis of the suprahepatic vena cava with a running 10-0 suture and by the anastomosis of the portal vein and inferior vena cava with the cuff technique. The average anhepatic time was 19.8 ± 1.7 min. The bile duct was connected via ligation over the stent.

2.15 STATISTICS

Statistical significance was determined by unpaired Student's 't' test or two-way ANOVA where appropriate, using GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA). A value of $p \leq 0.05$ was considered significant. All experiments were carried out independently for a minimum of 3 times, unless indicated otherwise in the figure legends.

3.0 PLASMACYTOID DC REGULATE ALLOIMMUNE RESPONSES VIA B7-H1

3.1 ABSTRACT

pDC play important roles in innate immunity and in the regulation of adaptive immune responses. There is recent evidence that host pDC play a crucial role in regulation of alloreactive T cell responses and the promotion of organ transplant tolerance. We evaluated the contribution of the inhibitory molecule B7-H1 to the regulation of alloimmune responses by murine pDC. Freshly-isolated, PDCA-1 immunobead-purified splenic pDC or $CD11c^+$ cDC were evaluated by flow cytometric analysis of surface marker expression and T cell allostimulatory ability was assessed in MLR. Splenic pDC from WT (+/- B7-H1 neutralizing mAb) or B7-$H1^{-/-}$ mice were pulsed with donor Ag (cell-free BALB/c splenocyte lysate) and T cell allostimulatory capacity was assessed MLR. Freshly-isolated WT or B7-$H1^{-/-}$ pDC pulsed with donor Ag were injected ($2x10^6$) i.v. into syngeneic recipients and splenic and LN $CD4^+$ T cells were purified 7 days later for assay in secondary MLR. T cell proliferation was assessed by ^{3}H-thymidine incorporation. pDC expressed low levels of CD40, CD80, CD86, and MHC class II compared to cDC, while both subsets expressed significant levels of B7-H1. In MLR, pDC were poor stimulators of normal $CD4^+$ T cells compared to cDC via both the direct and indirect pathways. However, mAb neutralization of B7-H1 on WT pDC and pDC from B7-$H1^{-/-}$ mice yielded greater T cell stimulatory capacity compared to untreated WT controls. T cells from mice given immature,

donor Ag-pulsed B7-H1$^{-/-}$ pDC showed enhanced activation and greater proliferation in secondary MLR compared to mice given WT pDC and to untreated naïve controls. These results demonstrate that B7-H1 is a critical molecule on pDC that contributes to the regulation of alloreactive T cell responses via the indirect pathway *in vitro* and *in vivo*. These data suggest that pDC and B7-H1 may be important therapeutic targets in settings such as autoimmunity and transplantation, where immune regulation and tolerance is warranted.

3.2 INTRODUCTION

3.2.1 Transplantation tolerance: Cell-based therapies to target chronic allograft rejection

Continued advances in the field of transplantation (surgical techniques and IS drug therapy) have vastly improved the short-term survival of vascularized grafts; however, the development of graft arterial disease (GAD) and chronic allograft vasculopathy (CAV) leading to late rejection still occurs at high rates [85, 250]. Lack of progress in preventing chronic rejection or limiting vascular damage in clinical transplantation and a myriad of complications resulting from prolonged use of IS support the development and study of promising cell-based therapies in transplantation. Cell-based therapies offer the potential to promote tolerance to donor Ag while maintaining intact immune responses against opportunistic bacterial and viral pathogens as well as malignancies that transplant patients are normally susceptible to under treatment with global IS.

3.2.1.1 The pathways of allorecognition

T cell mediated allograft rejection can be initiated via several pathways of allorecognition (Figure 3.1) [251]: direct allorecognition – activation of recipient T cells by donor APC; indirect allorecognition – activation of recipient T cells by recipient APC that have acquired, processed, and present donor alloAg; and semi-direct allorecognition – recipient T cells are activated by intact donor MHC molecules on the surface of recipient APC. The precursor frequency of directly versus indirectly alloreactive T cells and the loss over time of donor DC versus the continuous re-population of recipient DC led to the belief that donor DC (via the direct pathway) were primarily responsible for acute allograft rejection, whereas recipient DC (via the indirect pathway) mediated chronic allograft rejection [252]. Since IS drug regimens have made significant strides in preventing acute allograft rejection episodes, and the indirect pathway has emerged as a major contributor to allograft rejection [253]. Therefore, the indirect pathway of allorecognition, and thus, recipient DC, serve as an important target for the development of DC-based therapies in transplantation.

3.2.2 pDC and transplantation

The involvement of pDC and transplantation tolerance is discussed in detail in section 1.5.2.4.

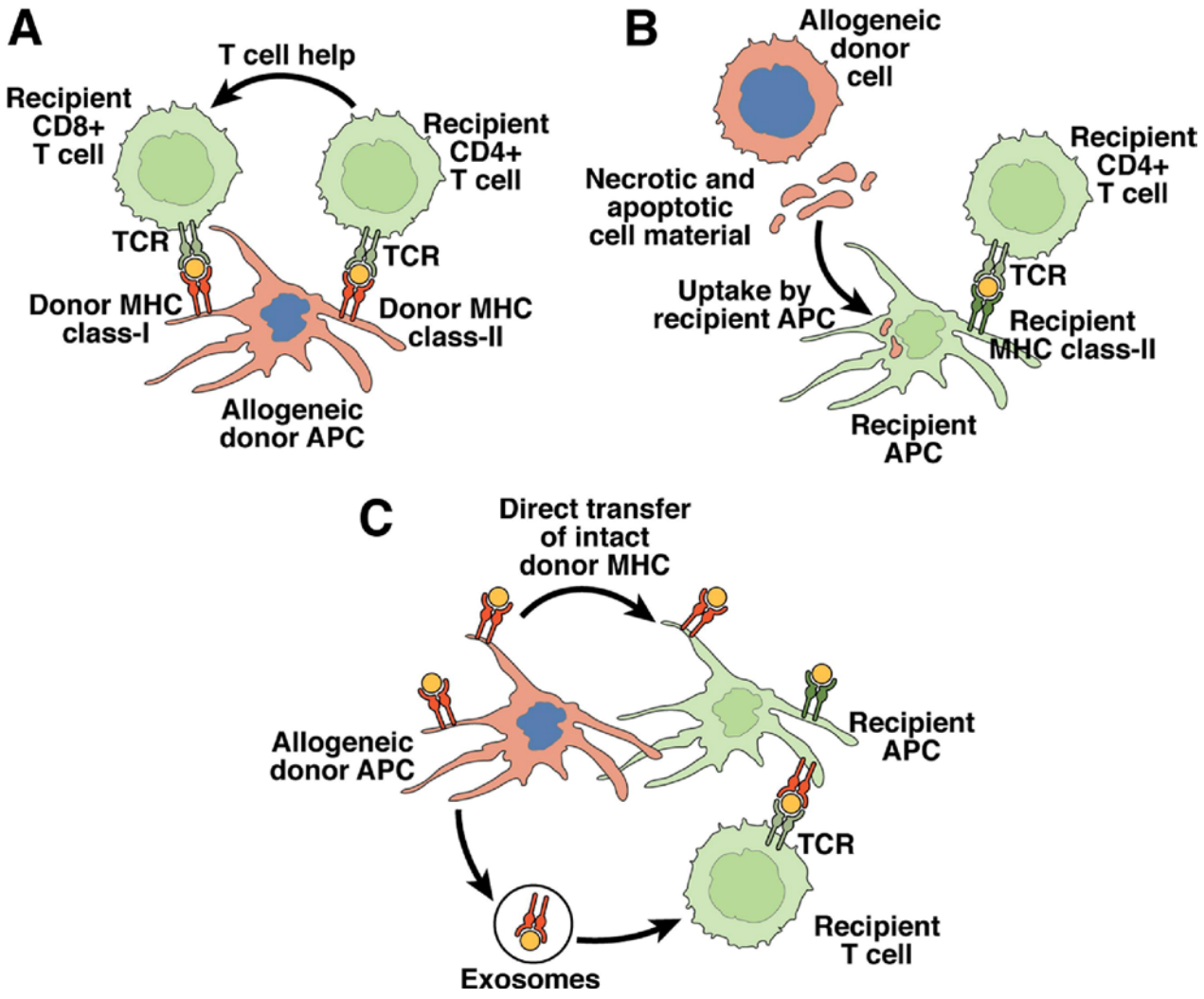


Figure 3.1 Pathways of alloantigen presentation.

Three nonmutually exclusive pathways of allorecognition have been described. (A) In the direct pathway, recipient T cells recognize intact allogeneic MHC molecules on the surface of donor APCs. The direct pathway is responsible for the large proportion of T cells that have reactivity against alloantigens due to cross-reactivity of the T-cell receptor (TCR) with self and foreign MHC molecules. (B) In the indirect pathway, recipient APCs trafficking through the allograft phagocytose allogeneic material shed by donor cells (mostly peptides derived from allogeneic MHC molecules) and present it to recipient T cells on recipient MHC molecules. (C) In the semidirect pathway, recipient APCs acquire intact MHC molecules following direct contact with donor APCs and/or through fusion with donor APC-derived exosomes. These chimeric recipient APCs stimulate recipient T cells through direct and indirect pathways. From Sánchez-Fueyo A and TB Strom. 2011. *Gastroenterology*. 140:51-64.e2. [254]

3.2.3 B7-H1: A co-inhibitory B7 family molecule with important implications for immune regulation

B7-H1 (PD-L1) is a recently-described B7 family molecule which acts as a negative regulator of T cell activation [191, 255]. B7-H1 is expressed by a variety of cells and tissues, including DC [194]. B7-H1 can deliver an inhibitory signal through PD-1 and CD80 (Figure 3.2) expressed on activated T cells (and CD80 on DC/APC), and disruption of B7-H1:PD-1 signaling in various models boosts T cell-mediated immunity and reduces disease pathogenesis [256-258]. B7-DC, or PD-L2, is another ligand for PD-1 that also has been reported to have regulatory function [259], however, its cellular distribution is more limited and it is not expressed on pDC [194]. Although B7-H1 is critical for modulating immune responses against pathogens, its expression also plays a role in regulating immune reactivity against self Ags. B7-H1 expressed on immune cells and non-lymphoid tissue serves to prevent T cell-mediated tissue destruction and contributes to self-tolerance which regulates autoimmune reactivity [260, 261]. The significance of B7-H1 expression in maintaining tolerance to self-Ags and ability to downregulate T cell responses serves as the basis for investigation into its' role in the ability of pDC to promote tolerance in transplantation.

3.2.3.1 The role of B7-H1 in transplantation

Using mice deficient in B7-H1 or PD-1, it has been demonstrated that B7-H1 inhibitory signaling is a key factor for allograft survival and tolerance [262, 263]. B7-H1 expressed by non-lymphoid cells is critical for regulating alloreactive T cells directly within the graft through the induction of apoptosis of both $CD4^+$ and $CD8^+$ T cells and through the induction of Treg [264-266]. In addition, we have reported that high B7-H1 expression relative to CD86 on pDC

positively correlates with tolerance in liver transplant patients [193]. Despite these findings, the significance of B7-H1 expression for the therapeutic capacity of DC in transplantation has not been well documented.

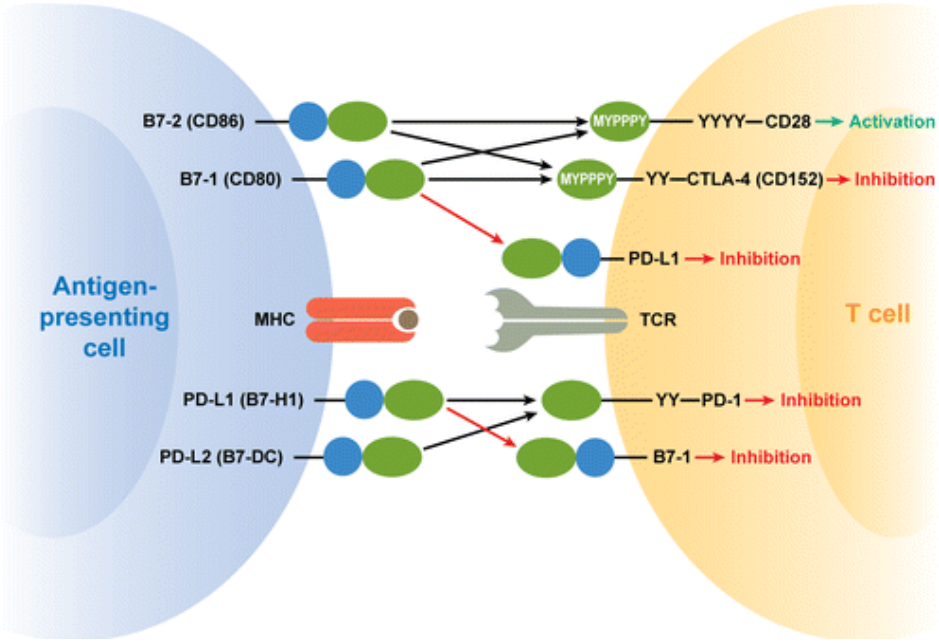


Figure 3.2 B7-1:PD-L1 interaction expands pathways in the B7:CD28 family.

Recent data demonstrate that PD-L1 and B7-1 productively interact on T cells and can deliver bidirectional inhibitory signals. The identification of these co-stimulatory molecules as binding partners increases our understanding of the interactions that can occur on T cells and APCs and raises the possibility that PD-L1:B7-1 binding may not only deliver signals when ligated, but may also serve to segregate binding away from previously identified receptors (PD-1, CD28, CTLA-4). IgV-like regions are depicted in blue and IgC-like regions in green, while tyrosine-containing signaling motifs are depicted by Ys. From Keir ME, Butte MJ, Freeman GJ, and AH Sharpe. 2007. *Annu Rev Immunol*. 26:677-704. [194]

3.3 MATERIALS AND METHODS

See chapter 2.0 for full description of materials and methods for experiments described in this chapter.

3.4 RESULTS

3.4.1 Splenic pDC are phenotypically immature compared to cDC

DC are important mediators of immune responses in the setting of transplantation, in both allograft rejection and alloAg-specific immune tolerance. In section 1.3.2, we described tolerogenic cDC, and how they may be manipulated to enhance their tolerogenic potential. The requirement for prior conditioning of these cells *ex vivo*, however, may add significant costs and concerns should these methods be considered for translation into a clinical setting. Using cells such as pDC that possess potent inherent tolerogenic properties may negate the need for *ex vivo* manipulation and makes the potential use of these cells as a therapeutic in transplantation an attractive prospect compared to cDC.

An important characteristic that influences the ability to DC to induce immunity or promote tolerance is their level of expression of Ag-presenting, co-stimulatory, and co-inhibitory molecules. We compared the expression of key molecules on unstimulated and stimulated PDCA-1^+ pDC and $CD11c^+$ cDC from spleens of Flt3L-mobilized B6 mice. As described in section 1.4.2, $B220^+CD11c^{low}$ pDC uniquely express Siglec-H and PDCA-1 (Figure 3.3), whereas $CD11c^+$ cDC do not express either molecule. pDC and cDC express similar levels of

MHC class I (K^b), however, compared to cDC, pDC express lower levels of MHC class II ($I\text{-}A^b$), CD40, CD80, and CD86 (Figure 3.3). Both DC subsets express B7-H1, although pDC express more B7-H1 relative to CD40/CD80/CD86. As discussed in section 1.5.1.2, the balance of co-inhibitory to co-stimulatory molecules on DC plays an important role in

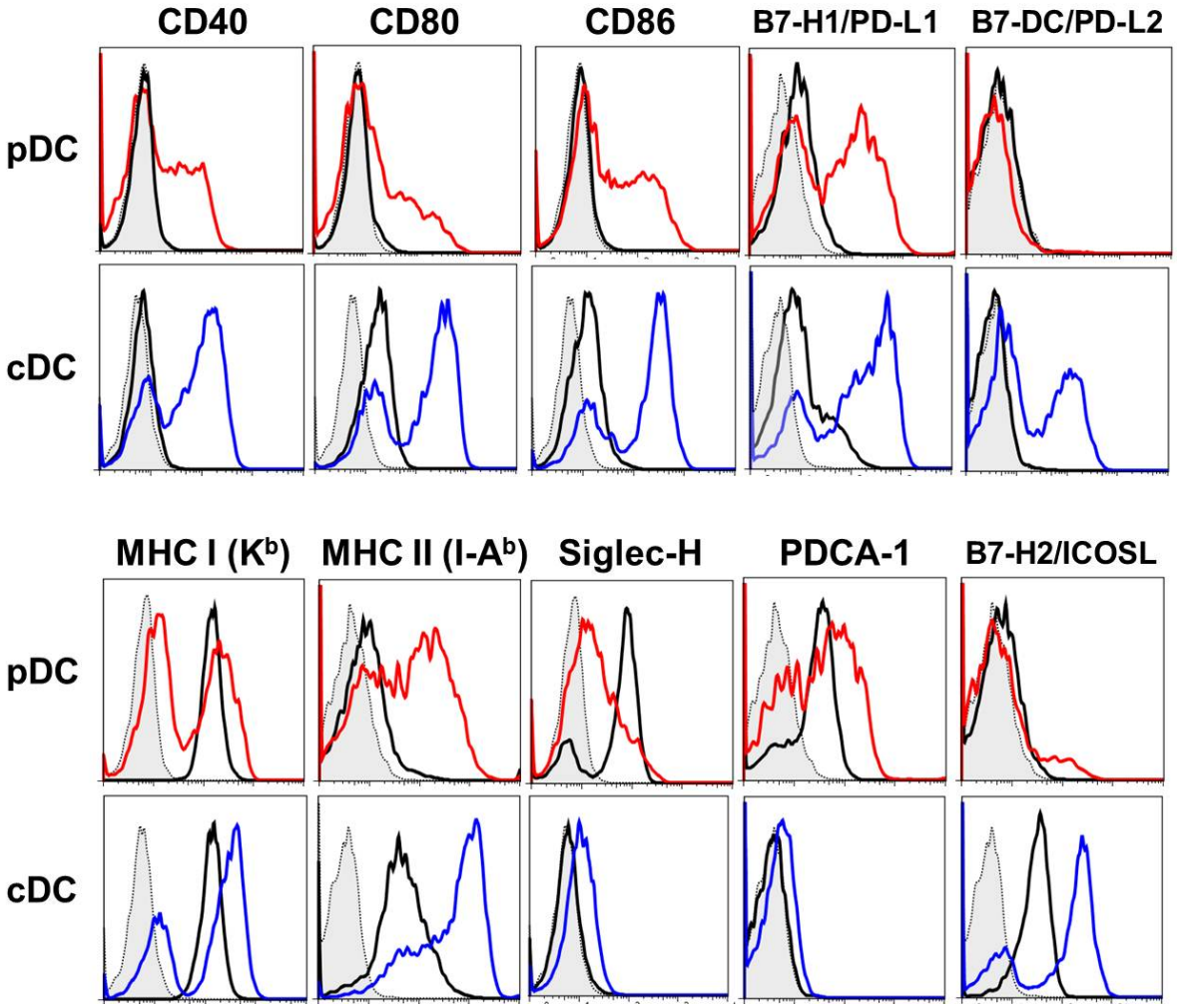


Figure 3.3 Phenotypic characterization of splenic pDC and cDC.

PDCA-1^+ pDC and $CD11c^+$ cDC from Flt3L-mobilized B6 mice were cultured for 18 h ± CpG B ODN (pDC) or LPS (cDC). Cells were harvested from culture and stained for multi-color flow cytometric analysis. Histograms represent surface molecule expression on $B220^+CD11c^{low}$ cells (as described in section 2.4 and shown in Figure 2.1) for pDC and $B220^-CD11c^+$ cells for cDC. Shaded histograms indicate isotype control, black line indicates unstimulated cells, and red (pDC) and blue (cDC) lines represent stimulated cells.

determining their functional ability to control immune responses, either positively or negatively. Thus, high expression of B7-H1 relative to co-stimulatory molecule (CD40, CD80, and CD86) expression on pDC suggests they may be uniquely designed to regulate immune responses in the absence of viral infection and type I IFN. Other key differences between pDC and cDC include lack of expression of B7-DC/PD-L2 and B7-H2/ICOSL on pDC, while both molecules are expressed, or are induced with LPS, on cDC.

3.4.2 Splenic pDC exhibit weak T cell allostimulatory capacity compared to cDC

We have shown previously that BM-derived pDC have weak T cell allostimulatory capacity (T cell proliferation, IFN-γ, and IL-2 cytokine production) via the direct pathway [128]. In correlation with their comparatively immature surface phenotype, Flt3L-mobilized, B6 splenic pDC are also poor stimulators of normal, allogeneic BALB/c T cells, compared to cDC via the direct pathway, either unstimulated or stimulated with CpG B ODN (Figure 3.4A). We also observed this trend when pDC and cDC were first pulsed with BALB/c cell lysate and incubated with syngeneic B6 T cells, for evaluation of T cell proliferative capacity via the indirect pathway (Figure 3.4B). These differences were statistically significant, and these results are summarized in Table 3.1. Several factors implicate the indirect pathway as a major contributor to chronic allograft rejection (section 3.2.1.1) and pDC clearly have a weak ability to stimulate allogeneic T cells. Thus, the indirect pathway and pDC will be the focus of the rest of the data presented in this chapter.

3.4.3 B7-H1 expression by splenic pDC is critical for their ability to regulate T cell responses

Based on the reported ability of B7-H1 to negative regulate immune responses and the high expression of B7-H1 relative to CD40/CD80/CD86 on pDC, we tested the effects of inhibiting B7-H1 signaling on pDC in MLR. Addition of B7-H1 neutralizing mAb to MLR resulted in significantly greater T cell proliferation compared to isotype control Ab (Figure 3.5A). Since B7-H1 can also be expressed on T cells (Figure 3.2), use of B7-H1 mAb in MLR does not specifically target B7-H1 on pDC, thus, we utilized B7-H1-deficient mice for isolation of pDC to eliminate potential inhibition of B7-H1 on T cells that may skew our results. B7-H1$^{-/-}$ splenic pDC also showed enhanced T cell stimulatory capacity compared to WT pDC (Figure 3.5B), confirming that B7-H1 on pDC contributes to their weak T cell allostimulatory capacity *in vitro* and is important for their inherent tolerogenicity.

3.4.4 B7-H1 regulates T cell activation *in vivo* induced by adoptively transferred splenic pDC

The significant contribution of B7-H1 to the weak ability of splenic pDC to stimulate alloreactive T cells *in vitro* suggests that it may be a critical molecule that regulates pDC function *in vivo*. To test this, we adoptively transferred freshly-isolated WT or B7-H1$^{-/-}$ splenic pDC that were pulsed with BALB/c cell-free lysate into syngeneic B6 recipient mice. After 7 days, T cells were purified from the spleens and LNs of B6 recipients. T cells were restimulated via the indirect pathway with B6 BMDC pulsed with cell-free BALB/c lysate, and proliferation was determined on day 3 by ^{3}H-thymidine incorporation. Mice that received B7-H1$^{-/-}$ splenic

pDC showed significantly greater levels of splenic and LN T cell proliferation upon restimulation compared to mice that received WT pDC and untreated naïve controls (Figure 3.6A).

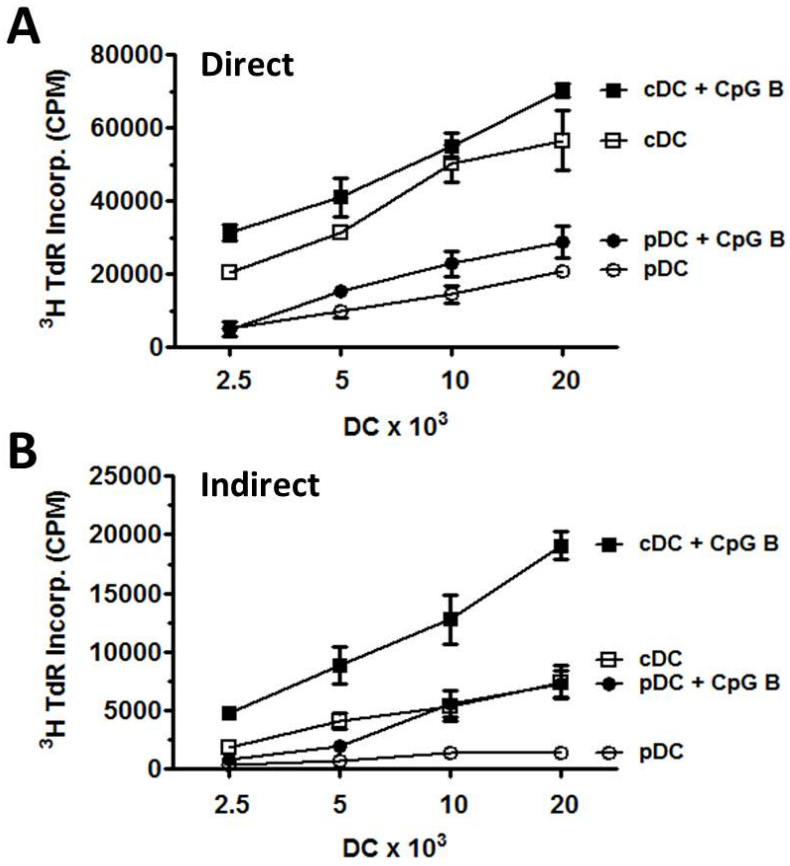


Figure 3.4 Splenic pDC exhibit weak T cell allostimulatory capacity compared to cDC.

A, Freshly-isolated, B6 spleen PDCA-1$^+$ pDC or CD11c$^+$ cDC were cultured for 18 h with 1 µg/ml CpG B ODN, washed, counted, and cultured in MLR with BALB/c splenic T cells. On day 2, 1 µCi/well ^{3}H-Thymidine was added for the final 18 h, and proliferation was quantified using a scintillation counter. **B**, B6 spleen pDC or cDC were cultured as described in **A** and pulsed with cell-free, BALB/c lysate. Cells were washed, counted, and cultured for 3 d with B6 T cells. Proliferation was quantified by ^{3}H-thymidine incorporation as described in **A**. Statistical significance is presented in Table 3.1.

Table 3.1 Statistical significance for data presented in Figure 3.4.

	DC x 10^3			
Direct	**2.5**	**5**	**10**	**20**
pDC vs. cDC	***	***	***	***
pDC vs. cDC + CpG B	***	***	***	***
pDC + CpG B vs. cDC	***	***	***	***
pDC + CpG B vs. cDC + CpG B	***	***	***	***
Indirect				
pDC vs. cDC	ns	**	***	***
pDC vs. cDC + CpG B	***	***	***	***
pDC + CpG B vs. cDC	ns	ns	ns	ns
pDC + CpG B vs. cDC + CpG B	***	***	***	***
ns = not statistically significant, ** $p \leq 0.01$, *** $p \leq 0.001$				

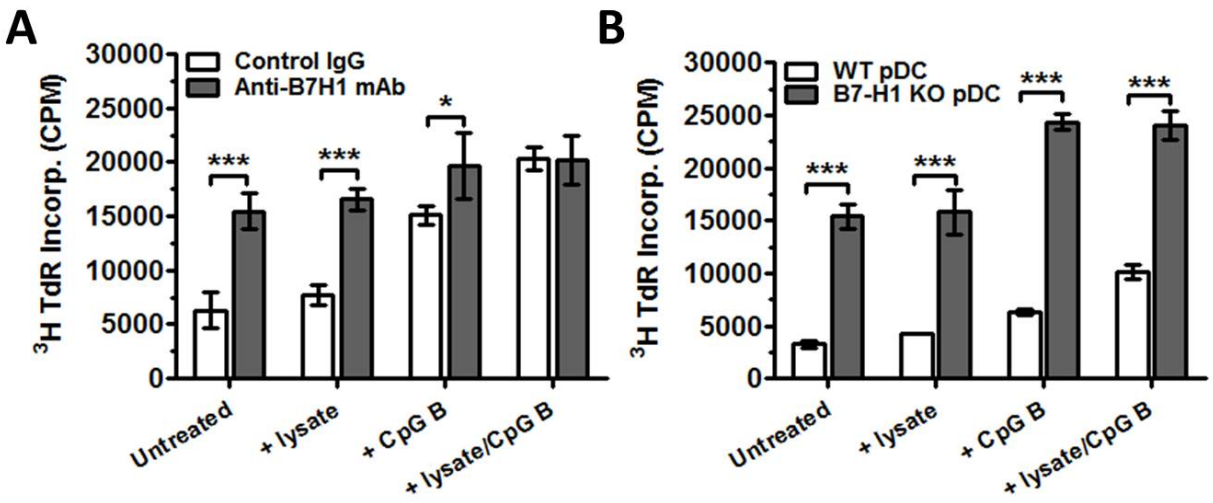


Figure 3.5 Inhibition of B7-H1 increases splenic pDC T cell allostimulatory capacity.

Freshly-isolated, B6 spleen PDCA-1$^+$ pDC were cultured for 18 h with 1 µg/ml CpG B ODN ± cell-free, BALB/c lysate, washed, counted, and cultured in MLR with B6 splenic T cells. On day 2, 1 µCi/well ^{3}H-Thymidine was added for the final 18 h, and proliferation was quantified using a scintillation counter. **A**, B7-H1 mAb (MIH5) or Rat IgG2b isotype control Ab was added 5 µg/ml for the duration of MLR. **B**, Experiment described in **A** was repeated with B6 WT pDC and B7-H1$^{-/-}$ pDC. No Abs were added to MLR in **B**. * $p \leq 0.05$, *** $p \leq 0.001$.

Flow cytometric analysis of purified T cells from spleens of recipient mice showed that $CD4^+$ T cells from mice that received B7-$H1^{-/-}$ pDC exhibited a greater frequency of $CD44^{high}CD62L^{low}$ activated cells (Figure 3.6B) compared to mice receiving WT pDC and untreated naïve controls. Together, these data suggest that B7-H1 is necessary for pDC to regulate T cell activation and immune responses *in vivo*.

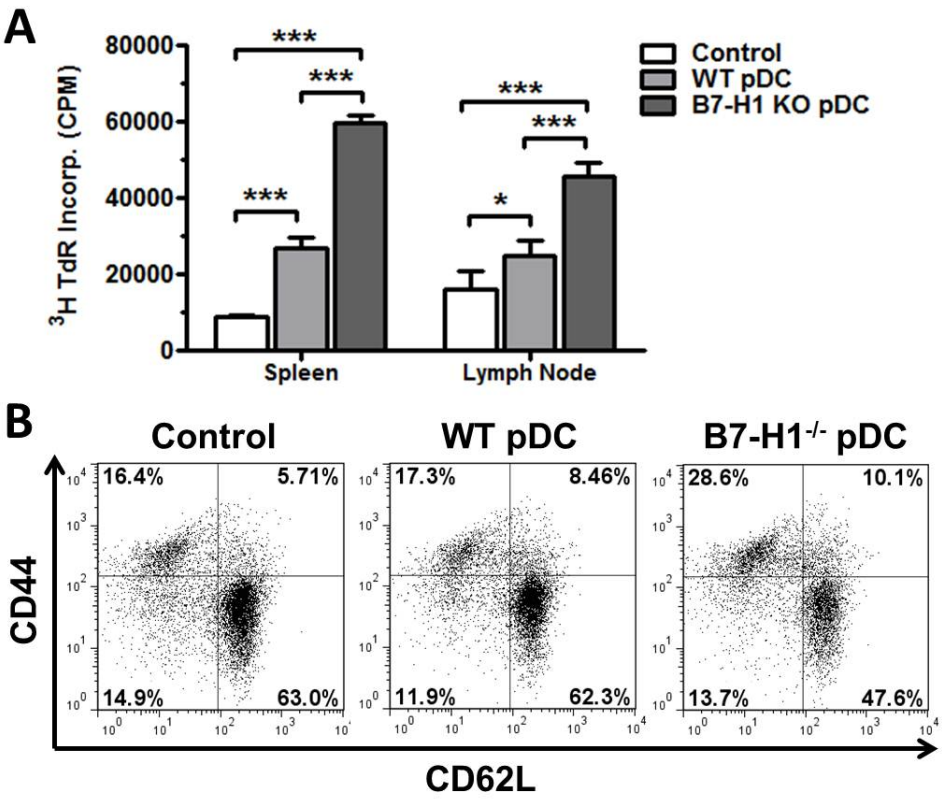


Figure 3.6 B7-H1 regulates splenic pDC function *in vivo*.

Freshly-isolated, splenic pDC ($2x10^6$) from B6 WT or B7-$H1^{-/-}$ mice were pulsed with cell-free BALB/c splenocyte lysate and adoptively transferred into syngeneic B6 recipient mice via tail vein injection. After 7 d, spleens and LNs were harvested and $CD4^+$ T cells were purified. **A**, Purified T cells were restimulated with B6 BMDC that were pulsed with cell-free BALB/c splenic lysate. 1 µCi/well ^{3}H-thymidine was added for the final 18 h of culture and proliferation was determined by quantification of radioisotope incorporation using a scintillation counter. Results are representative of 3 independent experiments. B, Purified splenic T cells were analyzed by multi-color flow cytometry for expression of activation markers. * $p \leq 0.05$, *** $p \leq 0.001$.

3.5 DISCUSSION

The data presented in this chapter show that within the splenic DC compartment, pDC have a less mature phenotype and exhibit a significantly reduced ability to stimulate allogeneic T cell proliferation compared to cDC. The high expression of B7-H1 compared to CD40/CD80/CD86 on pDC plays a critical role in this phenomenon, and disruption of B7-H1 signaling by pDC significantly enhances their ability to stimulate T cell responses, both *in vitro* and *in vivo*.

The role of DC in transplantation has been studied extensively and clearly identifies them as important mediators of allograft rejection through presentation of alloAgs to T cells, via multiple pathways of allorecognition (Figure 3.1). The ability to modulate DC function to promote tolerance enhanced their potential as a possible cell-based therapeutic in transplantation. Although much of the focus has been on cDC, pDC have received attention recently, based on their unique immunoregulatory properties in the steady state (see section 1.5). Our lab has reported on their therapeutic capacity in experimental cardiac transplantation models in mice, and we have shown pDC are capable of prolonging allograft survival [128, 267]. Although we show their efficacy *in vivo* in this model, the mechanisms utilized by pDC to promote tolerance to alloAgs are not known.

Our previous studies have described the tolerogenic nature of pDC precursors propagated from BM cultures using Flt3L [128], however, the ability of splenic pDC to modulate immune responses was not evaluated. Here we show that splenic pDC in the steady state exhibit an immature phenotypic profile compared to splenic cDC. This includes drastically reduced expression of co-stimulatory molecules and lower expression of MHC class II. Splenic pDC maintain high expression of B7-H1 compared to CD40, CD80, and CD86, resulting in a net phenotype that favors immune regulation. The relatively immature phenotype of pDC translates

to their functional deficiency to induce immune responses against alloAgs. Thus, when splenic pDC are cultured in an MLR, they induce significantly less T cell proliferation compared to cDC, via both the direct and indirect pathways of allorecognition. The inability of pDC to efficiently stimulate T cells relative to cDC is highlighted by the fact that even pDC that have been activated with CpG B induce less allogeneic T cell proliferation compared to unstimulated cDC.

High B7-H1 expression relative to CD80 and CD86 on splenic pDC compared to cDC stands out as a key characteristic that may regulate splenic pDC allostimulatory capacity, as we have described for BM-derived pDC precursors [128]. Blockade of B7-H1 by mAb in MLR enhances the T cell stimulatory capacity of splenic pDC via the indirect pathway, leading to greater levels of T cell proliferation compared to isotype Ab-treated controls, and confirming that B7-H1 expression negatively regulates T cell function. B7-H1 has multiple binding partners (PD-1 and CD80) and is expressed on T cells as well as DC (Figure 3.2). Thus, use of mAb blockade of B7-H1 in MLR does not definitively identify B7-H1 expression on pDC as the main source of inhibitory signals. We utilized B7-H1-deficient mice as a source of splenic pDC, thereby eliminating the possibility of B7-H1 signaling on the T cells. B7-H1$^{-/-}$ splenic pDC show enhanced T cell stimulatory capacity, which positively links B7-H1 expression to the immunoregulatory capacity of splenic pDC.

As mentioned above, pDC can extend experimental cardiac allograft survival time when adoptively transferred into recipients. Despite this, the mechanisms utilized by pDC to regulate immune responses *in vivo* are less clear. B6 CD4^{+} T cells isolated from the spleens and LNs of mice that received adoptive transfer of syngeneic, BALB/c alloAg-pulsed B7-H1$^{-/-}$ splenic pDC exhibit significantly greater secondary proliferative responses when restimulated *ex vivo* via the

indirect pathway compared to mice that received B6 WT splenic pDC or naïve, untreated controls. Enhanced secondary T cell responses correlate to a more activated phenotype, characterized by an elevated frequency of $CD62L^{lo}CD44^{hi}$ cells. Taken together with our *in vitro* data, B7-H1 emerges as a key molecule that controls splenic pDC allostimulatory function.

In summary, of the two main DC subsets in the spleen, pDC have a distinctly different phenotype compared to cDC, characterized by low expression of MHC class II and co-stimulatory molecules, even following TLR stimulation. This translates to their significantly weaker ability to stimulate allogeneic T cell proliferation. B7-H1 expression plays a critical role in regulating pDC allostimulatory capacity, both *in vitro* and *in vivo*, and may be an important target to exploit the therapeutic potential of pDC in prolonging experimental cardiac allograft, that has been previously observed.

4.0 IL-27 AND STAT3-DEPENDENT UPREGULATION OF B7-H1 MEDIATE IMMUNE REGULATORY FUNCTIONS OF LIVER PLASMACYTOID DC

4.1 ABSTRACT

Plasmacytoid (p) dendritic cells (DC) are highly-specialized APC that, in addition to their well-recognized role in anti-viral immunity, also regulate immune responses. Liver-resident pDC are considerably less immunostimulatory than those from secondary lymphoid tissues and are equipped to promote immune tolerance/regulation through various mechanisms. IL-27 is an IL-12-family cytokine that regulates the function of both APC and T cells, although little is known about its role in pDC immunobiology. In this study, we show that mouse liver pDC express higher levels of IL-27p28 and EBV-induced protein (Ebi)3 compared to splenic pDC. Both populations of pDC express the IL-27Rα/WSX-1; however, only liver pDC significantly upregulate expression of the co-inhibitory molecule B7 homolog-1 (B7-H1) in response to IL-27. Inhibition of STAT3 activation completely abrogates IL-27-induced upregulation of B7-H1 expression on liver pDC. Liver pDC treated with IL-27 increase the percentage of $CD4^{+}Foxp3^{+}$ T cells in MLR, which is dependent upon expression of B7-H1. pDC from Ebi3-deficient mice lacking functional IL-27, show increased capacity to stimulate allogeneic T cell proliferation and IFN-γ production in MLR. Liver but not spleen pDC suppress delayed-type hypersensitivity responses to OVA, an effect that is lost with $Ebi3^{-/-}$ and $B7\text{-}H1^{-/-}$ liver pDC compared to WT

liver pDC. These data suggest that IL-27 signaling in pDC promotes their immunoregulatory function and that IL-27 produced by pDC contributes to their capacity to regulate immune responses *in vitro* and *in vivo*.

4.2 INTRODUCTION

4.2.1 The liver: A unique immunologic organ

The liver is a unique immunologic organ (Figure 4.1) with inherent characteristics that promote immune tolerance [60, 268]. Constant exposure to gut-derived products via the portal venous blood conditions the liver microenvironment to suppress unwanted inflammatory responses to harmless or inert Ags, such as proteins from food or derived from commensal bacteria [60]. The liver contains a diversity of cells with Ag-presenting capacity, including liver sinusoidal endothelial cells, Kupffer cells or liver-resident macrophages, hepatic stellate cells, and DC. The distribution of these APC within the liver sinusoids leads to specialized functional interplay with one another and with NK cells, T cells, and hepatocytes, and helps shape this unique microenvironment [60].

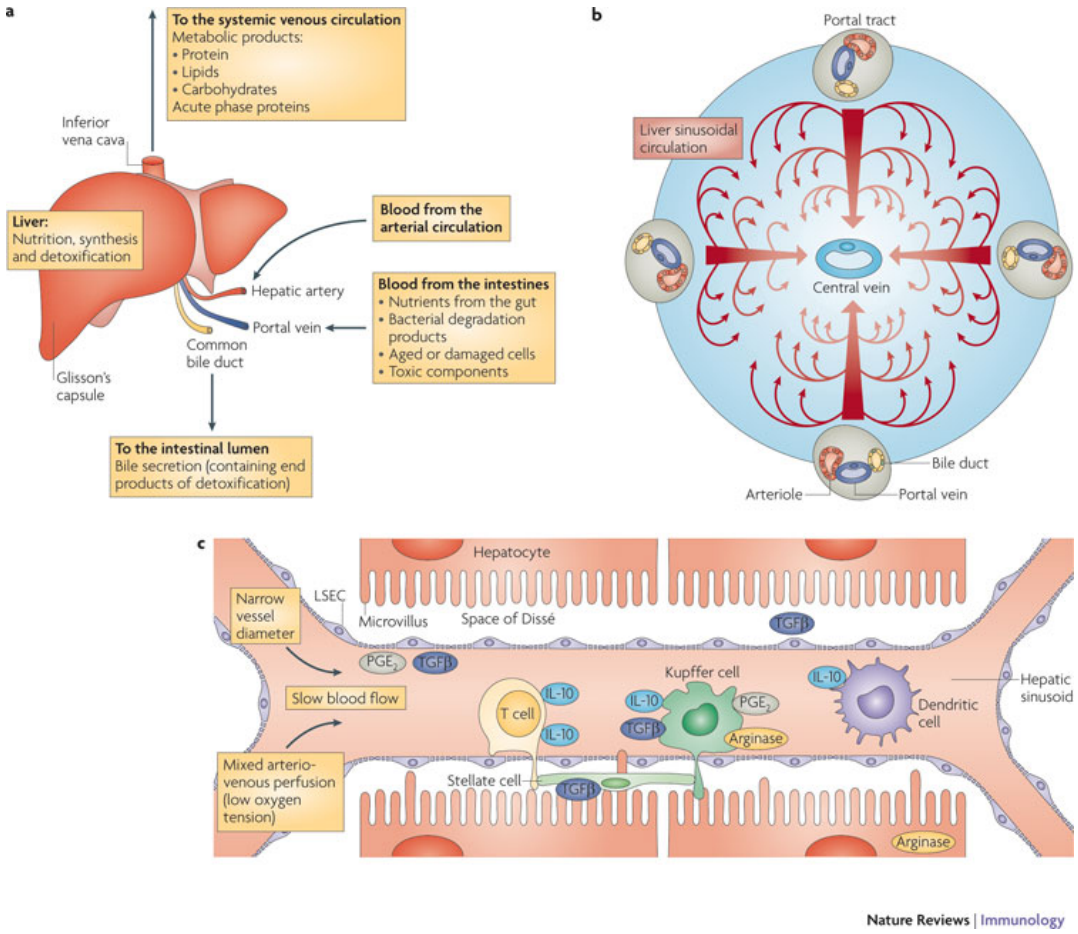


Figure 4.1 Anatomy, function, and location of liver and liver-resident APCs.

A, The metabolic functions of the liver and the flow of products through the portal vein, hepatic artery, inferior vena cava and common bile duct. **B**, General microanatomy of the liver, showing the location of central veins and portal tracts, as well as the direction of blood flow. **C**, Anatomical location of hepatic antigen-presenting cells (APCs) and the factors that regulate their function. Branches of the hepatic artery merge with sinusoidal vessels carrying blood from the portal vein in the liver, resulting in a mixed arterio-venous perfusion of the liver with low oxygen tension. Owing to extensive branching of portal vessels into liver sinusoids, and the accompanying increase in cumulative vessel diameter, the hepatic microcirculation is characterized by low pressure and slow, sometimes irregular, blood flow. Together with the narrow diameter of hepatic sinusoids, this facilitates the interaction of circulating leukocytes with hepatic sinusoidal cell populations. The hepatic sinusoids are lined by a population of microvascular liver sinusoidal endothelial cells (LSECs) that separate hepatocytes and stellate cells (all of which function as APCs) from leukocytes circulating through the liver in the blood. Fenestrations in the LSEC lining allow the passive exchange of molecules between the space of Dissé and the blood, as well as direct contact of lymphocyte filopodia with hepatocyte microvilli. The liver interstitium is highly enriched in cells of the innate immune system (such as antigen-presenting dendritic cells, Kupffer cells, natural killer (NK) cells and NKT cells (not shown)) and in T cells, which participate in adaptive immune responses. Mediators produced by both parenchymal and non-parenchymal cells, including interleukin-10 (IL-10), transforming growth factor-β (TGFβ), arginase and prostaglandin E2 (PGE2), regulate immune function within the liver. From Thomson AW and PA Knolle. 2010. *Nat Rev Immunol*. 10:753-766. [60]

4.2.2 pDC hold significant immunoregulatory potential

DC are BM-derived, professional APC that are the most potent activators of T cell-mediated immune responses, constituting a critical link between innate and adaptive immunity (section 1.2). The main subsets in the mouse are cDC ($CD11c^{+}CD11b^{+}NK1.1^{-}$) and pDC ($CD11b^{-}CD11c^{low}B220^{+}CD8\alpha^{-}$). Steady-state pDC express PDCA-1/BST-2/CD317 [147], and the endocytic receptor Siglec-H [151] (see section 1.4.2 for full description of pDC). They express high levels of endosomal TLR 7, 8 and 9 that sense single-stranded viral RNA (ssRNA; TLR7 and 8) and unmethylated, CpG-rich viral and bacterial ssDNA (TLR9) [173]. pDC are the primary type-I IFN producers in response to viral infection [140, 173, 269]. They are weaker T cell stimulators than cDC, and can promote T cell hyporesponsiveness and Treg function [128, 197, 270]. Consequently, pDC have been linked to the development of tolerance in various experimental models and disease states [240].

Liver pDC (Figure 4.2) express low levels of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86) compared to their counterparts in secondary lymphoid tissues, such as the spleen [73, 271]. They also express comparatively high levels of the immunoregulatory cytokine IL-10 and B7-H1 (PD-L1) [69, 72], which are known to negatively regulate immune responses [96, 272-274]. Despite the implications of these findings for the function of liver pDC, the molecular mechanisms that regulate liver pDC function and the expression of immunoregulatory molecules by these cells remain underexplored.

4.2.3 IL-27 is an emerging cytokine with immunoregulatory function

IL-27 is a heterodimeric, IL-12-family cytokine (Figure 4.3) comprised of the p40-like molecule EBV-induced protein (Ebi)3 and the p35-like molecule p28 [275, 276]. It is produced by APC, including macrophages and DC, and signals through the heterodimeric IL-27R that consists of T cell cytokine receptor (TCCR)/WSX-1 and the shared gp130 chains [277]. Signaling through the IL-27R activates STAT 1, 3, 4, and 5, where STAT1 and STAT3 appear to be the primary downstream targets, especially in T cells [278]. Activation of STAT1 by IL-27 has been shown to drive Th cell responses toward a Th1/IFN-γ-producing phenotype that favors immune activation and pathogen clearance [279]. By contrast, there is considerable evidence [244, 280, 281] that IL-27 plays an important role in the induction of IL-10-producing, $Foxp3^-$, T-regulatory 1 (Tr1) cells [282]. In support of an immunoregulatory influence on the outcome of T cell responses, IL-27 has been found to suppress the development of inflammatory Th17 cells [283, 284].

The IL-27R is expressed widely on hematopoietic cells and plays distinct roles in their function, depending on the cell type on which it is expressed. Mouse splenic $CD11c^+$ DC lacking the IL-27Rα/WSX-1 exhibit prolonged upregulation of activation markers and enhanced T cell stimulatory capacity following LPS stimulation [285], suggesting that IL-27 exerts immunoregulatory effects on DC. In addition, exposure of human monocyte-derived DC to IL-27 decreases their T cell stimulatory capacity in a B7-H1-dependent manner [286]. It was reported recently that mouse splenic pDC express Ebi3 and that it is upregulated following TLR ligation [287].

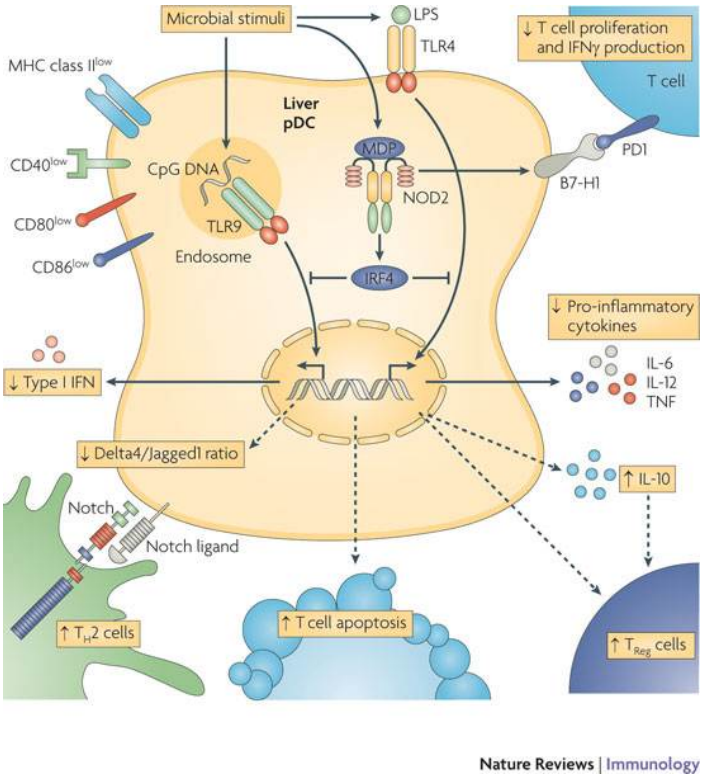


Figure 4.2 Functional biology of liver plasmacytoid DCs.

Unlike conventional myeloid dendritic cells (DCs), plasmacytoid DCs (pDCs) are characterized by their ability to secrete type I interferons (IFNs) in response to microbial stimuli that ligate Toll-like receptors (TLRs) (such as CpG-containing DNA or lipopolysaccharide (LPS)) or nucleotide-binding oligomerization domain (NOD) receptors (such as muramyl dipeptide (MDP)). In vivo exposure of liver pDCs to the NOD2 ligand MDP upregulates expression of IFN regulatory factor 4 (IRF4), a negative regulator of TLR signaling, and impairs the ability of pDCs to secrete pro-inflammatory cytokines in response to TLR ligands and their capacity to induce T cell proliferation and IFNα production. These MDP-stimulated liver pDCs have increased cell surface B7-H1 expression, the absence of which (for example, on B7-H1-deficient liver pDCs) reverses the inhibitory effect of MDP on liver pDC function. The ability of liver pDCs to rapidly delete oral antigen-specific T cells is consistent with the liver as a site of CD4+ and CD8+T cell apoptosis. The mechanisms by which liver pDCs may regulate T cell responses include the expression of B7-H1, modulation of Notch ligand expression (which promotes T helper 2 (TH2) cell differentiation), production of interleukin-10 (IL-10) (which promotes regulatory T (Treg) cell differentiation) and T cell apoptosis. PD1, programmed cell death 1; TNF, tumor necrosis factor. From Thomson AW and PA Knolle. 2010. *Nat Rev Immunol*. 10:753-766. [60]

Moreover, pDC exposed to exogenous TGF-β increase IL-27 production [245] and cDC cultured with T cells that constitutively express TGF-β acquire a "plasmacytoid-like" phenotype and increase production of IL-27 [244]. TGF-β is an important cytokine in the biology of the liver, and its ability to induce IL-27 production by DC suggests a novel mechanism that may contribute to the inherent tolerogenicity of the liver.

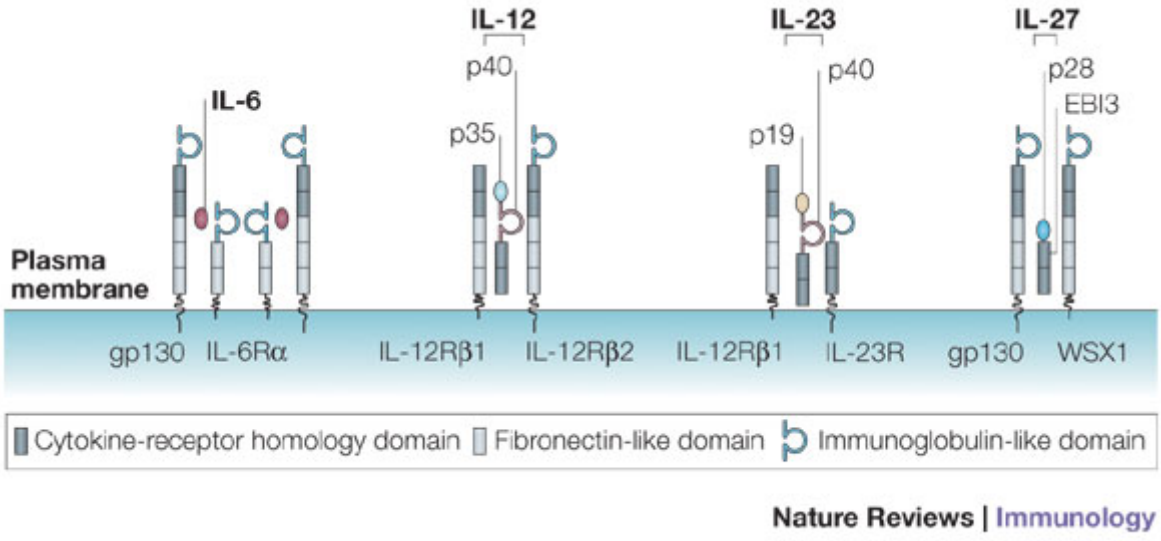


Figure 4.3 The interleukin-6 and interleukin-12 family of cytokines and their receptors.

Interleukin-6 (IL-6) is a monomeric cytokine that forms a symmetrical complex with gp130 (glycoprotein 130) and the IL-6 receptor -chain (IL-6R), and this complex is required for the propagation of intracellular signals. IL-12 is a covalently linked heterodimer composed of a light chain (IL-12p35) and a heavy chain (IL-12p40). The IL-12 receptor comprises IL-12R1 and IL-12R2, both of which have homology to gp130. The IL-12p40 component of IL-12 can also dimerize with IL-23p19 to form IL-23. The receptor for this heterodimer is formed by the association of IL-12R1 and IL-23R. The last member of this family of cytokines is IL-27, which is composed of EBI3 (Epstein–Barr-virus-induced molecule 3) and IL-27p28. IL-27 binds a receptor composed of gp130 and WSX1. The evolutionary relationship between these cytokines is shown by the homology of IL-12p35, IL-23p19 and IL-27p28 to other single-chain cytokines such as IL-6, whereas IL-12p40 and EBI3 more closely resemble the extracellular domains of members of the haematopoietic cytokine-receptor family, such as IL-6R. This similarity indicates that these heterodimeric cytokines are derived from an early precursor of the IL-6 family and from a chain of its receptor. From CA Hunter. 2005. *Nat Rev Immunol*. 5:521-531. [288]

4.2.4 IL-27 and immune regulation in the liver

As mentioned above, TGF-β, which can be present at relatively high levels within the liver microenvironment, induces IL-27 production by DC. Thus, the IL-27/IL-27R axis could play a significant regulatory role in hepatic immunobiology. In support of this possibility, IL-27 produced by liver cDC induces effector T cell hyporesponsiveness and expands $CD4^+Foxp3^+$ Treg [289]. Moreover, in different models of infection and inflammation, it was demonstrated that mice lacking the IL-27R exhibited severe liver pathology [290, 291] and necrosis [292]. Despite these important findings, studies linking IL-27, pDC and the liver immunobiology have not been reported.

4.2.5 Liver pDC suppress unwanted immune responses *in vivo*

Oral tolerance is a phenomenon whereby feeding of Ag induces Ag-specific hyporesponsiveness upon subsequent exposure to the same Ag. It has been shown that induction of oral tolerance is dependent on liver and portal vein blood flow [293]. DC play an important role in oral tolerance through the capture and presentation of orally administered Ag, and various immunologic sites have been implicated in this process [50, 51, 294]. Liver pDC are reported as the key DC subset that mediates oral tolerance and that can also suppress Ag-specific delayed-type hypersensitivity (DTH) responses [209, 295]. Systemic depletion of $PDCA\text{-}1^+$ pDC blunts the effects of Ag feeding in oral tolerance, and adoptive transfer of $PDCA\text{-}1^+$ liver pDC from OVA-fed mice at the time and site of immunization with OVA-pulsed DC suppresses Ag-specific DTH responses upon rechallenge [209]. Additionally, IL-27 production is elevated in mesenteric LN DC ($CD11c^+CD11b^+$ and $CD11c^+CD11b^-$) from OVA-fed mice [296]. These data indicate a

regulatory role for liver pDC in promoting Ag-specific tolerance and suggest that upregulation of IL-27 may contribute to this phenomenon. To date, however, there are no reports that link IL-27, liver pDC, and Ag-specific immune regulation.

4.3 MATERIALS AND METHODS

See chapter 2.0 for full description of materials and methods for experiments described in this chapter.

4.4 RESULTS

4.4.1 Liver pDC phenotype and cytokine profile correlates with weak T cell allostimulatory ability

Due to their overall paucity in normal liver, we used Flt3L to expand liver DC [65, 66, 71, 271, 297, 298], in particular pDC. We found that freshly-isolated, PDCA-1-immunobead-purified liver pDC from B6 mice exhibited an immature phenotype, characterized by low cell surface expression of MHC II/I-A^b and the co-stimulatory molecules CD40, CD80, and CD86 (Figure 4.4A). The level of expression of these molecules was generally lower than on pDC isolated similarly and concomitantly from the spleen, even following overnight stimulation with the TLR9 ligand CpG B ODN (Figure 4.4A). By contrast, liver pDC exhibited higher levels of the co-inhibitory molecule B7-H1 compared with similarly-isolated splenic pDC. The ratio of co-

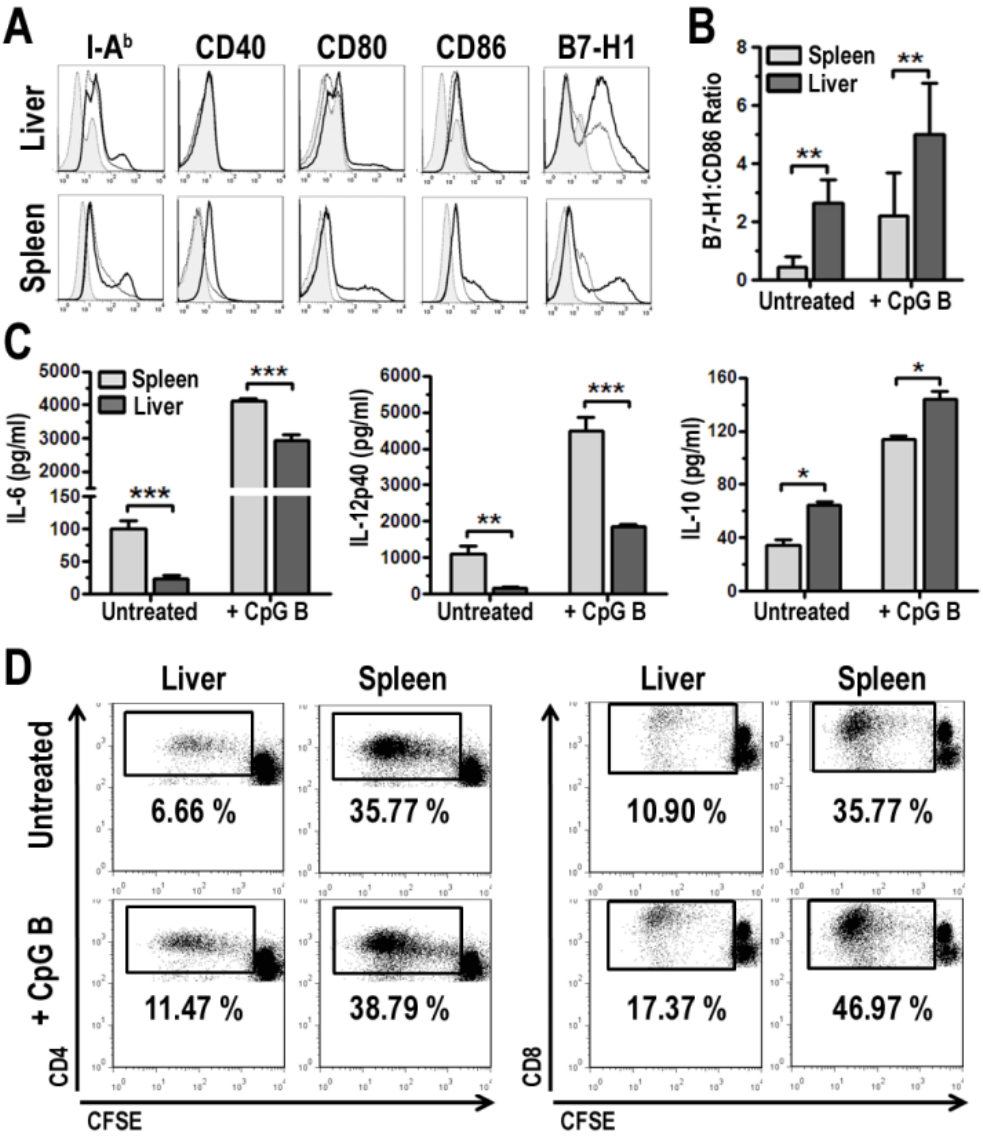


Figure 4.4 Liver and spleen pDC differ in phenotype and function.

A, B6 pDC were mobilized *in vivo* using Flt3L and enriched from total liver non-parenchymal cells or splenocytes as described in the Materials and Methods. PDCA-1-purified pDC were cultured in the absence or presence of 1 μg/ml CpG B ODN for 18 h, collected and analyzed by 5-color flow cytometry. Surface molecule expression was analyzed on $B220^{+}CD11c^{low}$ cells. Isotypes are represented by the gray-filled histograms, unstimulated cells (thin line), and CpG B-stimulated (bold line). Data are representative of 6 independent experiments; **B**, The relative mean fluorescence intensities (MFI) [Sample MFI – Isotype MFI] for B7-H1 was divided by the MFI for CD86 to generate the B7-H1:CD86 ratio. Data were averaged from 4 independent experiments; **C**, Culture supernatants from cells analyzed in **A** were harvested and cytokines quantified by ELISA. Data represent 3 independent experiments; **D**, Liver or spleen pDC were cultured with CFSE-labeled, allogeneic BALB/c T cells for 3 d. Cultures were harvested and proliferation quantified by flow cytometry. Data represent 5 independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

inhibitory to co-stimulatory molecules expressed on the surface of APC can strongly influence the functional outcome of their interaction with T cells and subsequent immune reactivity [299]. We found that liver pDC expressed a significantly higher B7-H1:CD86 ratio relative to splenic pDC, both in the steady-state and following TLR9 stimulation (Figure 4.4B).

The immature cell surface phenotype of liver pDC was associated with lower pro-inflammatory (IL-6 and IL-12p40) and higher anti-inflammatory (IL-10) cytokine production compared to splenic pDC, either in the absence of or following CpG stimulation (Figure 4.4C). Moreover, liver pDC were inferior in their ability to stimulate allogeneic $CD4^+$ or $CD8^+$ T cell proliferation compared to pDC from the spleen in 3 d CFSE MLR (Figure 4.4D). These data are consistent with observations we have reported previously regarding the phenotype and function of murine liver pDC before and after stimulation with LPS *in vitro* [72], or with CpG B or the NOD2 ligand muramyl dipeptide (MDP) *in vivo* [69].

4.4.2 IL-27p28 and the IL-27R/WSX-1 are expressed at comparatively high levels by liver pDC

IL-27 is a recently identified IL-12-family member comprised of the p40-like molecule Ebi3 and the p35-like molecule p28 [288]. Early reports on the impact of IL-27 on T cells [279] suggested that IL-27 was important for driving Th1-mediated immune responses. It was reported subsequently that IL-27 signaling could drive the induction of IL-10-producing, $Foxp3^-$ Tr1 cells, as well as inhibit the induction of $Foxp3^+$ Treg and IL-17-producing Th17 cells [244, 280, 300-302]. Interestingly, the p28 subunit alone possesses immune regulatory function. Thus, it was shown recently [303] that IL-27p28 can act as an antagonist of gp130-mediated signaling, indicating a unique role for this molecule independently of Ebi3. Although it is known that IL-27

is produced by APC (primarily activated macrophages but also DC [275, 304]), less is understood about the biology of IL-27 in relation to DC function compared to T cells.

Semi-quantitative RT-PCR analysis of freshly-isolated PDCA-1^{+} pDC shows similar levels of IL-27p28 and Ebi3 mRNA transcripts in liver and spleen pDC (Figure 4.5A). Interestingly, Western blot analysis revealed greater levels of Ebi3 protein in liver compared to spleen pDC (Figure 4.5B), and intracellular flow cytometry (Figure 4.5C) and quantification in culture supernatants by ELISA (Figure 4.5D) revealed greater IL-27p28 levels in liver pDC, suggesting possible post-transcriptional differences between pDC in these organs. It was reported previously that cDC from the liver produce more IL-27 compared to spleen cDC [289]. We did observe this phenomenon; however, we detected greater levels of IL-27p28 production by pDC compared to cDC from the liver and spleen, especially following activation (Figure 4.5D). Since Ebi3 is a shared component of both IL-27 and IL-35, an additional regulatory cytokine [305], we also tested IL-12p35 levels in these cells. We observed lower IL-12p35 mRNA transcripts (Figure 4.5A) by RT-PCR and protein expression by Western blot (Figure 4.5B) and flow analysis (Figure 4.5C) in liver pDC compared to spleen, suggesting IL-35 is not contributing to the known immune regulatory function of liver pDC.

IL-27 signals through the heterodimeric receptor TCCR/WSX-1 and the gp130 subunit, which is a shared component in the IL-6R sub-family [277]. In addition to T cells, IL-27R/WSX-1 is expressed on other hematopoietic cells, including DC, at high levels in the resting state [277]. Semi-quantitative RT-PCR (Figure 4.6A) and flow cytometric analysis (Figure 4.6B) showed that WSX-1 was expressed at significantly higher levels on liver pDC compared to splenic pDC. Together, these data suggest pDC not only produce IL-27, but have the capacity to respond to it as well.

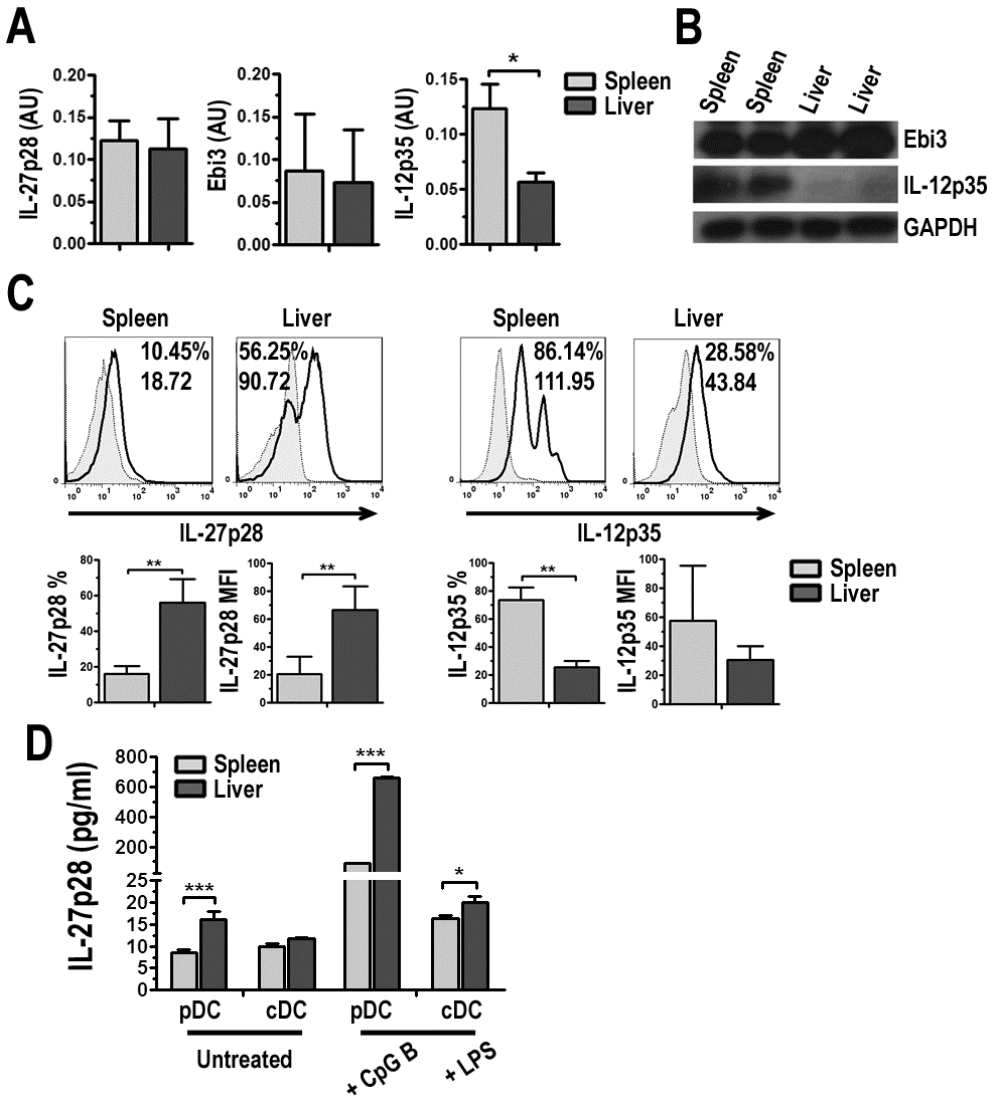


Figure 4.5 Liver pDC produce comparatively high IL-27p28 but low IL-12p35.

A, RNA was purified from freshly-isolated liver or spleen PDCA-1-purified pDC as described in Materials and Methods. RNA was reverse transcribed into cDNA and semi-quantitative PCR was performed. mRNA transcript levels of IL-27p28, Ebi3, and IL-12p35 were calculated relative to β-actin and data are presented as arbitrary units (AU). **B**, Total protein was extracted from freshly-isolated liver or spleen PDCA-1$^+$ pDC as described in Materials and Methods and Western blot performed. The membranes were probed for Ebi3 or IL-12p35 and then stripped and reprobed for GAPDH. **C**, pDC were cultured for 18 h and Golgi Plug protein transport inhibitor was added for the final 5 h of culture. Cell surface proteins were stained and cells were fixed and permeabilized prior to intracellular staining for IL-27p28 or IL-12p35. Values indicate percent positive cells and relative MFI compared to isotype controls (gray-filled histograms). Percentage of IL-27p28 or IL-12p35 positive cells and MFI were averaged from 5 and 4 independent experiments, respectively; **D**, Liver and spleen pDC and cDC were cultured in the absence or presence of 1 μg/ml CpG B ODN or LPS for 18 h. Cell culture supernatants were collected and IL-27p28 quantified by ELISA. The change in scale from "Untreated" to "+ CpG B" eliminates visibility of error bars for liver pDC. Data represent 3 independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

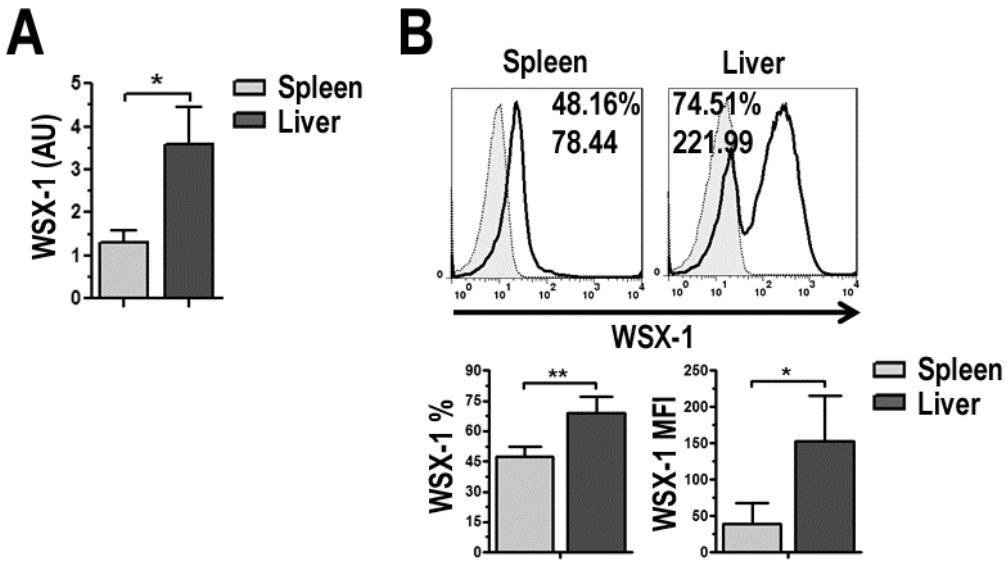


Figure 4.6 pDC express the IL-27Rα/WSX-1.

A, WSX-1 mRNA was determined by semi-quantitative RT-PCR for liver and spleen pDC and WSX-1 gene expression relative to β-actin was calculated. Data are expressed as AU; **B**, Liver and spleen pDC were stained for expression of IL-27Rα/WSX-1 and analyzed by flow cytometry. Values indicate percent positive cells and relative MFI compared to isotype controls (gray-filled histograms). Percentage of WSX-1 positive cells and MFI were averaged from 3 independent experiments, * $p < 0.05$, ** $p < 0.01$.

4.4.3 IL-27 augments B7-H1 expression on liver pDC

Previous studies on mouse [285] and human monocyte-derived DC [286] suggested that IL-27 can downregulate DC function. The high expression of WSX-1 we observed on pDC (Figure 4.6) suggests they have the capacity to respond to IL-27 in the steady-state. Therefore, we cultured liver and spleen pDC overnight in the presence of IL-27. After 18 h, cells were harvested and their phenotype examined by flow cytometry. We found that liver pDC significantly upregulated cell surface expression of B7-H1 but concomitantly downregulated expression of CD86, whereas there was no significant change in surface molecule expression of corresponding splenic pDC (Figure 4.7A and Figure A.1). As a result of increased B7-H1 and decreased CD86 expression, there was a significant increase in the B7-H1:CD86 ratio in IL-27-conditioned liver pDC

compared to untreated control liver pDC, which displayed high baseline expression of B7-H1 (Figure 4.4A) and a high B7-H1:CD86 ratio (Figure 4.4B). Interestingly, at a lower concentration of IL-27 (10 ng/ml), there was less of an increase in B7-H1 MFI by splenic pDC, but a similar response by liver pDC compared to the higher concentration of IL-27 (25 ng/ml). Despite upregulation of B7-H1, intracellular flow cytometric analysis and ELISA showed that conditioning in IL-27 did not alter production of IL-6, IL-10, IL-12p70 or IFN-α by liver or spleen pDC (data not shown).

pDC also produce IL-6 and IL-10, which are two cytokines abundant in the liver. Therefore, we tested whether exposure of pDC to these cytokines would also upregulate B7-H1 as was observed following exposure to IL-27. As shown in Figure 4.7B, only IL-27 significantly upregulated the expression of B7-H1 on liver pDC. Subsequent analysis revealed that pDC expressed low levels of the IL-6Rα and IL-10Rα (Figure 4.7C), especially relative to the high expression of WSX-1 (Figure 4.6 and Figure 4.7C). These data suggest a unique role for IL-27 and WSX-1 in the regulatory function of liver pDC.

4.4.4 STAT3 regulates IL-27-mediated upregulation of B7-H1 on liver pDC

It has been reported that STAT3 nuclear activity is greater in the liver than in other organs [306]. As a result, a higher basal level of STAT3 activation contributes to the inherent immaturity of liver DC. IL-27R/WSX-1 signaling in T cells has shown that STAT3 is a significant downstream signaling molecule that regulates the effects of IL-27 [280, 307]. Moreover, there is recent evidence that B7-H1 is upregulated on tumor cells through STAT3 activation [308]. Based on the observations that activated STAT3, B7-H1 and IL-27 are elevated

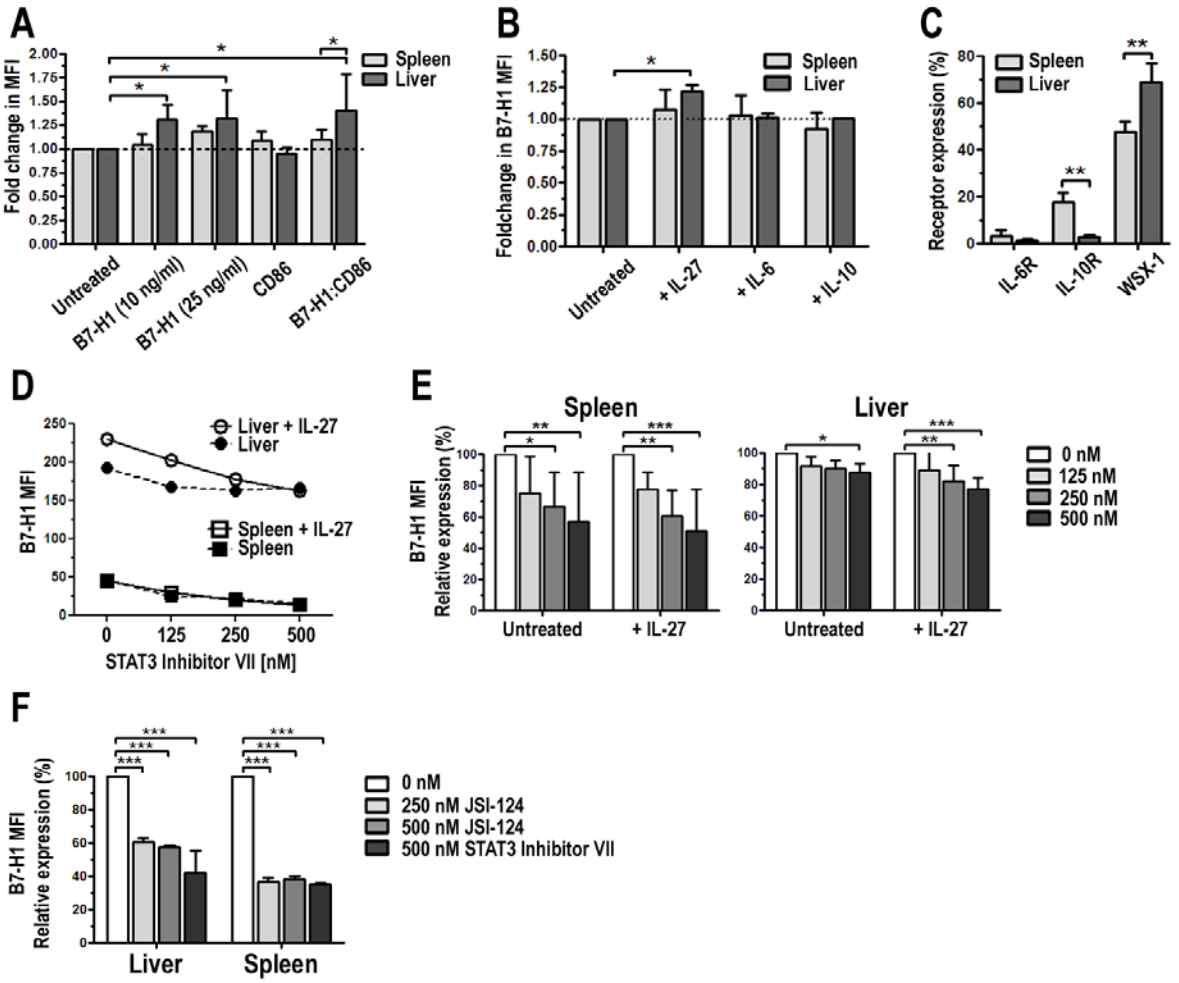


Figure 4.7 IL-27 augments B7-H1 expression on liver pDC.

A, PDCA-1-purified liver or spleen pDC were cultured for 18 h in the presence of 10 or 25 ng/ml IL-27. Cells were harvested, stained, and the expression of B7-H1 and CD86 examined by flow cytometry. Results are presented as fold change in B7-H1 MFI on IL-27-conditioned cells compared with untreated cells (set to 1.0); **B**, Liver pDC were cultured with 25 ng/ml IL-27, IL-6, or IL-10 for 18 h and B7-H1 expression was analyzed. Results represent fold change in B7-H1 MFI; **C**, Surface expression of the IL-6Rα, IL-10Rα and WSX-1/IL-27Rα were analyzed on liver and spleen pDC. Data represent percent positive cells, **D**, Experiments in A were repeated in the absence or presence of increasing concentrations of a STAT3 inhibitor (STAT3 Inhibitor VII). Data represent 4 independent experiments; **E**, Percent change in B7-H1 expression in the presence of the STAT3 inhibitor compared to untreated cells was calculated and averaged from 4 independent experiments; **F**, Experiments in **D** and **E** were repeated with a second STAT3 inhibitor JSI-124/Cucurbitacin at the indicated concentrations and compared to STAT3 Inhibitor VII. Results represent percent change in B7-H1 MFI in the presence of the STAT3 inhibitors and are an average of two independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

in the liver in the steady state, we postulated that STAT3 might be involved in upregulation of B7-H1 by IL-27 on liver pDC. To test this, liver and spleen pDC were cultured overnight in the presence of IL-27 and a highly selective STAT3 inhibitor at increasing concentrations. We observed a concentration-dependent decrease in B7-H1 expression following STAT3 inhibition (Figure 4.7D and Figure 4.7E) in both liver and spleen pDC. Additionally, we found that inhibition of STAT3 reduced IL-27-induced upregulation of B7-H1 on liver pDC (Figure 4.7D) back to or below baseline levels. To confirm this was indeed a STAT3-dependent effect, we tested a second STAT3-specific inhibitor, JSI-124/Cucurbitacin, and obtained similar results (Figure 4.7F).

4.4.5 IL-27-conditioned liver pDC increase percentage of $CD4^+Foxp3^+$ T cells which is dependent on B7-H1

Next we tested whether there were any functional consequences, in addition to the phenotypic changes observed in pDC, following their exposure to exogenous IL-27. pDC conditioned overnight in IL-27 were washed then cultured in MLR with CFSE-labeled, allogeneic BALB/c splenic T cells for 3 d. Cultures were harvested and stained for flow cytometric analysis of T cell proliferation by CFSE dilution. IL-27-conditioned liver pDC showed minimal differences in $CD4^+$ T cell allostimulatory capacity compared to untreated cells (Figure 4.8A). However, further analysis of the proliferating $CD4^+$ T cells (cells gated in Figure 4.8A) revealed an increased percentage of $Foxp3^+$ cells in cultures stimulated with IL-27-conditioned liver, but not spleen pDC (Figure 4.8B). When the percent change in Foxp3 expression in $CD4^+$ T cells cultured with IL-27-conditioned pDC relative to untreated pDC was calculated, we found a marked difference in the ability of IL-27-conditioned liver and spleen pDC to influence Foxp3

expression (Figure 4.8C): thus, liver pDC favored a 30% increase and spleen pDC favored a modest decrease in Foxp3 expression. We next tested if this increase in $CD4^+Foxp3^+$ T cells was due to the upregulation of B7-H1 by IL-27. When WT or B7-$H1^{-/-}$ liver pDC were conditioned in IL-27 and then cultured in MLR, we observed that the frequency of $CD4^+Foxp3^+$ T cells was significantly reduced in cultures with B7-$H1^{-/-}$ liver pDC (Figure 4.8D), suggesting a critical link between IL-27 and B7-H1 expression by liver pDC, and $Foxp3^+$ Treg. The increase in the proportion of $Foxp3^+$ cells was restricted to proliferating cells, as we did not observe any differences in MLR between untreated and IL-27-conditioned liver or spleen pDC in the non-dividing $CD4^+$ ($CFSE^{hi}$) population (Figure 4.8E).

4.4.6 $Ebi3^{-/-}$ mice develop oral tolerance to OVA

Recent data implicate liver pDC as critical mediators that promote the induction of oral tolerance [209], although specific mechanisms were not reported. Shiokawa and colleagues reported that IL-27 is upregulated in gut DC following Ag feeding [296], and Tong et al reported exacerbated DTH responses in $Ebi3^{-/-}$ mice [309]. Based on our data implicating IL-27 in the inherent tolerogenic function of liver pDC, we sought to determine if $Ebi3^{-/-}$ mice could develop oral tolerance to the model Ag OVA, and if they failed to do so, could adoptive transfer of WT liver pDC that produce IL-27 rescue this phenotype.

Mobilization of DC *in vivo* using Flt3L enhances the development of oral tolerance [51], therefore, we fed OVA (or PBS as a control) to B6 WT or $Ebi3^{-/-}$ mice by gavage on day 8 of Flt3L treatment, and continued Flt3L for 2 days after gavage. Seven days after Ag feeding, all mice were immunized s.c. at the base of the tail with OVA in a PBS:CFA emulsion. Another

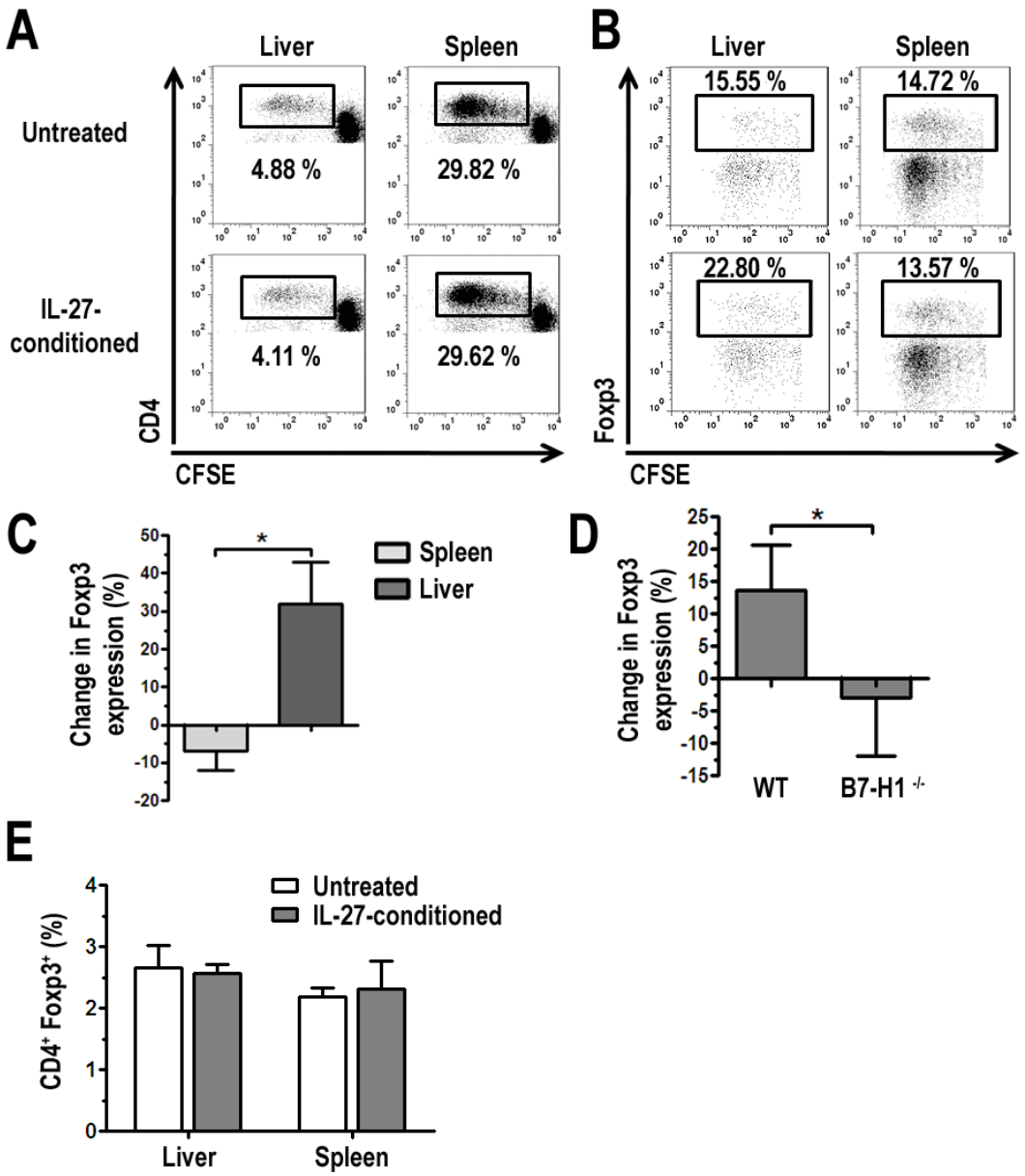


Figure 4.8 IL-27-conditioned liver pDC increase $CD4^+Foxp3^+$ T cells in MLR.

A, Freshly-isolated liver or spleen $PDCA\text{-}1^+$ pDC were cultured for 18 h with 25 ng/ml IL-27, washed, counted, and cultured in MLR with CFSE-labeled BALB/c splenic T cells. After 3 d, cells were harvested and stained for analysis of CD4+ T cell proliferation (**A**) and expression of Foxp3 (**B**) by flow cytometry. Data represents 3 independent experiments for both **A** and **B**; **C**, The percent change in Foxp3 expression in proliferating $CD4^+$ T cells (cells gated in **A**) in cultures with IL-27-conditioned liver or spleen pDC compared to untreated pDC was calculated and average across 3 independent experiments; **D**, WT or B7-$H1^{-/-}$ liver pDC were cultured with IL-27 for 18 h and subsequently cultured with BALB/c T cells. Intracellular expression of Foxp3 was analyzed by flow cytometry after 3 d MLR and results were averaged across 3 independent experiments; **E**, Foxp3 expression was quantified in non-dividing $CD4^+$ cells ($CD4^+$ CFSEhi cells) from experiments shown in **A**, and averaged across 3 independent experiments, * $p < 0.05$.

seven days after immunization, all mice were challenged with heat-aggregated OVA (HA-OVA) in the hind footpad and footpad swelling measured at 24-96 h. PBS was injected into the opposite footpad as a control.

As has been shown previously, Flt3L-mobilized B6 WT mice develop oral tolerance to OVA (Figure 4.9A and Figure 4.9B) and show significantly less footpad swelling upon challenge compared to PBS-fed controls. To our surprise, Ebi3$^{-/-}$ mice also developed oral tolerance (Figure 4.9B), with no significant difference in footpad swelling at any of the time points we evaluated. These data suggest that IL-27 is dispensable for the development of oral tolerance, and we could not proceed with this model to test the functional relevance of liver pDC-derived IL-27 *in vivo*. To simplify our experiment, we adopted only the DTH portion of the oral tolerance model, which also would allow us to more directly test the tolerogenic function of liver pDC and IL-27 *in vivo*.

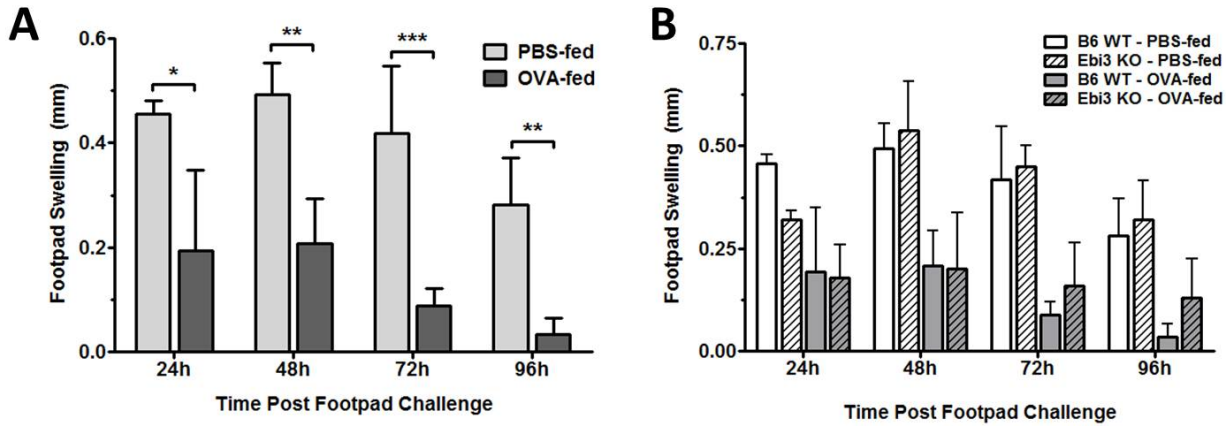


Figure 4.9 Ebi3$^{-/-}$ develop oral tolerance.

DC were mobilized in B6 WT (**A** and **B**) or Ebi3$^{-/-}$ (**B**) mice for 10 d. On day 8 of Flt3L treatment, mice were gavaged with 25 mg OVA or PBS as a control. Seven days later, all mice were immunized with 50 µg OVA in a PBS:CFA emulsion. On day 14, all mice were challenged in the footpad with 12.5 mg HA-OVA and footpad swelling measured at 24-96 h, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4.7 IL-27 produced by pDC impairs their T cell allostimulatory capacity

Before testing the tolerogenic function of IL-27 and liver pDC in vivo, we wanted to evaluate the contribution of IL-27 to the function of pDC in vitro. Utilizing Ebi3$^{-/-}$ mice that lack functional IL-27, liver and spleen pDC were purified and stimulated with CpG B and phenotypic analysis of WT and Ebi3$^{-/-}$ pDC was performed. These studies revealed few differences in the expression of key cell surface molecules (Figure A.2). Although not statistically significant, Ebi3$^{-/-}$ liver and spleen pDC showed a trend towards lower B7-H1 and WSX-1 expression following CpG B stimulation. Ebi3$^{-/-}$ spleen pDC showed slightly reduced CD86 MFI, but a significantly greater percentage of MHC class II/I-A^{b+} cells following overnight exposure to CpG B. We did not observe a change in WSX-1 expression on WT pDC following exposure to exogenous IL-27 (data not shown), however, pDC from Ebi3$^{-/-}$ mice did express a slightly, but not statistically significant lower level of WSX-1 (Figure A.2), which suggested that IL-27 signaling through WSX-1 may partially upregulate the receptor in an autocrine manner.

To determine if lack of functional IL-27 had an impact on pDC-stimulated allogeneic T cell responses, WT or Ebi3$^{-/-}$ pDC were cultured in MLR with CFSE-labeled BALB/c T cells for 3 days. T cell proliferation was quantified by flow cytometry and IFN-γ production in culture supernatants was analyzed by ELISA. Analyses of MLR cultures showed that Ebi3$^{-/-}$ liver and spleen pDC induced more CD4^{+} T cell proliferation compared to WT pDC, when either untreated or following overnight stimulation with CpG B (Figure 4.10A). No significant differences in CD8^{+} T cell proliferation were observed (data not shown). Increased CD4^{+} T cell proliferation correlated with higher levels of IFN-γ in culture supernatants, (Figure 4.10B), suggesting that IL-27 produced by pDC regulates their T cell stimulatory capacity. When T cells were stimulated in the absence of pDC (anti-CD3/anti-CD28 Ab stimulation), IL-27 did not

suppress T cell proliferation or promote Foxp3 expression (Figure A.3). These data suggest that the elevated T cell stimulatory capacity of Ebi3$^{-/-}$ pDC, and therefore, the regulatory effect of IL-27, is due to a pre-conditioning of the pDC by IL-27 *in vivo* and not a direct effect on the T cells.

4.4.8 IL-27 and B7-H1 mediate liver pDC ability to suppress DTH responses

Despite their ability to promote anti-viral immunity, pDC also exert tolerogenic effects and modulate immune responses [240]. Specifically, systemic depletion of PDCA-1^{+} cells blunts the development of oral tolerance, and adoptive transfer of liver pDC from Ag-fed mice at the site and time of immunization suppresses Ag-specific DTH responses [209]. Thus, we hypothesized that IL-27 expressed by liver pDC might mediate their capacity to suppress DTH to fed Ag.

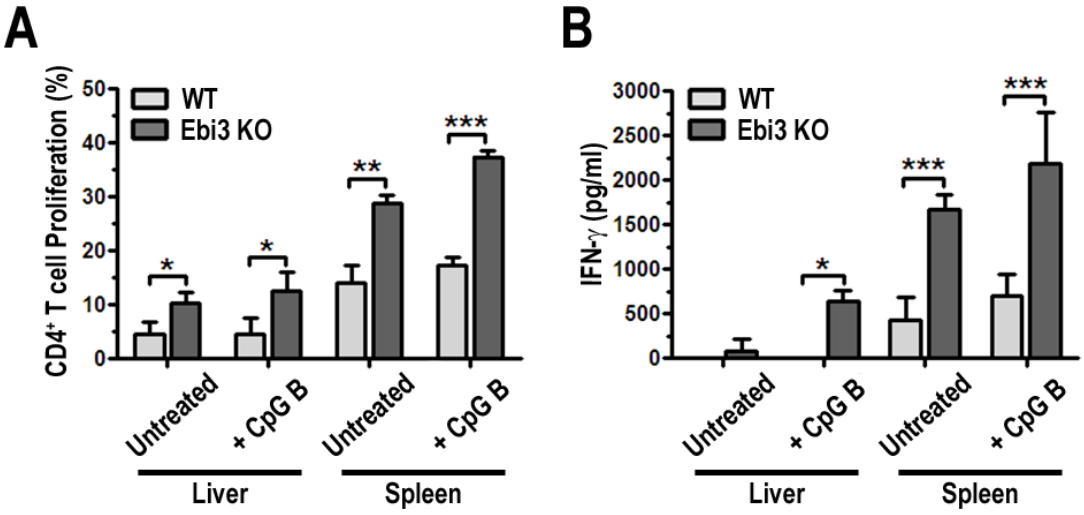


Figure 4.10 Ebi3$^{-/-}$ pDC exhibit greater CD4^{+} T cell stimulatory capacity compared to WT.

A, Unstimulated or CpG B ODN-stimulated (18 h) PDCA-1+ WT and Ebi3-/- liver or spleen pDC were cultured with allogeneic, CFSE-labeled BALB/c T cells. After 3 d MLR, cells were harvested and stained for analysis of T cell proliferation by CFSE dilution; **B**, IFN-γ was quantified in supernatants from cultures in 6A by ELISA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

First, we tested whether suppression of DTH responses was limited to pDC from the liver, and confirmed their ability to do this in an Ag-dependent manner. Liver and spleen PDCA-1^{+} pDC were isolated from mice fed OVA by gavage, or PBS as a control. pDC were co-injected s.c. at the base of the tail at a 1:1 ratio with syngeneic BM-derived DC (BMDC) that were pulsed with OVA (BMDC-OVA). BMDC or BMDC-OVA were injected alone as controls. After seven days, mice were challenged in the footpad with HA-OVA and footpad swelling was quantified. Compared to BMDC alone, BMDC-OVA induced a significant level of footpad swelling upon challenge with HA-OVA at 24 h (Figure 4.11A). Liver pDC from OVA-fed mice, but not from PBS-fed controls significantly suppressed footpad swelling when co-injected at the same site and time of immunization with BMDC-OVA (Figure 4.11A). Spleen pDC from OVA-fed mice or PBS-fed controls failed to suppress DTH responses (Figure 4.11A). Together, these results confirm that suppression of DTH responses is specific to liver pDC and is dependent upon Ag acquired though feeding.

To test whether IL-27 was required for the regulatory effect of liver pDC in the model of DTH to OVA, WT or Ebi3$^{-/-}$ liver pDC from mice fed OVA were co-injected with BMDC-OVA as described above. Mice were challenged in the footpad with HA-OVA and footpad swelling quantified at 24 h post-challenge. Liver pDC from Ebi3$^{-/-}$ mice failed to suppress DTH responses compared to WT liver pDC (Figure 4.11B). When splenocytes from these mice were restimulated *ex vivo* with OVA, we detected greater levels of IFN-γ in cultures supernatants from mice that received Ebi3$^{-/-}$ liver pDC compared to WT liver pDC (Figure 4.11C). To highlight the significance of these findings and the importance of IL-27 in the functional biology of liver pDC, we compared these results to liver pDC from mice lacking B7-H1, which is a known potent regulator of immune responses.

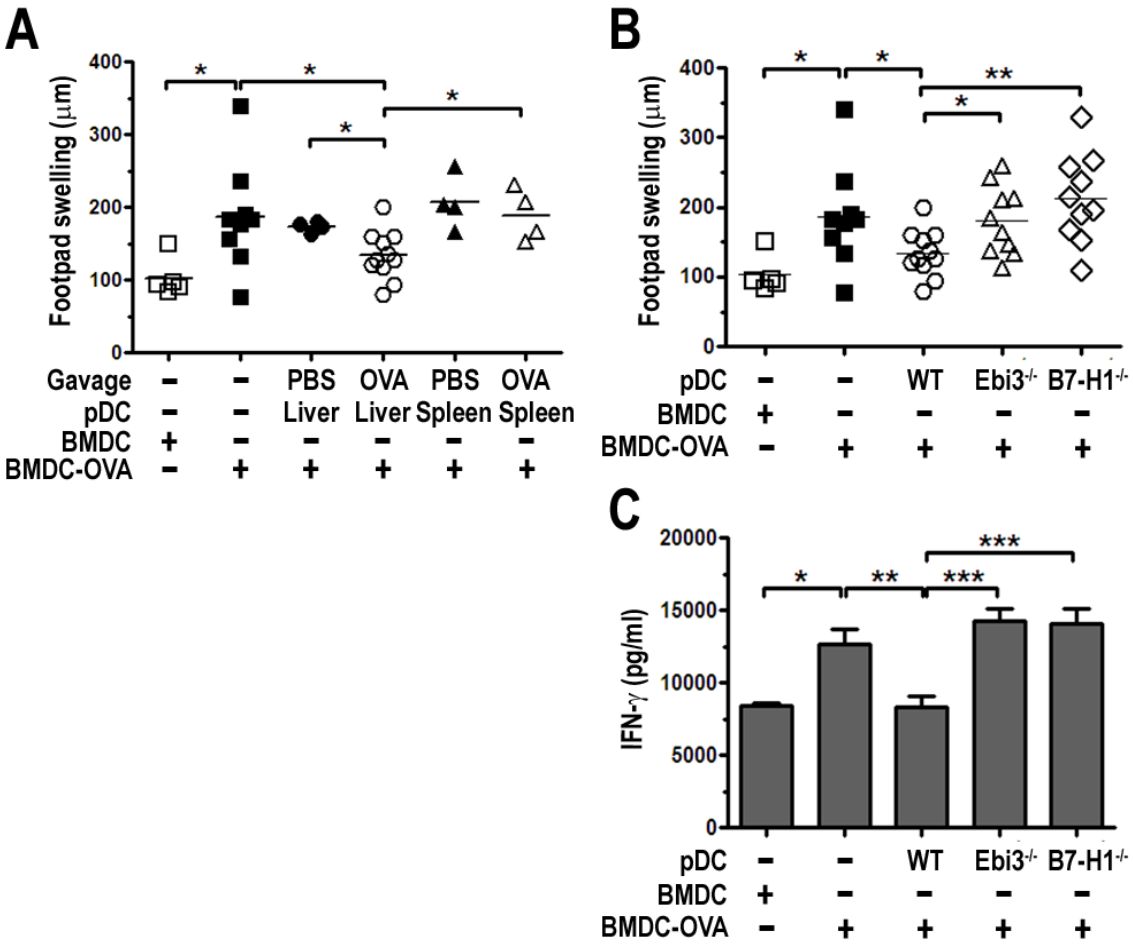


Figure 4.11 WT, but not Ebi3$^{-/-}$ or B7-H1$^{-/-}$ liver pDC suppress DTH responses to OVA.

B6 mice were immunized with B6 BMDC-OVA ± liver or spleen pDC from PBS-fed control or OVA-fed mice (**A**), or WT, Ebi3$^{-/-}$ or B7-H1$^{-/-}$ liver pDC from OVA-fed mice (**B**) by s.c. injection at the base of the tail. 7 d later, mice were challenged in the footpad with HA-OVA and footpad swelling measured at 24 h; **C**, Total splenocytes from challenged mice were cultured for 48 h with 1 mg/ml OVA and supernatants harvested for quantification of IFNγ by ELISA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

B7-H1$^{-/-}$ liver pDC also failed to suppress DTH responses compared to WT pDC. Footpad swelling (Figure 4.11B) and splenocyte production of IFN-γ (Figure 4.11C) were at a similar level as observed for mice receiving Ebi3$^{-/-}$ liver pDC, suggesting IL-27 and B7-H1 play a significant role in the immunoregulatory capacity of liver pDC.

4.5 DISCUSSION

The data presented in this chapter show that pDC express IL-27 as well as the IL-27R/WSX-1. Liver pDC express higher levels of both proteins compared to pDC from the spleen, and also exhibit a unique capacity to respond to IL-27 through upregulation of the co-inhibitory molecule B7-H1. We found STAT3 to be critical for this phenomenon, as its pharmacologic inhibition completely abrogated the effect of IL-27. A functional consequence of liver pDC conditioning by IL-27 was an ability to increase the incidence of $CD4^{+}Foxp3^{+}$ cells in allogeneic MLR. IL-27 contributes to the ability of pDC to regulate T cell responses *in vitro*, and our data also identify IL-27 and B7-H1 as critical molecules utilized by liver pDC to suppress DTH responses to OVA *in vivo*.

Although studied typically for their potent anti-viral function through production of type I IFNs [140, 269], pDC have garnered significant attention for their immunosuppressive function, and multiple mechanisms of immune regulation and tolerance by pDC have been studied by our lab and others [240]. Important features of pDC that shape their immunoregulatory function include their low level of Ag-presenting (MHC class II) and co-stimulatory molecules (CD80 and CD86) and unique regulation of MHC class II expression [31] which lead to poor T cell stimulatory function compared to cDC [128]. As we show here, pDC residing in the liver microenvironment show even lower expression of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86) compared to pDC isolated concomitantly from the spleen. Both pDC populations exhibit high baseline expression of the co-regulator B7-H1, however levels on liver pDC are greater than on spleen pDC. Liver-resident APC, including pDC, are continuously exposed to bacterial degradation products (e.g., endotoxin) via the portal blood [306] leading to constant stimulation of TLRs and other pattern recognition receptors (PRRs), which may

contribute to their comparatively high baseline expression of B7-H1. We have demonstrated previously that an elevated B7-H1:CD86 ratio on pDC is positively correlated with successful weaning of IS drug therapy or operational tolerance in liver transplant patients, suggesting that this may be an important characteristic associated with the tolerogenic capacity of pDC [310]. Analysis of the ratio of B7-H1 to CD86 MFI on liver and spleen pDC in this study clearly highlights the regulatory potential of liver pDC, based on their significantly elevated ratio of co-inhibitory to co-stimulatory molecule expression compared to pDC from the spleen.

In addition to their more immature phenotype compared with splenic pDC, liver pDC produce lower levels of the pro-inflammatory cytokines, IL-6, IL-12p40 and IL-12p70 [69], but greater levels of the immunoregulatory cytokine IL-10 in the steady state, or after stimulation with LPS [72] or CpG B. Similar to the fact that pDC are poor T cell allostimulators relative to cDC, liver pDC are comparatively weak stimulators compared with pDC from the spleen, of both $CD4^+$ and $CD8^+$ T cells.

Previous studies have identified IL-27 to play a role in the functional biology of DC, in addition to its known effects on T cell polarization and function. Liver cDC, mobilized through hydrodynamic injection of plasmids encoding GM-CSF, showed elevated levels of IL-27p28 compared to their counterparts in the spleen [289]. We observed a similar difference between liver and spleen cDC following LPS stimulation, but also found that pDC have a greater capacity for IL-27p28 production relative to cDC, and we detected higher levels of both p28 and Ebi3 in liver pDC compared to spleen pDC. Our detection of the p28 subunit in the culture supernatants of liver pDC (and intracellularly by flow cytometry) lends support to the tolerogenic potential of liver pDC, as p28 has been suggested to exert immunoregulatory function when secreted independently of Ebi3 [303, 311]. In support of our findings on IL-27, PDCA-1^+ splenic pDC

exposed to exogenous TGF-β (which is produced by multiple cell types in the liver, [60]) show elevated IL-27 and decreased IL-6 production compared to untreated controls [245], suggesting that the immunoregulatory molecules found within the normal liver microenvironment (i.e. TGF-β) may contribute to elevated IL-27 production by liver pDC. pDC also express p35, which can heterodimerize with Ebi3 to form the regulatory cytokine IL-35. However, low expression of p35 by liver pDC, which we have shown exhibit greater regulatory phenotype and function compared to pDC in the spleen, suggests that p35 and Ebi3 may not be forming functional IL-35 in these cells. The higher expression of p35 by spleen pDC, which have greater immunostimulatory capacity compared to liver pDC, supports this observation. The presence of two distinct populations of IL-12p35^{+} cells from the spleen is an interesting observation and may indicate phenotypically and functionally different subpopulations. The IL-12p35^{hi} spleen pDC may be responsible for the greater levels of IL-12p70 we have reported previously in these cells [72].

IL-27 binds to the heterodimeric IL-27R comprised of IL-27Rα/WSX-1/TCCR and gp130 [277]. Although gp130 is a shared subunit for multiple cytokines, WSX-1 is specific to IL-27 and is expressed by pDC, suggesting they have the capacity to respond to IL-27. Indeed, in the presence of exogenous IL-27, liver but not spleen pDC upregulated B7-H1 but not CD86 expression. Consequently, IL-27-conditioned liver pDC have a significantly elevated B7-H1:CD86 ratio compared to untreated cells. Furthermore, liver pDC are more sensitive to lower concentrations of IL-27, highlighting the significance of higher WSX-1 expression on these cells. Studies on T cells have revealed important roles for STAT1 and STAT3 signaling in mediating downstream effects of IL-27 [279, 280]. Wölfle et al [312] recently showed that STAT3 directly binds the B7-H1 (PD-L1) promoter and drives its expression. When we used a soluble inhibitor to block STAT3 activation *in vitro*, upregulation of B7-H1 by IL-27 was abrogated.

Additionally, B7-H1 levels on liver and spleen pDC in the absence of IL-27 also decreased, confirming that STAT3 promotes B7-H1 expression on pDC. IL-6 and IL-10 also signal through STAT3, however, the increase in B7-H1 expression we observed was unique to IL-27. Subsequent analysis showed low expression of both the IL-6R and IL-10R. These data not only suggest that these cytokine receptors may not critically influence pDC function, but also highlight the significance of high expression of the IL-27Rα/WSX-1 in the functional biology of these cells. Interestingly, we noted that exposure to IL-27 did not alter IFN-α, IL-6, IL-10, or IL-12p70 production by pDC, despite a report that IL-27 blunts TNF-α and IL-12p40 production in activated peritoneal macrophages via a STAT3-dependent mechanism [313].

Liver pDC [209, 295] and IL-27 [296] have been implicated in the development of oral tolerance, providing a model to test the importance of these two molecules together *in vivo*. We hypothesized that Ebi3$^{-/-}$ would fail to develop oral tolerance, thus allowing us to rescue this phenotype using WT liver pDC which produced functional IL-27. Using Flt3L-mobilized mice which were shown previously to exhibit enhanced development of oral tolerance [51], we observed that Ebi3$^{-/-}$ mice were still able to develop oral tolerance to OVA. Based on these data, we would not be able to directly test the contribution of IL-27 to the tolerogenic function of liver pDC. We simplified our approach by using only the DTH portion of the protocol, as described previously [209], which would directly allow us to test the immunoregulatory function of IL-27 produced by liver pDC.

We first needed to evaluate the functional immunobiology of Ebi3$^{-/-}$ liver pDC compared to WT. In studies using WSX-1-deficient mice, Yoshida et al [285] showed that, in the absence of IL-27 signaling, spleen cDC exhibited enhanced T cell stimulatory capacity in MLR, suggesting that IL-27 negatively regulates DC function. In agreement with these data, human monocyte-

derived DC exposed to exogenous IL-27 at high levels show a reduction in T cell stimulatory capacity, which is dependent on B7-H1 expression [286]. Although we did not observe any significant phenotypic differences in Ebi3$^{-/-}$ pDC compared to WT pDC, a trend towards lower B7-H1 and WSX-1 expression and a greater incidence of Ebi3$^{-/-}$ MHC class II (I-A^{b})$^{+}$ cells supports the existence of an autocrine feedback loop by IL-27 in pDC. When IL-27-conditioned pDC were cultured with allogeneic T cells, there was a greater proportion of proliferating CD4^{+} T cells expressing the Treg transcription factor Foxp3 in cultures with liver, but not spleen pDC. This effect was lost when IL-27-conditioned B7-H1$^{-/-}$ liver pDC were used as stimulators. Supporting a regulatory role for IL-27 in pDC immune function, Ebi3$^{-/-}$ pDC showed an enhanced ability to stimulate allogeneic T cell proliferation and subsequent IFN-γ production. The addition of IL-27 to these cultures did not suppress T cell proliferation to levels observed with WT pDC, suggesting that the regulatory effect of IL-27 in WT pDC, that is absent in Ebi3$^{-/-}$ pDC, is due to a pre-conditioning by IL-27 *in vivo*, and not a direct effect of IL-27 produced by pDC on T cells. Overall, IL-27 may influence liver pDC regulatory function by upregulating B7-H1 expression and enhancing their ability to promote Foxp3^{+} Treg while reducing their T cell allostimulatory capacity.

In a series of DTH and contact hypersensitivity (CHS) experiments, Goubier et al [209] showed recently that liver pDC (CD11c^{+}CD11b^{-}NK1.1^{-}) accounted for the suppressive capacity of total CD11c^{+} hepatic DC. Liver pDC suppressed both Ag-specific DTH and CHS responses to model Ags, whereas they reported spleen pDC failed to inhibit immune reactivity in their CHS model. Furthermore, systemic depletion of pDC blunted the induction of oral tolerance to OVA. Our data provide strong evidence that liver but not spleen pDC suppress DTH responses to OVA in an Ag-dependent manner. Moreover, we have identified novel mechanisms contributing to this

phenomenon. IL-27, as evidenced using Ebi3$^{-/-}$ liver pDC, and B7-H1, as evidenced using B7-H1$^{-/-}$ liver pDC, are critical for the ability of liver pDC to significantly suppress DTH responses. These data suggest that liver pDC can sufficiently acquire fed Ag and limit priming of the immune response during the immunization phase. Since pDC can inhibit cDC activation [160], it remains to be seen in this model whether pDC: suppress BMDC-OVA directly to inhibit the priming phase; migrate to the LNs to inhibit T cell activation; or both. The capacity of liver pDC to partially but not significantly suppress DTH responses in an Ag-independent manner (PBS-Liver pDC, Figure 4.11A) in addition to their significant suppression in an Ag-dependent fashion (Figure 4.11B and 4.11C) suggests they are able to actively regulate immunity through multiple mechanisms.

In summary, our studies show that IL-27 plays a critical regulatory role in the functional biology of liver pDC. First, IL-27 promotes cell surface expression of B7-H1 in liver pDC which, in turn, increases the incidence of CD4^{+} T cells expressing Foxp3. The effects of IL-27 on liver compared to spleen pDC suggest the potential for unique signaling molecules downstream of the IL-27R in these cells, which may be critical in regulating their tolerogenic capacity. Second, IL-27 is critical for liver pDC ability to regulate immune responses *in vitro* and *in vivo*, with the potential for regulation of cDC, T cells, or both. Together, these results suggest that IL-27 and liver pDC may be important targets or tools for therapeutic intervention to limit immune reactivity or promote tolerance.

5.0 DONOR pDC CONTRIBUTE TO THE SPONTANEOUS ACCEPTANCE OF FULLY MHC-MISMATCHED MURINE LIVER ALLOGRAFTS

5.1 ABSTRACT

Liver pDC possess significant capacity to regulate immune responses. In experimental liver transplantation in rodents, liver allografts are accepted spontaneously from fully MHC-mismatched donors without the use of therapeutic intervention. We sought to determine how donor liver pDC, acting as "passenger leukocytes" during transplantation, may promote spontaneous acceptance of murine liver allografts relative to other DC subsets. Freshly-isolated, B6 liver and spleen immunobead-purified PDCA-1^+ pDC or $CD11c^+$ cDC were cultured in MLR with allogeneic, C3H T cells. Proliferation was quantified by ^{3}H-thymidine incorporation, and cytokine production in culture supernatants was determined by ELISA. Freshly-isolated B6 liver or spleen pDC or cDC were injected s.c. into C3H recipients. After 7 days, C3H mice were challenged in the footpad with B6 splenocytes and footpad swelling was quantified. Splenocytes from C3H recipient mice were restimulated *ex vivo* with donor (B6), third-party (BALB/c) or syngeneic (C3H) splenocytes, and proliferation and cytokine production were determined. Liver pDC exhibited weak allogeneic T cell stimulatory capacity *in vitro* and poor ability to prime the alloresponse *in vivo* compared to all other DC groups tested. Donor livers from mice depleted of pDC were not spontaneously accepted and did not induce tolerance in our model. In conclusion,

liver pDC exhibit a weak ability to stimulate alloresponses *in vitro* and *in vivo* compared to other DC subsets, however, the mechanisms they utilize to promote spontaneous acceptance of liver allografts remain to be defined.

5.2 INTRODUCTION

5.2.1 Hepatic DC and immune regulation

Our group has played a significant role in the characterization of liver DC (pDC and cDC; Figure 5.1 and Figure 5.2) and their ability to regulate immune responses and promote tolerance [65, 66, 68, 314]. To examine their tolerogenic ability, many studies from our lab and others have compared liver DC function to that of lymphoid (spleen) DC [69-73], which have been well characterized and are known to be potent inducers of immunity. Interestingly, liver DC possess a phenotype and function that is similar to what has been described for tolerogenic DC (see section 1.3.2). Thus, compared to spleen DC, liver DC exhibit: reduced expression of co-stimulatory (CD40, CD80, CD86) and Ag-presenting (MHC class II) molecules, and comparatively high expression of co-inhibitory molecules (B7-H1); reduced production of IL-12 and elevated production of IL-10; enhanced ability to induce/promote $Foxp3^+$ Treg; and an enhanced ability to induce effector T cell apoptosis and/or hyporesponsiveness. The composition of DC subsets (Figure 5.3) within the liver is also skewed compared to lymphoid tissue [73], with an elevated proportion of pDC and $CD11c^+NK1.1^+$ natural killer (NK) DC that exhibit APC as well as natural killer cell functions [315]. NKDC have only been described in the mouse, and a human equivalent has not yet been identified.

5.2.1.1 Molecular regulation of hepatic DC function

As discussed in section 4.2.1 and described in Figure 4.1, the liver is continuously exposed to bacterial degradation products (e.g. TLR ligands) via the portal venous blood coming from the gut, resulting in a constant state of TLR stimulation. To prevent unwanted activation and maturation, DC in the liver have a unique molecular profile that maintains them in a relatively immature state. Due to the constant basal level of TLR stimulation (e.g., TLR stimulation with LPS), liver DC show impaired activation (reduced upregulation of co-stimulatory molecules and production of pro- inflammatory cytokines) compared to DC from lymphoid tissues when stimulated *ex vivo*/*in vitro* with LPS, a phenomenon termed endotoxin tolerance [64].

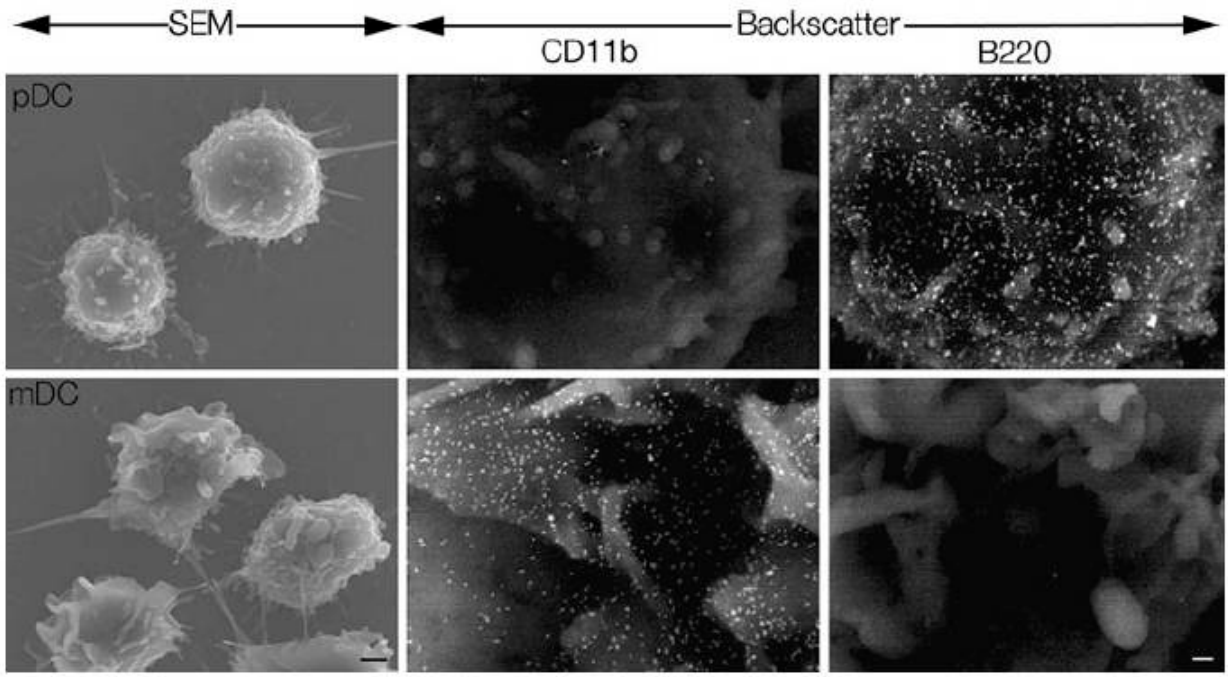


Figure 5.1 Liver DC morphologies and surface marker expression.

DC were isolated from murine liver as described [46]. Using immunoscanning electron microscopy (SEM), two liver DC populations have distinct morphologies that correlate with specific surface marker expression (B220 vs. CD11b when examined using backscatter analysis). These results indicate that the liver harbors both plasmacytoid (p) and conventional myeloid (m) DC (SEM black bars = 1 lm, backscatter white bars = 100 nm). From: Thomson AW, Geller DA, Gandhi C, Murase N, Demetris AJ, and D Beer-Stolz. 2011. *Immunol Res*. 50:221-227. [316]

Liver DC also exhibit cross-tolerance, where LPS-stimulated DC show impaired responses upon subsequent exposure to other TLR ligands such as CpG [65]. These data suggest regulation within intracellular signaling pathways in liver DC that differs from DC in other tissues that ultimately leads to the maintenance of their immature status.

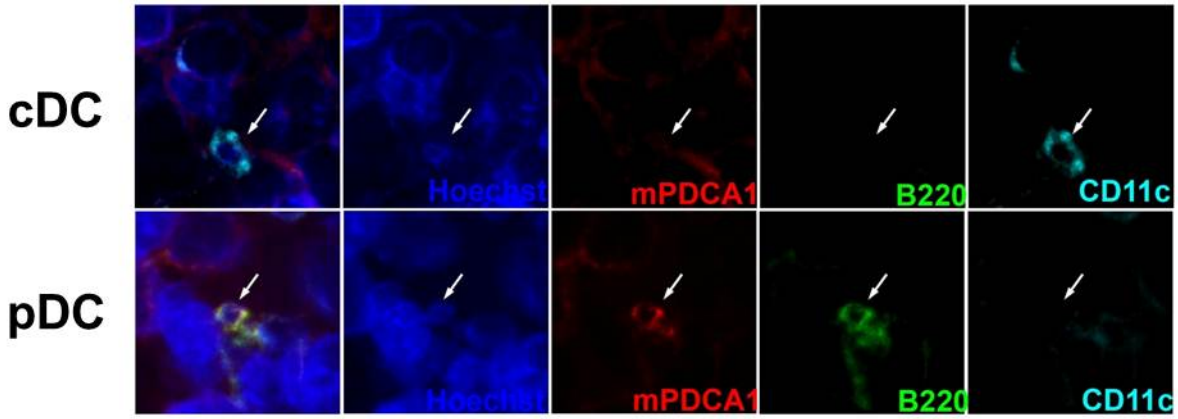


Figure 5.2 Mouse liver DC visualized using in situ multiplex Qdot immunostaining.

Investigation into the molecular mechanisms regulating liver DC maturation has produced several important observations (Figure 5.3) [60, 64]. Gut-derived bacterial degradation products present in the portal venous blood induce IL-6 production in liver DC leading to increased phosphorylation of STAT3 [306]. This leads to elevated expression of IL-1R-activated kinase (IRAK)-M, which is a known negative regulator of TLR signaling. Expanding on this study, we have reported recently [71] that the intracellular adaptor protein DAP12 plays a critical role in regulating liver DC maturation. DAP12 expression is enhanced in liver DC compared to other organs (spleen, lung, and kidney), and stimulation of liver DC with physiological concentrations of LPS (100 -1000 pg/ml; [317]) further increases DAP12 expression [71].

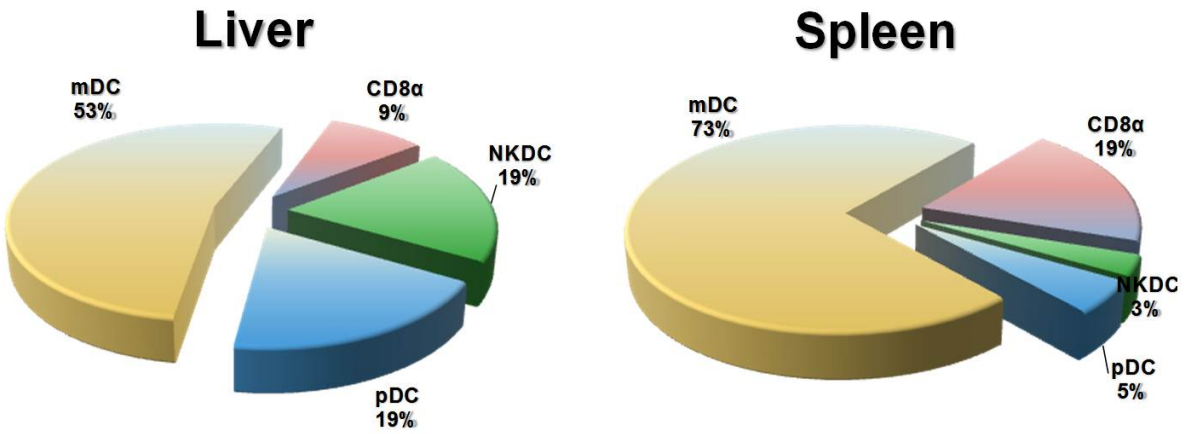


Figure 5.3 Representation of DC subsets in the liver and spleen.

Schematic representation of the proportion of DC subsets in the liver and spleen based on multi-color flow cytometric analysis. Adapted from: Pillarisetty VG, Shah AB, Miller G, Bleier JI, and RP DeMatteo. 2004. *J Immunol*. 172:1009-1017. [73]

In support of the findings mentioned above, DAP12 expression also promotes STAT3 activation and subsequent IRAK-M expression. Silencing of DAP12 using small-interfering (si)RNA technology results in: increased production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-12p70 and decreased production of IL-10; elevated expression of CD80 and CD86; enhanced T cell stimulatory capacity *in vitro* and *in vivo*; and decreased expression of IRAK-M. Moreover, as discussed in section 1.4.2.2, DAP12 associates with Siglec-H in pDC and plays an important role in their immunologic function. These important findings identify critical targets for future therapeutic intervention to target DC activation and promote or modulate their immune stimulatory capacity.

5.2.2 Liver allotransplantation

The most striking evidence supporting the tolerogenic nature of the liver is that in many rodent (mouse and rat) MHC-mismatched strain combinations [248, 318] and in human patients [319], liver allografts are spontaneously accepted (operationally tolerant) without the use of IS drug treatment, or in the clinical setting, without the continued use of IS. Moreover, in human transplantation, liver allografts that are not spontaneously accepted typically require reduced levels of IS compared to other solid organ allografts, and patients are often successfully weaned from IS drug therapy while maintaining allograft function [320-322].

5.2.2.1 Migration of donor hepatic DC following liver allotransplantation

"Passenger leukocytes" are cells resident in the donor organ that migrate to lymphoid tissues of the recipient following transplantation. This is evidenced by the fact that donor progenitor cells have been found in recipient BM following liver allotransplantation, and DC can be propagated from these BM cultures [323]. Moreover, following transplantation, inflammation and ischemia/reperfusion (I/R) injury can cause DC activation and maturation [324], leading to upregulation of CCR7 and subsequent migration to recipient secondary lymphoid tissues [325, 326]. In the setting of transplantation where hepatic lymph vessels are severed and inflammation is present, liver DC can migrate to spleen and celiac LNs [327] or portal tract-associated lymphoid tissue (PALT) [325], which may be an important step towards inducing immune tolerance in this model [328]. Collectively, these data suggest that trafficking of donor liver DC to lymphoid compartments in the recipient provides them with the opportunity for interactions with recipient cells that may contribute to the induction of tolerance to the transplanted liver.

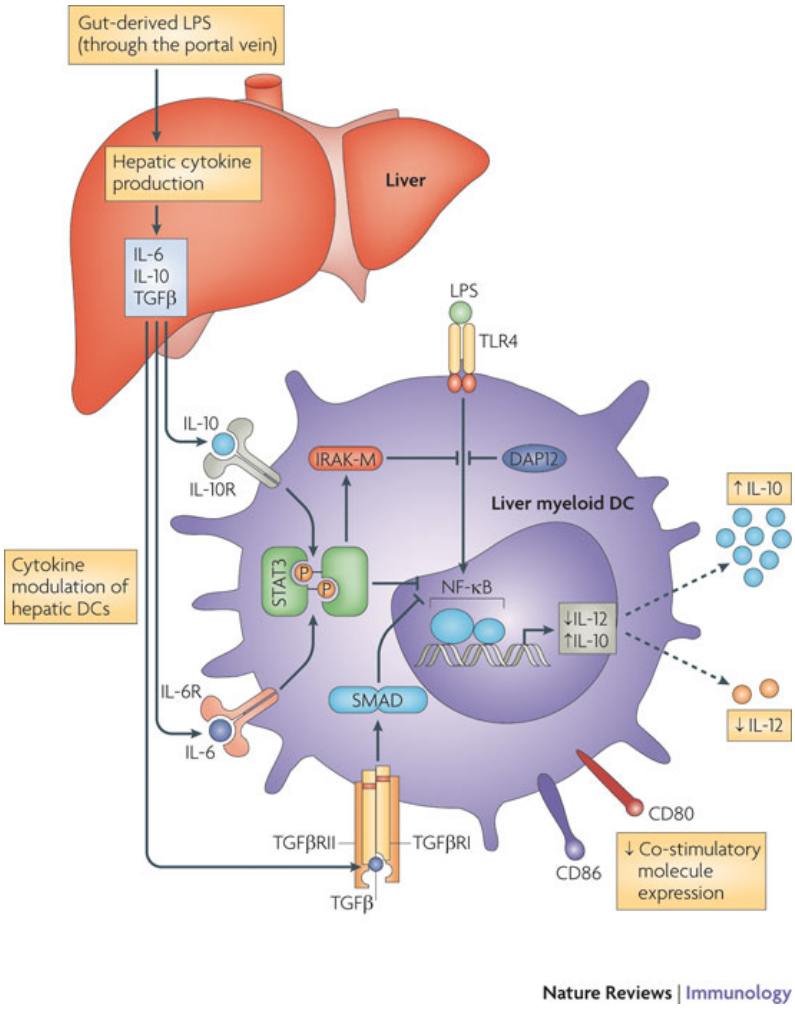


Figure 5.4 Mechanisms downmodulating the responsiveness of liver myeloid DC to LPS.

Recent evidence indicates that gut-derived commensal bacterial products, typified by lipopolysaccharide (LPS), inhibit mouse liver dendritic cell (DC) maturation by stimulating production of hepatic interleukin-6 (IL-6). In turn, this stimulates signal transducer and activator of transcription 3 (STAT3) activity downstream of the IL-6 receptor (IL-6R) in liver DCs. Hepatic DCs in normal, wild-type mice are phenotypically and functionally less mature than DCs from IL-6-deficient or STAT3-inhibited IL-6-sufficient mice. Moreover, IL-6-induced STAT3 signaling seems to increase the expression of IL-1 receptor-activating kinase M (IRAK-M), a negative regulator of Toll-like receptor (TLR) signaling (that would otherwise lead to DC maturation), in liver $CD11c^+$ DCs. IL-10 and transforming growth factor-β (TGFβ), which are produced by various liver cell populations, signal through STAT3 and SMAD proteins, respectively, to repress activation of nuclear factor-κB (NF-κB), thus inhibiting hepatic DC maturation. In addition, there is new evidence that the transmembrane adaptor protein DNAX-activating protein of 12 kDa (DAP12), which has been implicated in the negative regulation of TLR responses in DCs, is expressed at comparatively high levels by liver myeloid DCs and negatively regulates their maturation in association with IRAK-M expression. This results in impaired production of the T helper 1 (TH1) cell-inducing cytokine IL-12, but increased secretion of IL-10. From: Thomson AW and PA Knolle. 2010. *Nat Rev Immunol*. 10:753-766. [60]

5.2.2.2 pDC and liver allotransplantation

In an effort to understand the mechanism surrounding tolerance in liver allotransplantation, extensive studies have been performed to identify key biomarkers [319] that are associated with spontaneous acceptance/operational tolerance that may be used to identify patients who are candidates for weaning from IS, or that may be applied to other solid organ allografts to prevent rejection. Our group has observed an elevated ratio of pDC to cDC (Figure 5.4) in the circulation of operationally tolerant patients and those undergoing prospective weaning from IS but not those on maintenance IS [193] in pediatric liver transplantation. These pDC exhibited a high B7-H1:CD86 ratio on their surface, and this correlated with an increased frequency of $Foxp3^+$ Treg. Moreover, liver cDC are able to induce deleterious Th1 T cell responses that may contribute to liver allograft rejection [329], suggesting that pDC, and not cDC, may play a critical role in the overall success of liver allografts. To our knowledge, however, a direct role for donor pDC in promoting allograft tolerance in liver transplantation has not been evaluated.

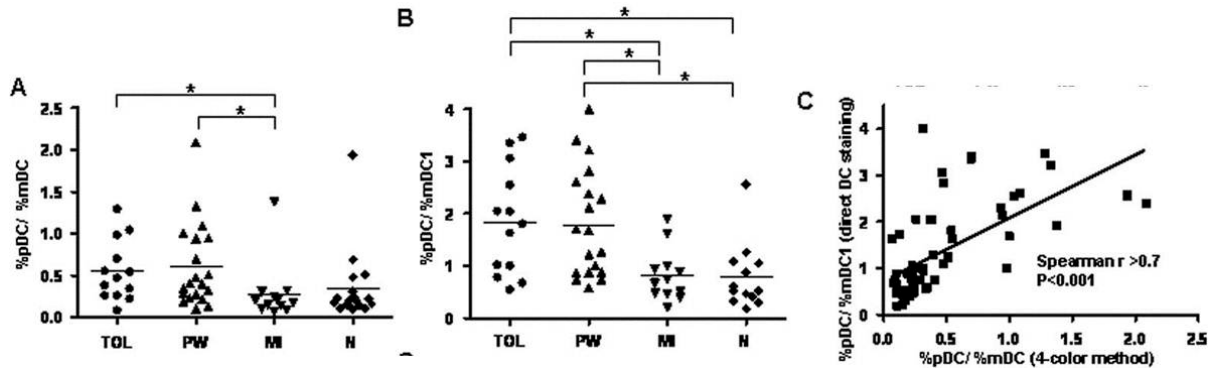


Figure 5.5 Circulating pDC/mDC ratios in pediatric liver transplant patients.

Tolerant (TOL) and prospective weaning patients (PW) exhibit higher circulating %precursor pDC/%precursor mDC ratios compared with patients on maintenance immunosuppression (MI) and controls (N). **A**, Historical four-color method. **B**, Direct DC subset staining method; values are expressed as percentage of PBMC. Data were generated as depicted in Figure 1. Individual patient or control values, arithmetic means, and significances of differences between groups (ascertained as described in the Materials and Methods by multiple comparisons using Bonferroni correction) are shown. *P<0.05. **C**, The precursor pDC/precursor mDC1 ratio obtained using the direct DC staining method shows a good correlation with the precursor pDC/precursor mDC ratio obtained using the historical four-color staining procedure. From: Tokita D, Mazariegos GV, Zahorchak AF, Chien N, Abe M, Raimondi G, and AW Thomson. 2008. *Transplantation*. 85:369-377. [193]

5.3 MATERIALS AND METHODS

See chapter 2.0 for full description of materials and methods for experiments described in this chapter.

5.4 RESULTS

5.4.1 Liver pDC exhibit weak ability to stimulate allogeneic T cell responses *in vitro*

Based on our observations that liver pDC exhibit potent immunoregulatory function, we sought to determine how they influenced allogeneic T cell responses compared to liver cDC. These two populations of cells (Figure 5.1 and Figure 5.2) may play key roles in the spontaneous acceptance of murine liver allografts, therefore, their ability to regulate or stimulate allogeneic T cells warrants evaluation. These data will help determine if donor liver pDC (or cDC) might contribute to the induction of tolerance in liver allograft recipients, based on the outcome of recipient T cell responses.

B6 (donor strain) liver pDC or cDC were cultured with normal, allogeneic C3H (recipient strain) T cells, and proliferation was quantified by ^{3}H-thymidine incorporation. Liver pDC induced very low levels of allogeneic T cell proliferation compared to liver cDC (Figure 5.6A and Figure 5.7A). Moreover, liver pDC were the weakest T cell stimulators compared to liver cDC, splenic pDC, and splenic cDC. We observed these results using freshly-isolated, unstimulated, irradiated DC (Figure 5.6A), or non-irradiated DC +/- CpG B ODN (pDC) or LPS (cDC) (Figure 5.7A). Supernatants harvested from these cultures were assayed for cytokine production by ELISA. Liver pDC induced less T cell production of IFN-γ and IL-2 (Figure 5.6B and Figure 5.7B) compared to all other DC groups. Interestingly, pDC induce similar levels of IL-10 (Figure 5.6B) compared to the other DC groups. Analysis of the ratios of IFN-γ:IL-10 and IL-2:IL-10 (Figure 5.6C) shows that liver pDC induce very high levels of IL-10 relative to IFN-γ and IL-2. Moreover, when IL-10 levels are divided by the proliferation from Figure 5.6A, liver pDC show the greatest ratio, suggesting liver pDC may be inducing IL-10-producing Treg.

These data suggest that liver pDC can promote regulatory responses by T cells without inducing significant levels of effector T cell activation, and ultimately, liver pDC may be a critical subset of donor DC that could promote allograft tolerance in the recipient following liver transplantation.

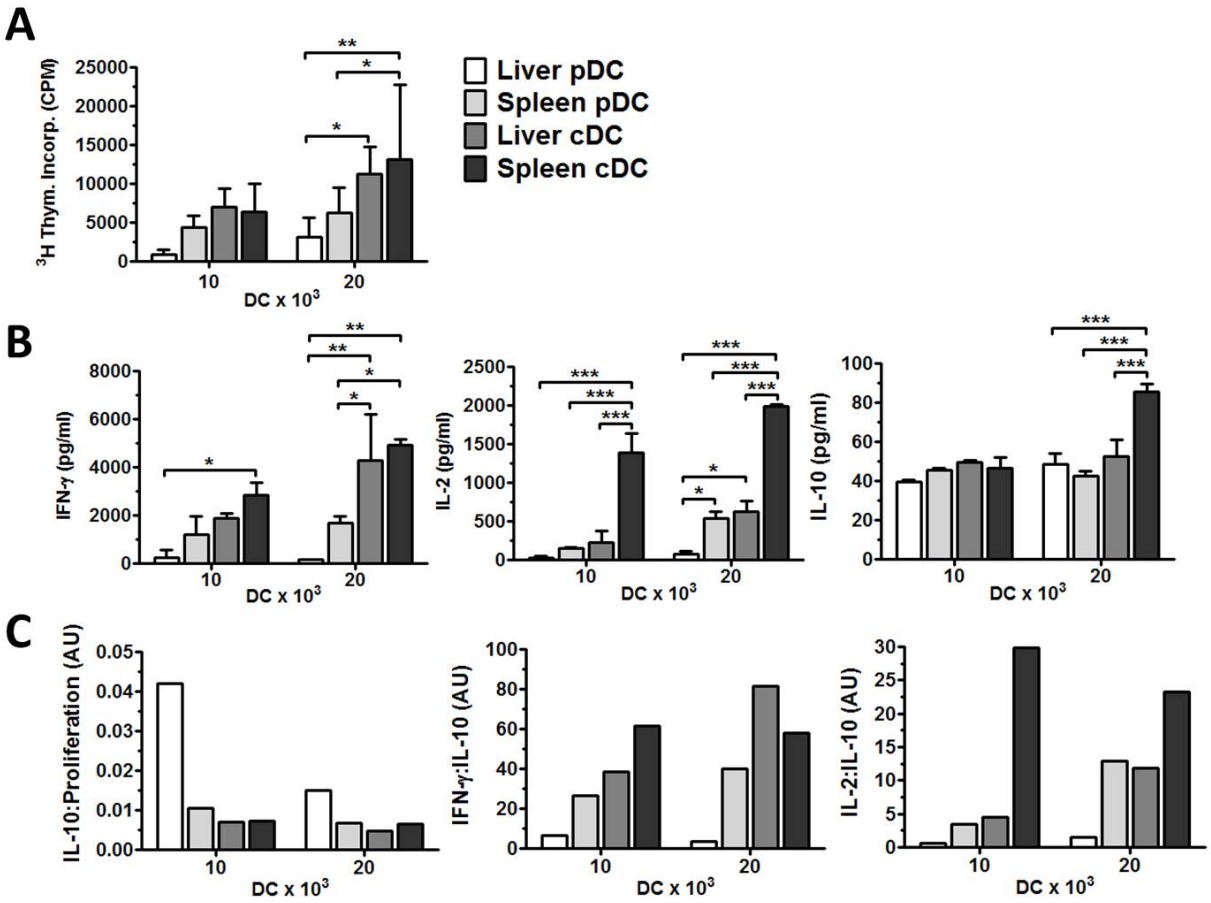


Figure 5.6 Liver pDC exhibit weak ability to stimulate allogeneic T cell responses.

Freshly-isolated PDCA-1$^+$ pDC or CD11c$^+$ cDC from livers and spleens of B6 mice were irradiated (2000 rad) and cultured with allogeneic C3H T cells (CD4$^+$ and CD8$^+$) for 5 days. **A**, 1 µCi 3H-thymidine was added to each well for the final 18 hours of culture. Proliferation was quantified as radioisotope incorporation using a liquid scintillation counter and shown as counts per minute (CPM). **B**, Culture supernatant from **A** was assayed by ELISA for IFN-γ, IL-2, and IL-10. **C**, The ratios of IL-10:proliferation, IFN-γ:IL-10 and IL-2:IL-10 were calculated from data in **B**. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

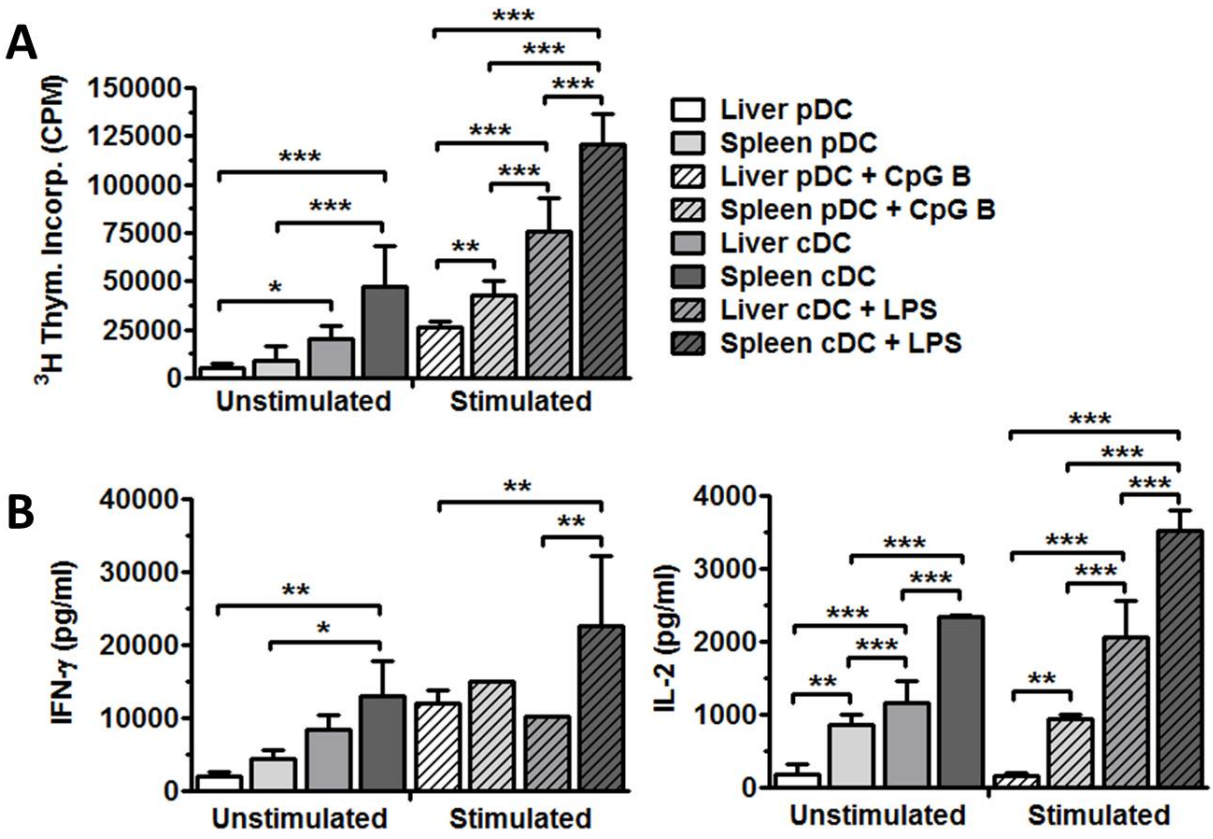


Figure 5.7 Liver pDC remain weak T cell allostimulators following activation.

Freshly-isolated PDCA-1$^+$ pDC or CD11c$^+$ cDC from livers and spleens of B6 mice were cultured with allogeneic C3H T cells (CD4$^+$ and CD8$^+$) for 5 days in the absence or presence of 10 ng/ml CpG B ODN (pDC) or LPs (cDC). **A**, 1 µCi 3H-thymidine was added to each well for the final 18 hours of culture. Proliferation was quantified as radioisotope incorporation using a liquid scintillation counter and shown as counts per minute (CPM). **B**, Culture supernatant from **A** was assayed by ELISA for IFN-γ and IL-2. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

5.4.2 Liver pDC fail to prime allogeneic immune responses *in vivo*

Due to their weak ability to stimulate allogeneic T cell responses *in vitro*, we next determined if liver pDC could prime donor-specific responses *in vivo*. B6 liver or spleen pDC or cDC were injected s.c. into C3H recipient mice. After 7 days, all mice were challenged in the hind footpad

with B6 splenocytes, and footpad swelling was quantified at 24, 48, and 72 hours post-challenge. We did not observe any significant differences in levels of footpad swelling between any of the groups (Figure 5.8A). Within individual experiments, some of the animals that were immunized with liver pDC showed lower levels of footpad swelling relative to the other groups (Figure 5.8B), however, an average of all animals tested did not reveal significant differences between any DC groups (Figure 5.8A).

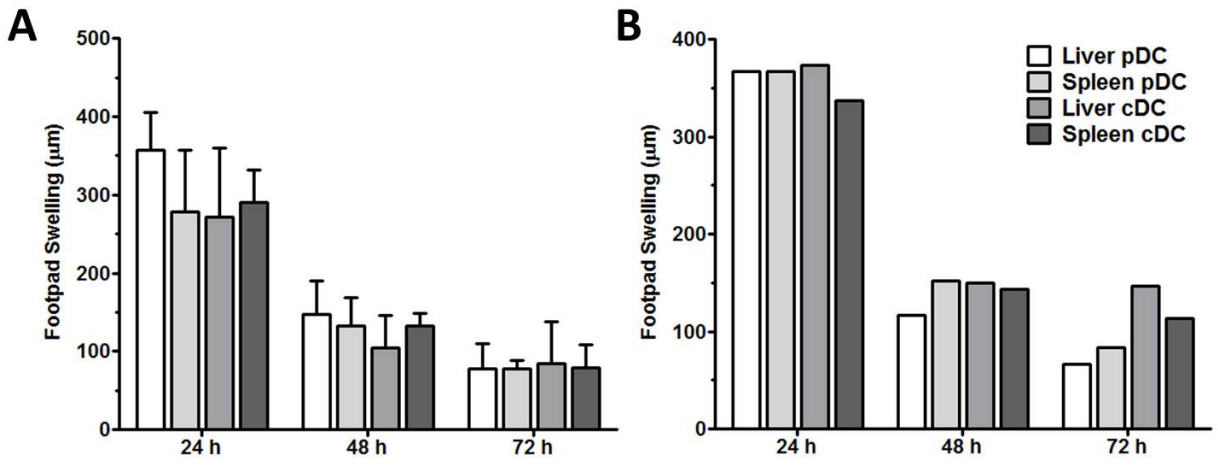


Figure 5.8 Use of Allo-DTH to assess DC ability to prime alloresponses *in vivo*.

Freshly-isolated PDCA-1$^+$ pDC or CD11c$^+$ cDC ($5x10^6$) from livers and spleens of B6 mice were injected s.c. into C3H recipients. After 7d, C3H mice were challenged in the footpad with $1x10^7$ B6 splenocytes. Footpad swelling was quantified at 24, 48, and 72 h post-challenge. **A**, Data are an average of 3 mice. **B**, Representative data of 3 independent mice.

Despite no significant differences in footpad swelling, we harvested spleens from C3H recipient mice at 96 h post-challenge and restimulated them with irradiated B6 (donor), BALB/c (third-party), or C3H (syngeneic) splenocytes at a 1:1 ratio for 4 days. Proliferation and cytokine production by C3H recipient cells were quantified by ^{3}H-thymidine incorporation and ELISA,

respectively. Naïve C3H mice that were not primed with B6 DC showed a weak proliferative response (Figure 5.9A) and produced very little IFN-γ (Figure 5.9B) when restimulated with B6 splenocytes. In correlation with our *in vitro* data (Figure 5.6 and Figure 5.7), liver pDC did not strongly prime the alloresponse *in vivo*, since C3H that were primed with all other DC groups exhibited greater secondary responses (Figure 5.9) compared to mice primed with liver pDC. A similar observation was noted for C3H responders restimulated with BALB/c splenocytes, suggesting a modest level of T cell activation not specific against B6 alloAgs. The response against syngeneic (C3H) splenocytes was minimal, and no significant differences were observed between all four DC groups tested.

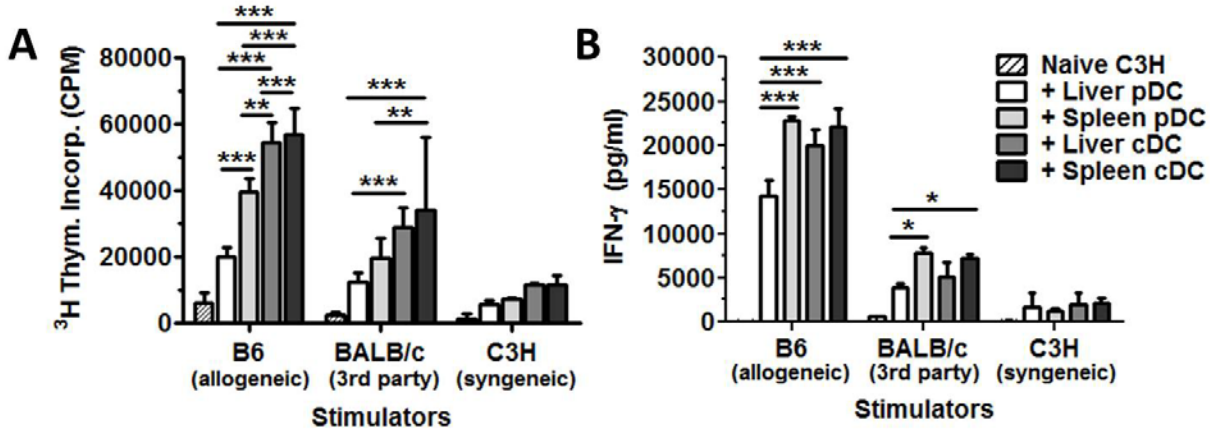


Figure 5.9 Liver pDC do not elicit strong alloresponses *in vivo*.

Freshly-isolated PDCA-1$^+$ pDC or CD11c$^+$ cDC ($5x10^6$) from livers and spleens of B6 mice were injected s.c. into C3H recipients. After 7d, C3H mice were challenged in the footpad with $1x10^7$ B6 splenocytes. After 96 h, spleens were harvested from C3H recipient mice, and restimulated *ex vivo* with irradiated splenocytes from B6 (donor), BALB/c (third-party), or C3H (syngeneic) mice for 4 d. **A**, 1 µCi ^{3}H-thymidine was added to each well for the final 18 hours of culture and proliferation was determined by quantification of radioisotope incorporation using a liquid scintillation counter. **B**, Culture supernatant from **A** was assayed by ELISA for IFN-γ. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

5.4.3 High expression of ICAM-1 and ICAM-2 on liver pDC may contribute to their tolerogenic capacity

Adhesion molecules play an important role in T cell trafficking and activation. The interaction of leukocyte function-associated Ag (LFA)-1 with intracellular adhesion molecule (ICAM)-1 is critical for both activation of T cells by APC/DC and for migration of activated T cells into inflamed tissues [330]. ICAM-1/LFA-1 interactions have also been associated with the immune regulatory function of several cell types, especially within the hepatic microenvironment. Hepatocytes are able to induce apoptosis of activated T cells [331] and hepatic stellate cells can inhibit T cell function [332]; both of which are dependent on expression of ICAM-1. ICAM-2 also binds to LFA-1, and although ICAM-1 expression may be more critical for interaction with T cells, ICAM-2 also plays a role in mediating T cell responses [333].

Based on the weak ability of liver pDC to stimulate allogeneic T cells (Figure 5.6 and Figure 5.7), we analyzed ICAM-1 and ICAM-2 expression on pDC. pDC isolated from both the liver and spleen showed positive expression of ICAM-1 and ICAM-2 (Figure 5.10). Interestingly, liver pDC expressed significantly greater levels of both molecules compared to spleen pDC, both before and after stimulation with CpG B. A greater difference in expression between liver and spleen pDC was observed for ICAM-2 compared to ICAM-1, however, both molecules were expressed at greater levels on liver pDC (Figure 5.10). These data suggest that liver pDC may be able to form stronger interactions with T cells compared to spleen pDC, and their known regulatory function and inability to adequately stimulate T cell responses suggests expression of these molecules may support other functions.

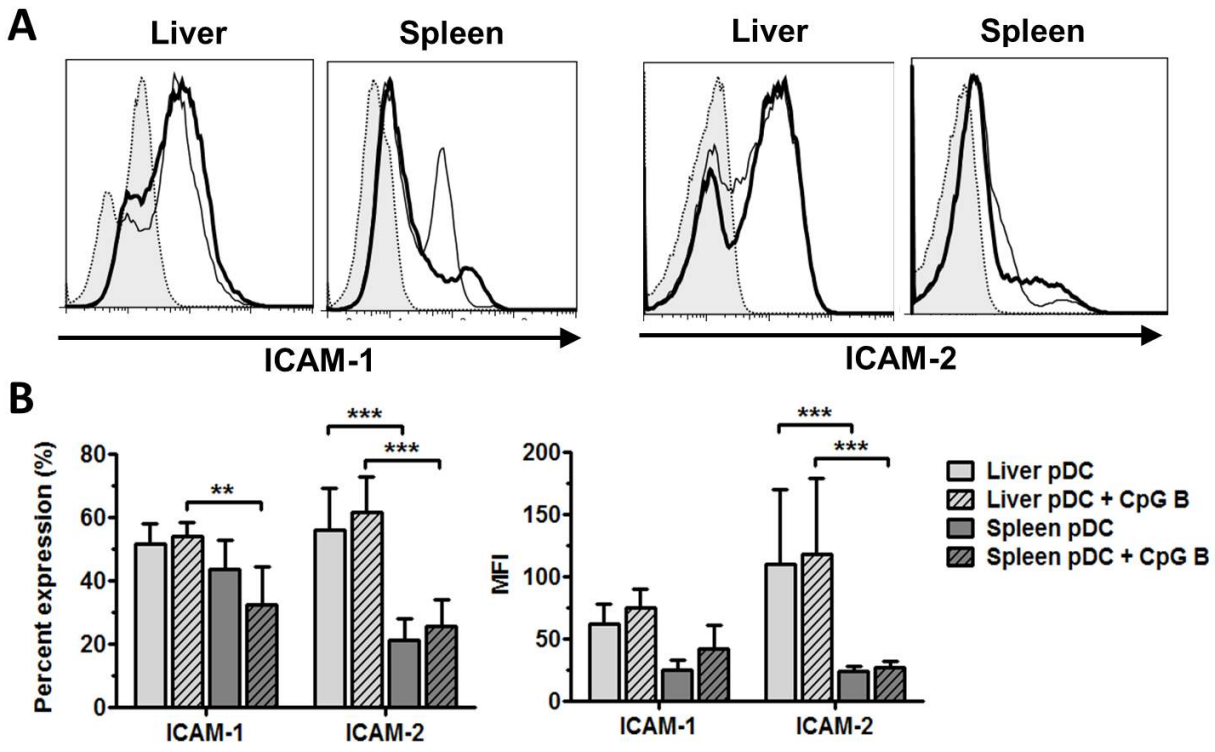


Figure 5.10 ICAM-1 and ICAM-2 expression on liver and spleen pDC.

PDCA-1$^+$ B6 liver or spleen pDC were cultured for 18 h with or without 1 µg/ml CpG B ODN. Cells were harvested from culture and stained for analysis of expression of ICAM-1 (CD54) and ICAM-2 (CD102) by flow cytometry. **A**, Representative histograms for ICAM-1 and ICAM-2 expression on liver and spleen pDC. Isotype control (shaded histogram), unstimulated pDC (thin line), pDC + CpG B (bolded line). **B**, Percent expression and MFI for ICAM-1 and ICAM-2 on pDC were averaged across 4 independent experiments. ** $p \leq 0.01$, *** $p \leq 0.001$.

5.4.4 Donor pDC promote long-term survival of fully MHC-mismatched liver allografts

In solid organ transplantation, the liver is regarded as a tolerogenic organ that typically requires less IS to prevent rejection, and can be accepted spontaneously across MHC barriers (spontaneous operational tolerance). This phenomenon may be largely dependent upon donor-derived cells (passenger leukocytes) [323, 327] that are transplanted into the recipient with the

allograft and can promote tolerance to alloAgs. Our group has clearly defined a tolerogenic role for pDC in the liver, however their role in spontaneous acceptance of liver allografts has not been reported.

We used the monoclonal antibody 120G8 to deplete pDC *in vivo* from B6 mice (Section 2.13 and Figure 5.11). Livers were harvested from pDC-depleted mice and transplanted orthotopically into C3H recipients. Graft survival was monitored based on animal survival. In the mouse model of B6 to C3H liver allotransplantation, livers are spontaneously accepted and the animals survive long-term (Mean survival time [MST] = 95 days) in the absence of therapeutic intervention (Figure 5.12) or IS treatment. When livers from pDC-depleted B6 mice were used as donors, mean survival time was much shorter (MST = 25 days). These data are the first to directly identify donor liver pDC as a critical cell population required for long-term survival and spontaneous acceptance of murine liver allografts.

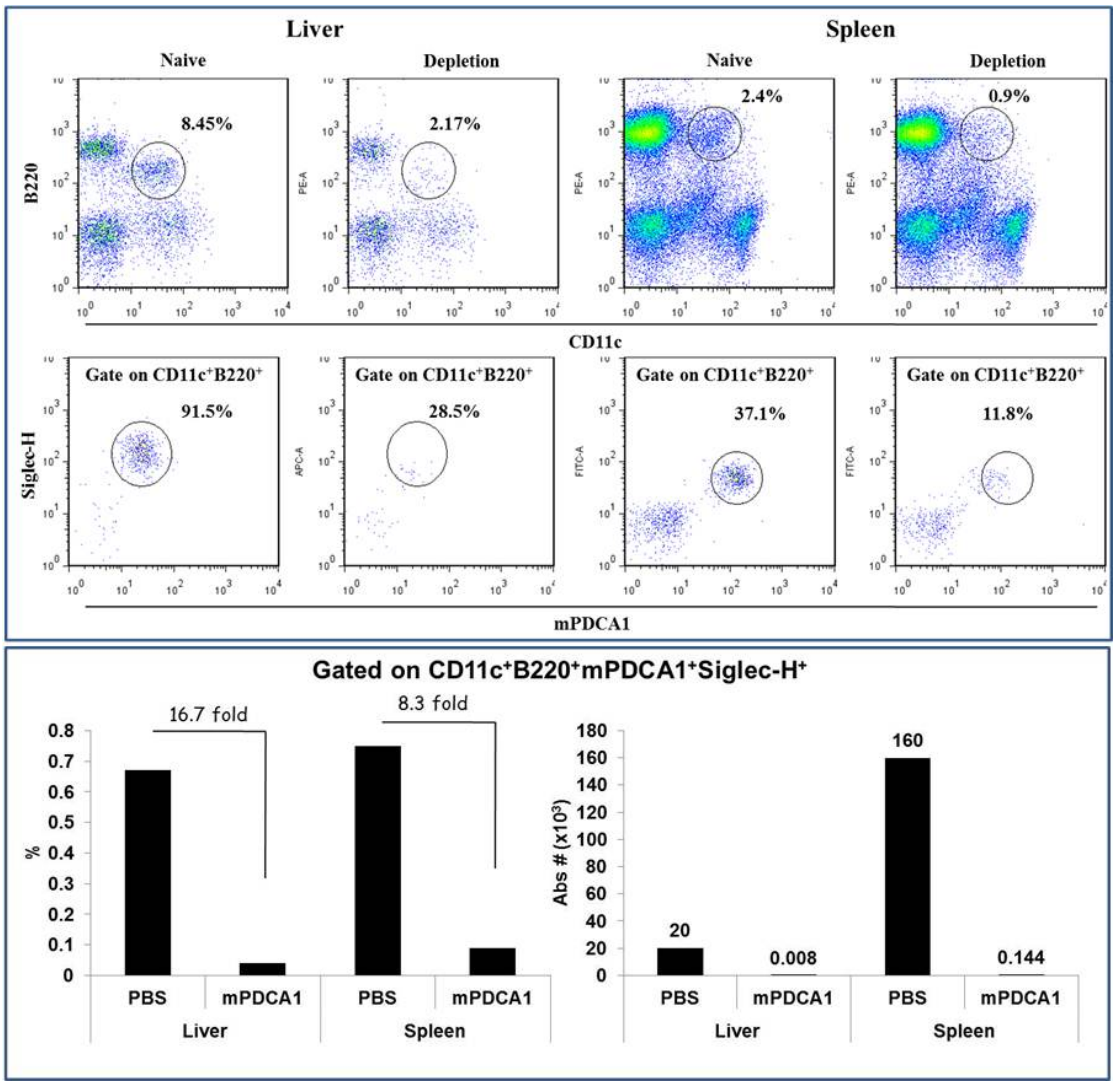
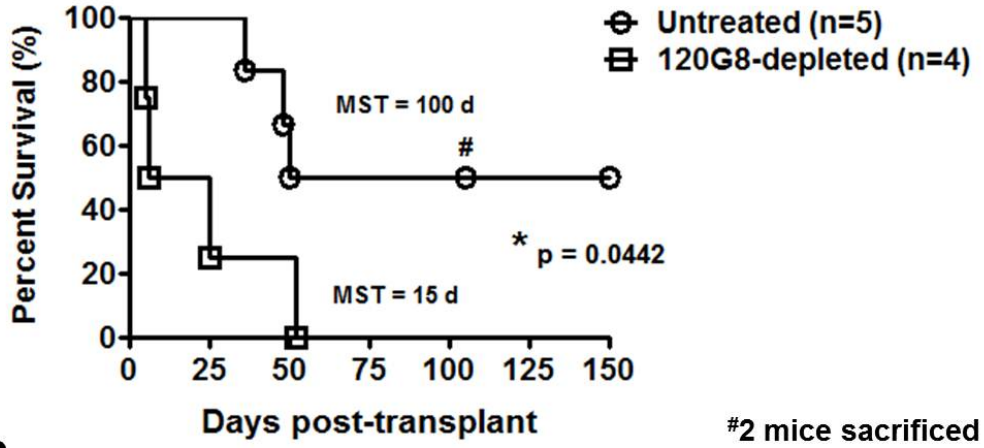


Figure 5.11 *In vivo* depletion of PDCA-1$^+$ pDC.

B6 mice were administered 120G8 monoclonal antibody i.p. and depletion of pDC was confirmed by flow cytometric analysis. Top panel shows representative data and bottom panel shows an average of 3 mice. Data courtesy of A. Castellaneta.

A



B

#2 mice sacrificed at day 105 for histological analysis

B6 to C3H	Survival (days)	Mean Survival Time (days)
Untreated	36, 48, 50, 105#, 105#, 150+	MST = 100 d
pDC (120G8)-depleted	5, 6, 25, 52	MST = 15 d

Figure 5.12 Donor liver pDC promote spontaneous acceptance of murine liver allografts.

Livers from normal (untreated) or pDC (120G8)-depleted B6 mice were transplanted orthotopically into C3H recipient mice and survival was monitored. Data are represented graphically (A) as percent survival or in tabular form (B) including individual mouse survival times. MST = mean survival time, # represents 2 mice that were alive and healthy that were sacrificed at day 105 post-transplant for histological analysis.

5.5 DISCUSSION

The data presented in this chapter clearly identify liver pDC as having a very weak ability to stimulate allogeneic immune responses. Their T cell allostimulatory capacity *in vitro* and ability to prime immune responses to alloAgs *in vivo* is weaker compared to cDC in the liver, and to both pDC and cDC from the spleen. Liver pDC exhibit an elevated expression of ICAM-1 and

ICAM-2 compared to spleen pDC and their expression may govern interactions with T cells and other APC that contributes to their ability to regulate immune responses. The significant tolerogenic potential of liver pDC is highlighted by the fact that when pDC are depleted from the donor, liver allografts are not spontaneously accepted as observed with normal, WT donor livers.

Liver DC have garnered significant attention in recent years for their profound ability to regulate immune responses [60]. Recent studies from our lab and others have made significant progress in elucidating the mechanisms that regulate their inherent tolerogenic capacity compared to DC in lymphoid tissues [65, 69-73, 306]. Thus, DC residing in the liver microenvironment are conditioned such that in the steady state and certain pathological conditions, their function favors the development of immunological tolerance.

In chapter 3.0, we present data that show spleen pDC are phenotypically immature and possess weaker T cell allostimulatory capacity compared to spleen cDC. In chapter 4.0, our data show that liver pDC are more immature and possess weaker T cell allostimulatory compared to spleen pDC. Comparison of all four DC subsets in the present chapter identifies liver pDC as the being the weakest T cell allostimulators in *in vitro* MLR assay compared to liver cDC, and spleen pDC and cDC. B6 liver pDC induce significantly less proliferation, and IFN-γ and IL-2 production from C3H T cells. They do however, induce similar levels of IL-10 production, and comparison of IFN-γ and IL-2 to IL-10 shows that liver pDC are able to induce high levels of IL-10, despite failure to promote effector T cell function. Further highlighting these data, analysis of the ratio of IL-10 to proliferation shows that liver pDC promote the induction of IL-10 producing T cells to a greater degree than any other DC group evaluated here. We showed in Chapter 4.0 that liver pDC produce high levels of IL-27 which limits their T cell allostimulatory ability, and IL-27 is also known to induce IL-10-producing Tr1 Treg [244]. Thus, liver pDC may

preferentially induce the generation of IL-10-producing Tr1 Treg over effector T cells through production of IL-27. Further investigation into the phenotype of the T cells in these cultures could provide additional support for this hypothesis.

In solid organ transplantation, donor DC or "passenger leukocytes", are believed to play a significant role in rejection as they migrate from the graft to secondary lymphoid tissues in the recipient and initiate immune responses against alloAgs via the direct pathway of allorecognition [334, 335]. Although they traffic from the allograft to secondary lymphoid tissues, donor liver DC persist in the recipient following liver transplantation [248], and their distinct regulatory function may play a significant role in spontaneous liver allograft acceptance in experimental animal models. In correlation with their weak ability to stimulate allogeneic T cells *in vitro*, liver pDC exhibit a very limited capacity to prime immune responses to alloAgs *in vivo*. Compared to C3H primed with B6 liver cDC, mice primed with liver pDC exhibited weaker secondary responses (proliferation and cytokine production) when restimulated *ex vivo* with B6 alloAgs. We did not, however, detect differences between experimental groups in levels of footpad swelling following challenge with B6 splenocytes in these mice. This may be due in part, to the large number of B6 splenocytes injected in the footpad ($1x10^7$). The level of swelling induced by the presence of these cells in the footpad may not allow us to observe subtle, yet significant differences in the recall response between the groups. In support of this, we typically did not observe any differences until nearly 72 hours following footpad challenge for each experiment (Figure 5.3B), whereas in the DTH model using OVA, we could detect significant differences after only 24 hours when using OVA protein for footpad challenge (Figure 4.10). Repeated experiments using fewer cells for footpad challenge may allow for the observation of more subtle differences between the groups. Conversely, an increase in the number of DC injected during the

immunization/priming phase may also result in a greater initial response during the priming phase, thus resulting in a greater degree of footpad swelling upon challenge with splenocytes.

It was recently reported in human liver transplantation [329], that donor liver cDC are capable of stimulating allogeneic Th1 T cell responses in recipients of liver allografts and may contribute to liver allograft rejection. Thus, liver pDC, and not cDC, may be a critical cell population that can promote spontaneous acceptance of liver allografts in experimental models and in the clinical setting. Overall, our data evaluating the ability of liver pDC to prime immune responses to alloAgs *in vivo* suggest that in the setting of liver transplantation, donor liver pDC that migrate from the graft into the recipient may not elicit strong responses to alloAgs.

As mentioned earlier, many mechanisms have been attributed to the tolerogenic capacity of liver pDC, including molecular regulation of key immunomodulatory proteins. This results in in skewed expression of key molecules, at lower levels (CD40, CD80, CD86, MHC class II, IL-6, and IL-12) or higher levels (B7-H1 and IL-10) compared to spleen pDC [70]. Interestingly, we show that liver pDC also express higher levels of ICAM-1/CD54 and ICAM-2/CD102 compared to spleen pDC. These two adhesion molecules are typically thought of as being important for activation of T cell responses [330], however a role for them in regulating immune responses has also been reported. Hepatic stellate cells are known to inhibit T cell responses, and recent data show that elevated expression of ICAM-1 is critical for their ability to restrict T cell activation, including regulation of expression of the high affinity IL-2R and production of IL-2 [336]. Thus, high ICAM-1 expression on liver pDC may contribute to their weak T cell allostimulatory capacity and failure to induce significant amounts of IL-2 production. It is also possible that ICAM-1 regulates liver pDC function. ICAM-1/LFA-1 interactions between $Foxp3^+$ Treg and DC can downregulate CD80 and CD86 expression on DC and their subsequent ability to activate

allogeneic T cell responses [337]. Our data that show liver pDC express low levels of co-stimulatory molecules CD80 and CD86 (Figure 4.4), and they promote Foxp3^{+} Treg expression (Figure 4.8) suggesting specific interactions between these two cell populations. Consequently, the elevated expression of ICAM-1 on liver pDC compared to spleen pDC may be contributing to these phenomena.

More marked than differences in ICAM-1 expression, ICAM-2 is expressed at significantly greater levels on liver pDC compared to spleen pDC. Like ICAM-1, ICAM-2 is also a ligand for LFA-1, however, it binds at higher affinity to DC-SIGN [338], an Ag uptake molecule which is expressed by cDC (see section 1.2.1.1). DC-SIGN downstream signaling events can promote DC maturation [339], thus, it remains to be seen if ligation of DC-SIGN on cDC by ICAM-2 can inhibit cDC maturation. It was recently shown that pDC are able to inhibit cDC maturation via B7-H1 [160], and the ability of pDC to bind to cDC with high affinity via ICAM-2/DC-SIGN may facilitate regulation by B7-H1, suggesting an additional mechanism through which that pDC might be able to regulate cDC function.

Their immature and regulatory phenotype, weak ability to prime immune responses to alloAgs *in vitro* and *in vivo*, and migratory ability of donor DC following liver transplantation suggests that liver pDC may be a critical cell population contributing to the spontaneous acceptance of fully MHC-mismatched murine liver allografts. In the strain combination of B6 to C3H liver transplantation in mice, liver allografts are 'spontaneously' accepted with long-term graft survival (MST = 95 days) and minimal histological evidence of rejection (at 105 days post-transplant). Strikingly, when B6 donor mice were depleted of pDC prior to surgery, liver allografts were rejected with an MST of 25 days. Based on our *in vitro* data, this could be caused by failure to induce IL-10-producing Tr1 Treg that may be necessary to prevent rejection.

In summary, our data represents the first report to directly link donor liver pDC as being a critical cell population that long-term allograft survival and tolerance in a mouse model of liver allotransplantation. Coupled with their weak ability to prime immune responses to alloAgs, these data suggest that as liver pDC migrate from the donor liver into recipient lymphoid tissues, they may be interacting with recipient cells to promote tolerance to the transplanted organ. Investigation into the mechanisms that are required for pDC to promote tolerance in this model is warranted and will be the focus of future studies. We have previously shown that IL-27 and B7-H1 are two important molecules that regulate the tolerogenic capacity of liver pDC, and B7-H1 expression has already been defined as an important molecule that regulates cold I/R injury [340] following liver transplantation. Thus, these represent two key molecules that may be essential for the regulatory role of donor liver pDC in liver transplantation.

6.0 SUMMARY

The concept of cell-based therapies for clinical application to treat disease pathologies has been around for a long time. They have been investigated as a means to bolster immune responses such as in the case of cancer or chronic infection, as well as to regulate unwanted immune reactivity in the case of autoimmunity and transplantation. A leading area of research is focused on DC for their ability to promote or modulate these responses in an Ag-specific fashion, leaving the remaining immune system intact to function normally. Moreover, pDC have emerged as an important cell population with the ability to modulate immune responses in favor of regulation and tolerance [240], especially in experimental transplantation models [128, 188, 229]. Recent data from the group led by Dr. Suzanne Ildstad [219] have brought tolerogenic pDC to the forefront with exciting results utilizing a population of facilitating cells containing pDC precursors [218, 341, 342] that promote chimerism and donor-specific tolerance with successful withdrawal from immunosuppression in combined kidney and hematopoietic stem cell transplant patients. These are important findings that highlight the regulatory capacity of pDC and strongly support investigation of pDC as a means for promoting tolerance induction in the clinical setting.

The data presented herein clearly indicate that pDC exhibit significant capacity to modulate immune responses *in vitro* and *in vivo*. Moreover, pDC residing in the liver microenvironment possess potent immune regulatory function, even more than their counterparts in the spleen and compared to cDC. Based on these data and results from clinical studies

mentioned above, we conclude that pDC may be a powerful, and more suitable alternative to cDC, and should be considered as a potential cell-based therapeutic in disease settings where regulation of immune responses is merited.

6.1 CAVEATS AND FUTURE DIRECTIONS

One of the major caveats of utilizing pDC as a cell-based therapy to promote tolerogenic immune responses is their ability to secrete large amounts of type I IFN (up to 1,000 times more than any other cell type). Type I IFN can activate just about every other cell type, which is critical for mounting potent anti-viral immune responses and limiting viral replication and the spread of infection. This may have detrimental effects for use in transplantation and autoimmunity, since TLR activation may prevent induction of transplant tolerance [343], and type I IFN can promote cDC and T cell function, exacerbate disease pathologies in many autoimmune disorders and may contribute to solid organ allograft rejection. It has been shown, however, that even CpG-activated human pDC induce functionally suppressive $CD4^+CD25^+Foxp3^+$ Treg [344], suggesting they are still capable of promoting tolerance following activation. Further study into the tolerogenic properties of TLR-activated pDC are warranted in this case.

Our novel data on the functional immunobiology of liver pDC highlight their significant capacity for modulating immune responses. Unfortunately, the feasibility of obtaining pDC from the liver for use in the clinical setting seems improbable in most scenarios. Therefore, DC derived from human PBMC may be conditioned using certain tolerogenic factors found within thin the liver microenvironment (e.g., IL-27, TGF-β, PGEs, or IL-10) to enhance their

tolerogenicity. TGF-β has been shown to upregulate IL-27 in pDC [245], and IL-27 upregulates B7-H1 expression on DC [70, 106], thus providing support for such studies. Fortunately, in the case of human pDC, IL-3 can be used as a survival factor and makes their study *in vitro* easier than that of mouse pDC, which do not express the IL-3R and fare poorly in culture for extended periods of time. Alternatively, pDC from other tissues that are more readily obtainable (e.g., pre-pDC present in the BM facilitating population [219]) may present an alternative to the need for conditioning *ex vivo*.

We have identified pDC as having significant tolerogenic potential compared to cDC, and that pDC in the liver are even more tolerogenic than those in the spleen. Our data using the DTH model to OVA and our data demonstrating that livers from pDC-depleted donors fail to induce tolerance and are rejected in a model where liver allografts are accepted spontaneously in the absence of IS intervention support these findings. These studies will serve as the basis for future investigation into the mechanisms utilized by donor liver pDC that contribute to spontaneous allograft acceptance using our mouse liver transplant model. The observation that liver pDC induce very little allogeneic T cell proliferation, IFN-γ and IL-2 production, yet still induce relevant amounts of IL-10 production provides strong evidence for mechanistic involvement of liver pDC in promoting tolerance in liver allotransplantation. The trafficking and migration patterns of donor liver pDC into host lymphoid tissues, interaction with host lymphocytes, and characterization of important molecules and signaling pathways, in addition to the ones identified here, are also important questions that remain unanswered and warrant further investigation.

APPENDIX A

SUPPLEMENTARY DATA

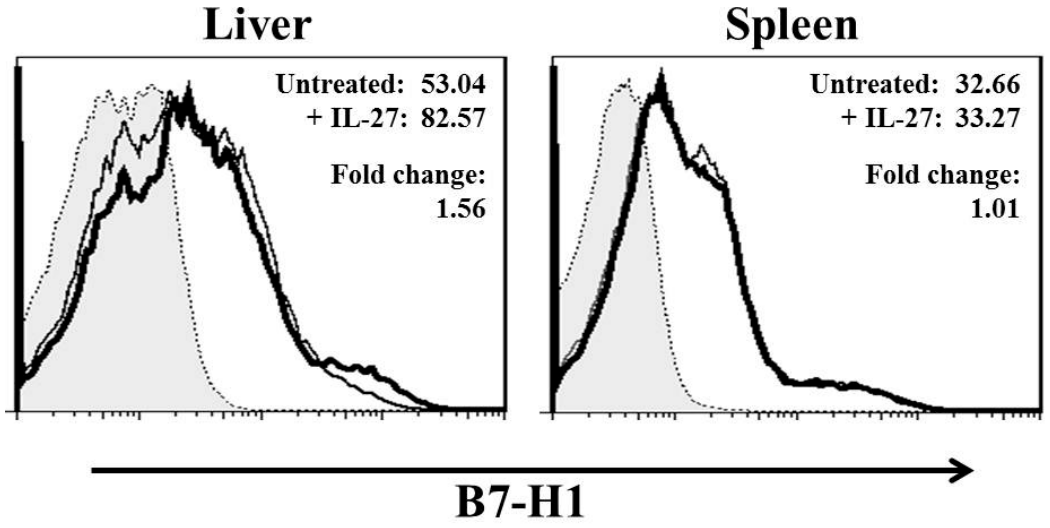


Figure A.0.1 Histograms depicting B7-H1 expression on liver pDC in response to IL-27

Freshly-isolated liver and spleen PDCA-1^+ pDC from Flt3L-mobilized, C57BL/6J WT mice were cultured in the presence of 25 μg/ml CpG B ODN for 18 h, collected and analyzed by 5-color flow cytometry. Surface expression of B7-H1 was analyzed on B220+CD11clow gated cells as previously described in Materials in Methods. Relative MFI of untreated (thin line) and IL-27-conditioned (bold line) liver and spleen pDC is shown, along with the fold change in B7-H1 MFI following exposure to IL-27.

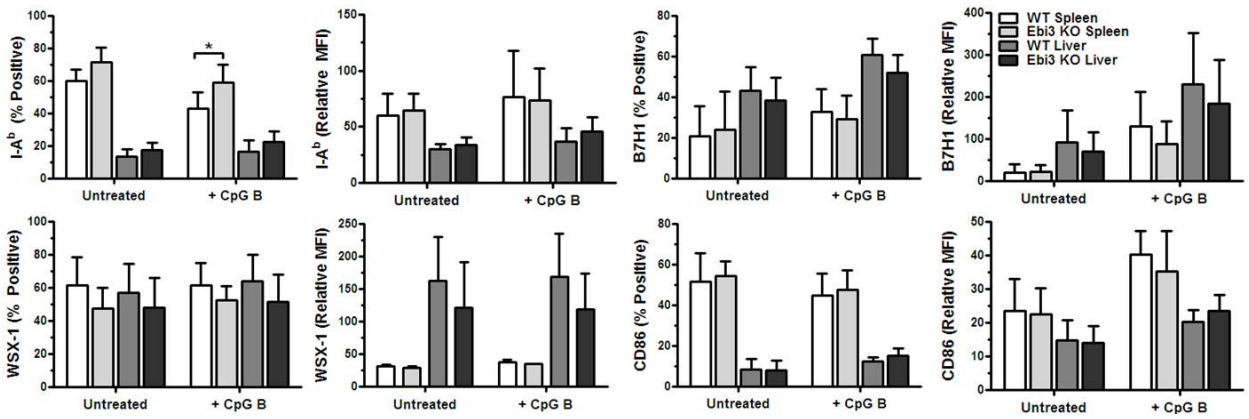


Figure A.0.2 Ebi3$^{-/-}$ pDC are phenotypically similar to WT pDC

Freshly-isolated liver and spleen PDCA-1^{+} pDC from Flt3L-mobilized, WT or Ebi3$^{-/-}$ mice were cultured in the presence of 1 μg/ml CpG B ODN for 18 h, collected and analyzed by 5-color flow cytometry. Surface protein expression was analyzed on B220^{+}CD11c^{low} gated cells (Figure A1) as previously described in section 2.4 of the Materials in Methods. Relative MFI and percent positive cells were average from 3 independent experiments, * $p < 0.05$.

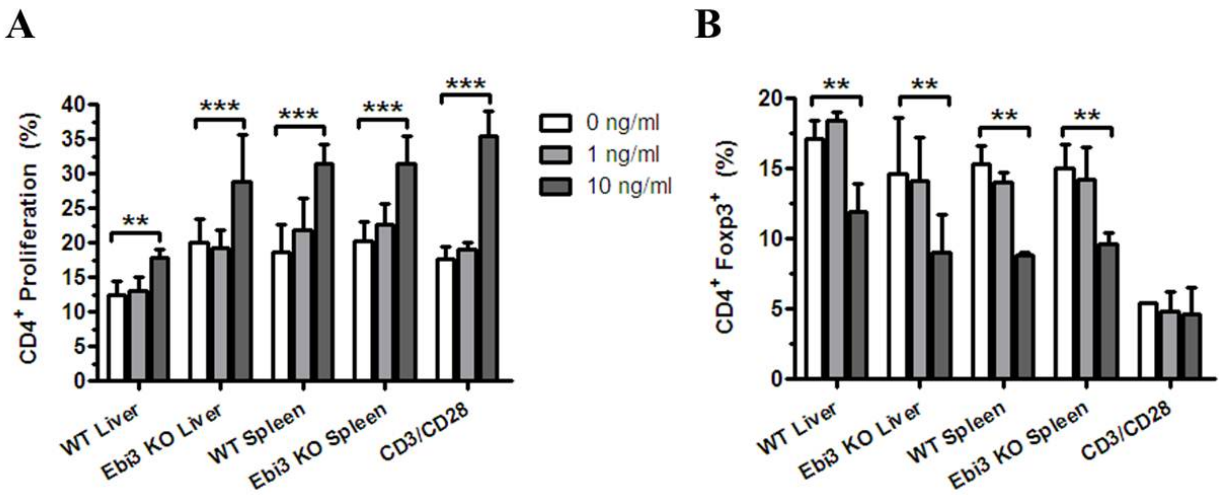


Figure A.0.3 Exogenous IL-27 enhances T cell proliferation and reduces Foxp3 expression

Freshly-isolated liver and spleen PDCA-1^{+} pDC from Flt3L-mobilized, C57BL/6J WT or Ebi3$^{-/-}$ mice were cultured with allogeneic BALB/c splenic T cells in the absence (white bars) or presence of 1 (light gray bars) or 10 (dark gray bars) ng/ml IL-27 for 3 d. Cultures were harvested and analyzed by flow cytometry for proliferation by CFSE dilution (A) and Foxp3 expression (B). T cells were also stimulated with plate-bound anti-CD3 and soluble CD28 (A-B). Results are an average of n =2 independent experiments, ** $p < 0.01$, *** $p < 0.001$.

APPENDIX B

PUBLICATIONS

Colvin BL, Matta BM, Thomson AW. Dendritic cells and Chemokine-Directed Migration in Transplantation: Where are we headed? *Clin Lab Med*. 2008 Sep;28(3):375-84, v.

Sumpter TL, Lunz III, JG, Castellaneta A, Matta B, Tokita D, Turnquist HR, Mazariegos GV, Demetris AJ, Thomson AW. Dendritic cell immunobiology in relation to liver transplant outcome. *Front Biosci*. (Elite Ed). 2009. Jun 1;1:99-114.

Raimondi G, Sumpter TL, Matta BM, Pillai M, Corbitt N, Vodovotz Y, Wang Z, Thomson AW. Mammalian target of rapamycin inhibition and alloantigen-specific regulatory T cells synergize to promote long-term graft survival in immunocompetent recipients. *J Immunol*. 2010 Jan 15;184(2):624-636.

Matta BM and AW Thomson. (2010) Natural killer cells and transplantation. In: Lotze MT and AW Thomson, (eds.) Natural Killer Cells. Elsevier. United Kingdom, London. pp 445-459.

Matta BM, Castellaneta A, Thomson AW. Tolerogenic plasmacytoid DC. *Eur J Immunol*. 2010 Oct; 40(10):2667-2676.

Matta BM, Raimondi G, Rosborough BR, Sumpter TL, Thomson AW. IL-27 production and STAT3-dependent upregulation of B7-H1 mediate immune regulatory functions of liver plasmacytoid DC. *J Immunol*. 2012. Jun 1; 188(11):5227-5237.

Sumpter TL, Dangi A, Matta BM, Huang C, Stolz DB, Vodovotz Y, Thomson AW, Gandhi C. Hepatic stellate cells reduce the allostimulatory capacity of hepatic myeloid dendritic cells through STAT3-driven induction of indoleamine 2.3-dioxygenase. *J Immunol*.

Rosborough BR, Matta BM, Lee K, Gan B, DePinho RA, Boothby M, Turnquist HR, Thomson AW. Dendritic cell rapamycin-resistant and rictor-independent mTOR controls IL-10, B7-H1, and regulatory T cell induction. *Immunity*. Under review.

Matta BM, Kimura S, Yoshida O, Demetris AJ, Murase N, Thomson AW. Donor liver plasmacytoid DC migrate to recipient lymphoid tissue following transplantation and promote allograft tolerance through induction of regulatory T cells. In preparation.

PUBLISHED ABSTRACTS

Raimondi G, Matta B, Wang Z, Thomson AW. Regulatory T cell Activity and Anergy Induction Lead to Allograft Tolerance after Transfer of AlloAg-Specific Treg in Unmanipulated Hosts. (2008). American Transplant Congress. *Am J Transplant* 8 (Suppl 2):481.

Raimondi G, Matta B, Wang Z, Thomson AW. Pro-Tolerogenic Properties of Rapamycin That Favor Indefinite Heart Allograft Survival Mediated by Regulatory T Cells. (2008). American Transplant Congress. *Am J Transplant* 8 (Suppl 2):623.

Matta BM, Colvin BL, Sumpter TL, Thomson AW. Host-derived plasmacytoid dendritic cells prolong cardiac allograft survival and regulate alloreactive T cell responses via B7 homolog-1. (2009) American Transplant Congress. *Am J Transplant*. 9 (Suppl 2):593. Oral presentation.

Matta BM, Colvin BL, Sumpter TL, Thomson AW. Host-derived plasmacytoid dendritic cells prolong cardiac allograft survival and regulate alloreactive T cell responses via B7 homolog-1. (2009) American Association of Immunologists. *J Immunol*. 182 (Meeting Abstracts 1): 141.28.

Matta BM, Sumpter TL, Raimondi G, Thomson AW. Liver DC modulate Foxp3 expression in allogeneic $CD4^+$ T cells via IL-27. (2010). American Association of Immunologists. *J Immunol*. 184 (Meeting Abstracts 1):145.2.

Matta BM, Sumpter TL, Raimondi G, Thomson AW. Liver DC modulate Foxp3 expression in allogeneic $CD4^+$ T cells via IL-27. (2010). American Transplant Congress. *Am J Transplant*. 10 (Suppl 4):178. Oral presentation.

Matta BM, Raimondi G, Thomson AW. IL-27 Promotes IL-10 Production and B7-H1 Expression By Plasmacytoid DC and Augments Their Expansion of $Foxp3^+$ Allogeneic $CD4^+$ T Cells. (2011). American Association of Immunologists. *J Immunol*. 186 (Meeting Abstracts 1): 147.2. Oral presentation.

Matta BM, Raimondi G, Thomson AW. IL-27 Promotes IL-10 Production and B7-H1 Expression By Plasmacytoid DC and Augments Their Expansion of $Foxp3^+$ Allogeneic $CD4^+$ T Cells. (2011) American Transplant Congress. *Am J Transplant*. 11 (Suppl s2):620.

Matta BM, Raimondi G, Rosborough BR, and AW Thomson. IL-27 and B7-H1 Mediate Liver Plasmacytoid DC Ability to Suppress Delayed-Type Hypersensitivity Responses. (2012). American Association of Immunologists. *J Immunol*. 188 (Meeting Abstracts): 48.9.

Yoshida O, Matta BM, Sumpter TL, and AW Thomson. The Transmembrane Adaptor Protein DAP12 Regulates T Cell Allostimulatory Function of Liver Dendritic Cells. (2012). American Transplant Congress. *Am J Transplant*. 12 (Suppl s3):630.

Matta BM, Raimondi G, Rosborough BR, and AW Thomson. IL-27 and B7-H1 Mediate Liver Plasmacytoid DC Ability to Suppress Delayed-Type Hypersensitivity Responses. (2012). American Transplant Congress. *Am J Transplant*. 12 (Suppl s3):236. Oral presentation.

Rosborough BR, Matta B, Turnquist H, and A Thomson. mTORC2 Negatively Regulates DC PD-L1 and IL-10 Through SOCS3 and STAT3. (2012). American Association of Immunologists. *J Immunol*. 188 (Meeting Abstracts): 172.34.

BIBLIOGRAPHY

1. Belz, G.T. and S.L. Nutt. 2012. Transcriptional programming of the dendritic cell network. *Nat Rev Immunol*. 12:101-113.

2. Stenger, E.O., H.R. Turnquist, M.Y. Mapara, and A.W. Thomson. 2012. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. *Blood*. 119:5088-5103.

3. Carotta, S., A. Dakic, A. D'Amico, S.H. Pang, K.T. Greig, S.L. Nutt, and L. Wu. 2010. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity*. 32:628-641.

4. Lyman, S.D., L. James, T. Vanden Bos, P. de Vries, K. Brasel, B. Gliniak, L.T. Hollingsworth, K.S. Picha, H.J. McKenna, R.R. Splett, and et al. 1993. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell*. 75:1157-1167.

5. McKenna, H.J., K.L. Stocking, R.E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C.R. Maliszewski, D.H. Lynch, J. Smith, B. Pulendran, E.R. Roux, M. Teepe, S.D. Lyman, and J.J. Peschon. 2000. Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood*. 95:3489-3497.

6. Brawand, P., D.R. Fitzpatrick, B.W. Greenfield, K. Brasel, C.R. Maliszewski, and T. De Smedt. 2002. Murine plasmacytoid pre-dendritic cells generated from Flt3 ligand-supplemented bone marrow cultures are immature APCs. *J Immunol*. 169:6711-6719.

7. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X.L. Xu, G. Trinchieri, A. O'Garra, and Y.J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 195:953-958.

8. Brasel, K., T. De Smedt, J.L. Smith, and C.R. Maliszewski. 2000. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood*. 96:3029-3039.

9. Maraskovsky, E., K. Brasel, M. Teepe, E.R. Roux, S.D. Lyman, K. Shortman, and H.J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J Exp Med*. 184:1953-1962.

10. Hackstein, H., T. Taner, A.F. Zahorchak, A.E. Morelli, A.J. Logar, A. Gessner, and A.W. Thomson. 2003. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood*. 101:4457-4463.

11. Liu, K. and M.C. Nussenzweig. 2010. Origin and development of dendritic cells. *Immunol Rev*. 234:45-54.

12. Colonna, M., G. Trinchieri, and Y.J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol*. 5:1219-1226.

13. Liu, Y.J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol*. 23:275-306.

14. Reizis, B., A. Bunin, H.S. Ghosh, K.L. Lewis, and V. Sisirak. 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol*. 29:163-183.

15. Kuipers, H., F.M. Schnorfeil, and T. Brocker. 2010. Differentially expressed microRNAs regulate plasmacytoid vs. conventional dendritic cell development. *Mol Immunol*. 48:333-340.

16. Shimizu, K. and S. Fujii. 2008. An adjuvant role of in situ dendritic cells (DCs) in linking innate and adaptive immunity. *Front Biosci*. 13:6193-6201.

17. Fooksman, D.R., S. Vardhana, G. Vasiliver-Shamis, J. Liese, D.A. Blair, J. Waite, C. Sacristan, G.D. Victora, A. Zanin-Zhorov, and M.L. Dustin. 2010. Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol*. 28:79-105.

18. Nimmerjahn, F. and J.V. Ravetch. 2007. Fc-receptors as regulators of immunity. *Adv Immunol*. 96:179-204.

19. Kawai, T. and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 11:373-384.

20. Kawai, T. and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 34:637-650.

21. Birkholz, K., M. Schwenkert, C. Kellner, S. Gross, G. Fey, B. Schuler-Thurner, G. Schuler, N. Schaft, and J. Dorrie. 2010. Targeting of DEC-205 on human dendritic cells results in efficient MHC class II-restricted antigen presentation. *Blood*. 116:2277-2285.

22. Cambi, A., I. Beeren, B. Joosten, J.A. Fransen, and C.G. Figdor. 2009. The C-type lectin DC-SIGN internalizes soluble antigens and HIV-1 virions via a clathrin-dependent mechanism. *Eur J Immunol*. 39:1923-1928.

23. Mizumoto, N. and A. Takashima. 2004. CD1a and langerin: acting as more than Langerhans cell markers. *J Clin Invest*. 113:658-660.

24. Berg, L.M., S.I. Gringhuis, and T.B. Geijtenbeek. 2012. An evolutionary perspective on C-type lectins in infection and immunity. *Ann N Y Acad Sci*.

25. Ackerman, A.L. and P. Cresswell. 2004. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat Immunol*. 5:678-684.

26. Ackerman, A.L., C. Kyritsis, R. Tampe, and P. Cresswell. 2005. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat Immunol*. 6:107-113.

27. Saveanu, L., O. Carroll, M. Weimershaus, P. Guermonprez, E. Firat, V. Lindo, F. Greer, J. Davoust, R. Kratzer, S.R. Keller, G. Niedermann, and P. van Endert. 2009. IRAP identifies an endosomal compartment required for MHC class I cross-presentation. *Science*. 325:213-217.

28. Landmann, S., A. Muhlethaler-Mottet, L. Bernasconi, T. Suter, J.M. Waldburger, K. Masternak, J.F. Arrighi, C. Hauser, A. Fontana, and W. Reith. 2001. Maturation of dendritic cells is accompanied by rapid transcriptional silencing of class II transactivator (CIITA) expression. *J Exp Med*. 194:379-391.

29. Otten, L.A., V. Steimle, S. Bontron, and B. Mach. 1998. Quantitative control of MHC class II expression by the transactivator CIITA. *Eur J Immunol*. 28:473-478.

30. De Gassart, A., V. Camosseto, J. Thibodeau, M. Ceppi, N. Catalan, P. Pierre, and E. Gatti. 2008. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. *Proc Natl Acad Sci U S A*. 105:3491-3496.

31. Young, L.J., N.S. Wilson, P. Schnorrer, A. Proietto, T. ten Broeke, Y. Matsuki, A.M. Mount, G.T. Belz, M. O'Keeffe, M. Ohmura-Hoshino, S. Ishido, W. Stoorvogel, W.R. Heath, K. Shortman, and J.A. Villadangos. 2008. Differential MHC class II synthesis and

ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat Immunol*. 9:1244-1252.

32. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe. 2005. The B7 family revisited. *Annu Rev Immunol*. 23:515-548.

33. Sharpe, A.H. and G.J. Freeman. 2002. The B7-CD28 superfamily. *Nat Rev Immunol*. 2:116-126.

34. McAdam, A.J., A.N. Schweitzer, and A.H. Sharpe. 1998. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunol Rev*. 165:231-247.

35. Van Gool, S.W., P. Vandenberghe, M. de Boer, and J.L. Ceuppens. 1996. CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model. *Immunol Rev*. 153:47-83.

36. Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol*. 27:485-517.

37. Liang, S.C., A.J. Long, F. Bennett, M.J. Whitters, R. Karim, M. Collins, S.J. Goldman, K. Dunussi-Joannopoulos, C.M. Williams, J.F. Wright, and L.A. Fouser. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J Immunol*. 179:7791-7799.

38. Laan, M., Z.H. Cui, H. Hoshino, J. Lotvall, M. Sjostrand, D.C. Gruenert, B.E. Skoogh, and A. Linden. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol*. 162:2347-2352.

39. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 441:235-238.

40. Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol*. 9:833-844.

41. Humblet, C., A. Rudensky, and B. Kyewski. 1994. Presentation and intercellular transfer of self antigen within the thymic microenvironment: expression of the E alpha peptide-I-Ab complex by isolated thymic stromal cells. *Int Immunol*. 6:1949-1958.

42. Bonasio, R., M.L. Scimone, P. Schaerli, N. Grabie, A.H. Lichtman, and U.H. von Andrian. 2006. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat Immunol*. 7:1092-1100.

43. Hadeiba, H., K. Lahl, A. Edalati, C. Oderup, A. Habtezion, R. Pachynski, L. Nguyen, A. Ghodsi, S. Adler, and E.C. Butcher. 2012. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity*. 36:438-450.

44. Doan, T., A. McNally, R. Thomas, and R.J. Steptoe. 2009. Steady-state dendritic cells continuously inactivate T cells that escape thymic negative selection. *Immunol Cell Biol*. 87:615-622.

45. Desch, A.N., G.J. Randolph, K. Murphy, E.L. Gautier, R.M. Kedl, M.H. Lahoud, I. Caminschi, K. Shortman, P.M. Henson, and C.V. Jakubzick. 2011. CD103+ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *J Exp Med*. 208:1789-1797.

46. del Rio, M.L., J.I. Rodriguez-Barbosa, E. Kremmer, and R. Forster. 2007. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol*. 178:6861-6866.

47. Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 204:1757-1764.

48. Matteoli, G., E. Mazzini, I.D. Iliev, E. Mileti, F. Fallarino, P. Puccetti, M. Chieppa, and M. Rescigno. 2010. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. *Gut*. 59:595-604.

49. Kohl, J. and M. Wills-Karp. 2007. Complement regulates inhalation tolerance at the dendritic cell/T cell interface. *Mol Immunol*. 44:44-56.

50. Mowat, A.M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol*. 3:331-341.

51. Viney, J.L., A.M. Mowat, J.M. O'Malley, E. Williamson, and N.A. Fanger. 1998. Expanding dendritic cells in vivo enhances the induction of oral tolerance. *J Immunol*. 160:5815-5825.

52. Iwasaki, A. and B.L. Kelsall. 1999. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. *J Exp Med*. 190:229-239.

53. Guilliams, M., K. Crozat, S. Henri, S. Tamoutounour, P. Grenot, E. Devilard, B. de Bovis, L. Alexopoulou, M. Dalod, and B. Malissen. 2010. Skin-draining lymph nodes

contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood*. 115:1958-1968.

54. Schulz, O., E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med*. 206:3101-3114.

55. Hill, J.A., J.A. Hall, C.M. Sun, Q. Cai, N. Ghyselinck, P. Chambon, Y. Belkaid, D. Mathis, and C. Benoist. 2008. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. *Immunity*. 29:758-770.

56. Mucida, D., K. Pino-Lagos, G. Kim, E. Nowak, M.J. Benson, M. Kronenberg, R.J. Noelle, and H. Cheroutre. 2009. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity*. 30:471-472; author reply 472-473.

57. Chen, W., W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, and S.M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 198:1875-1886.

58. Williamson, E., J.M. O'Malley, and J.L. Viney. 1999. Visualizing the T-cell response elicited by oral administration of soluble protein antigen. *Immunology*. 97:565-572.

59. Manicassamy, S. and B. Pulendran. 2011. Dendritic cell control of tolerogenic responses. *Immunol Rev*. 241:206-227.

60. Thomson, A.W. and P.A. Knolle. 2010. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol*. 10:753-766.

61. Ichikawa, S., D. Mucida, A.J. Tyznik, M. Kronenberg, and H. Cheroutre. 2011. Hepatic stellate cells function as regulatory bystanders. *J Immunol*. 186:5549-5555.

62. Chou, H.S., C.C. Hsieh, R. Charles, L. Wang, T. Wagner, J.J. Fung, S. Qian, and L.L. Lu. 2012. Myeloid-derived suppressor cells protect islet transplants by B7-H1 mediated enhancement of T regulatory cells. *Transplantation*. 93:272-282.

63. Xia, S., Z. Guo, X. Xu, H. Yi, Q. Wang, and X. Cao. 2008. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance. *Blood*. 112:3175-3185.

64. Sumpter, T.L., J.G. Lunz, 3rd, A.J. Demetris, and A.W. Thomson. 2009. Molecular regulation of hepatic dendritic cell function and its relation to liver transplant outcome. *Transplantation*. 88:S40-44.

65. Abe, M., D. Tokita, G. Raimondi, and A.W. Thomson. 2006. Endotoxin modulates the capacity of CpG-activated liver myeloid DC to direct Th1-type responses. *Eur J Immunol*. 36:2483-2493.

66. De Creus, A., M. Abe, A.H. Lau, H. Hackstein, G. Raimondi, and A.W. Thomson. 2005. Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. *J Immunol*. 174:2037-2045.

67. Drakes, M.L., L. Lu, H.J. McKenna, and A.W. Thomson. 1997. The influence of collagen, fibronectin, and laminin on the maturation of dendritic cell progenitors propagated from normal or Flt3-ligand-treated mouse liver. *Adv Exp Med Biol*. 417:115-120.

68. Thomson, A.W., P.J. O'Connell, R.J. Steptoe, and L. Lu. 2002. Immunobiology of liver dendritic cells. *Immunol Cell Biol*. 80:65-73.

69. Castellaneta, A., T.L. Sumpter, L. Chen, D. Tokita, and A.W. Thomson. 2009. NOD2 ligation subverts IFN-alpha production by liver plasmacytoid dendritic cells and inhibits their T cell allostimulatory activity via B7-H1 up-regulation. *J Immunol*. 183:6922-6932.

70. Matta, B.M., G. Raimondi, B.R. Rosborough, T.L. Sumpter, and A.W. Thomson. 2012. IL-27 Production and STAT3-Dependent Upregulation of B7-H1 Mediate Immune Regulatory Functions of Liver Plasmacytoid Dendritic Cells. *J Immunol*.

71. Sumpter, T.L., V. Packiam, H.R. Turnquist, A. Castellaneta, O. Yoshida, and A.W. Thomson. 2011. DAP12 promotes IRAK-M expression and IL-10 production by liver myeloid dendritic cells and restrains their T cell allostimulatory ability. *J Immunol*. 186:1970-1980.

72. Tokita, D., T.L. Sumpter, G. Raimondi, A.F. Zahorchak, Z. Wang, A. Nakao, G.V. Mazariegos, M. Abe, and A.W. Thomson. 2008. Poor allostimulatory function of liver plasmacytoid DC is associated with pro-apoptotic activity, dependent on regulatory T cells. *J Hepatol*. 49:1008-1018.

73. Pillarisetty, V.G., A.B. Shah, G. Miller, J.I. Bleier, and R.P. DeMatteo. 2004. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J Immunol*. 172:1009-1017.

74. Li, Q., Z. Guo, X. Xu, S. Xia, and X. Cao. 2008. Pulmonary stromal cells induce the generation of regulatory DC attenuating T-cell-mediated lung inflammation. *Eur J Immunol*. 38:2751-2761.

75. Kel, J.M., M.J. Girard-Madoux, B. Reizis, and B.E. Clausen. 2010. TGF-beta is required to maintain the pool of immature Langerhans cells in the epidermis. *J Immunol*. 185:3248-3255.

76. Contractor, N., J. Louten, L. Kim, C.A. Biron, and B.L. Kelsall. 2007. Cutting edge: Peyer's patch plasmacytoid dendritic cells (pDCs) produce low levels of type I interferons: possible role for IL-10, TGFbeta, and prostaglandin E2 in conditioning a unique mucosal pDC phenotype. *J Immunol*. 179:2690-2694.

77. Semmrich, M., M. Plantinga, M. Svensson-Frej, H. Uronen-Hansson, T. Gustafsson, A.M. Mowat, U. Yrlid, B.N. Lambrecht, and W.W. Agace. 2012. Directed antigen targeting in vivo identifies a role for CD103(+) dendritic cells in both tolerogenic and immunogenic T-cell responses. *Mucosal Immunol*. 5:150-160.

78. del Rio, M.L., G. Bernhardt, J.I. Rodriguez-Barbosa, and R. Forster. 2010. Development and functional specialization of CD103+ dendritic cells. *Immunol Rev*. 234:268-281.

79. Peron, J.P., A.P. de Oliveira, and L.V. Rizzo. 2009. It takes guts for tolerance: the phenomenon of oral tolerance and the regulation of autoimmune response. *Autoimmun Rev*. 9:1-4.

80. McDole, J.R., L.W. Wheeler, K.G. McDonald, B. Wang, V. Konjufca, K.A. Knoop, R.D. Newberry, and M.J. Miller. 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*. 483:345-349.

81. Cattaneo, D., N. Perico, F. Gaspari, and G. Remuzzi. 2004. Nephrotoxic aspects of cyclosporine. *Transplant Proc*. 36:234S-239S.

82. Ross, K. 2007. For organ transplant recipients, cancer threatens long-term survival. *J Natl Cancer Inst*. 99:421-422.

83. Buell, J.F., T.G. Gross, and E.S. Woodle. 2005. Malignancy after transplantation. *Transplantation*. 80:S254-264.

84. Fishman, J.A. and R.H. Rubin. 1998. Infection in organ-transplant recipients. *N Engl J Med*. 338:1741-1751.

85. Libby, P. and J.S. Pober. 2001. Chronic rejection. *Immunity*. 14:387-397.

86. Wang, Z., W.J. Shufesky, A. Montecalvo, S.J. Divito, A.T. Larregina, and A.E. Morelli. 2009. In situ-targeting of dendritic cells with donor-derived apoptotic cells restrains indirect allorecognition and ameliorates allograft vasculopathy. *PLoS One*. 4:e4940.

87. Morelli, A.E. and A.W. Thomson. 2007. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol*. 7:610-621.

88. Turnquist, H.R., J. Cardinal, C. Macedo, B.R. Rosborough, T.L. Sumpter, D.A. Geller, D. Metes, and A.W. Thomson. 2010. mTOR and GSK-3 shape the CD4+ T-cell stimulatory and differentiation capacity of myeloid DCs after exposure to LPS. *Blood*. 115:4758-4769.

89. Hackstein, H. and A.W. Thomson. 2004. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol*. 4:24-34.

90. Chung, D.J., M. Rossi, E. Romano, J. Ghith, J. Yuan, D.H. Munn, and J.W. Young. 2009. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. *Blood*. 114:555-563.

91. Sordi, V., G. Bianchi, C. Buracchi, A. Mercalli, F. Marchesi, G. D'Amico, C.H. Yang, W. Luini, A. Vecchi, A. Mantovani, P. Allavena, and L. Piemonti. 2006. Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. *Transplantation*. 82:826-834.

92. de Waal Malefyt, R., J. Haanen, H. Spits, M.G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J.E. de Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med*. 174:915-924.

93. Ding, L., P.S. Linsley, L.Y. Huang, R.N. Germain, and E.M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol*. 151:1224-1234.

94. de Waal Malefyt, R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. de Vries. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med*. 174:1209-1220.

95. Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J Immunol*. 147:3815-3822.

96. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol*. 146:3444-3451.

97. Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and A.H. Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol*. 159:4772-4780.

98. Steinbrink, K., E. Graulich, S. Kubsch, J. Knop, and A.H. Enk. 2002. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood*. 99:2468-2476.

99. Tai, N., H. Yasuda, Y. Xiang, L. Zhang, D. Rodriguez-Pinto, K. Yokono, R. Sherwin, F.S. Wong, M. Nagata, and L. Wen. 2011. IL-10-conditioned dendritic cells prevent autoimmune diabetes in NOD and humanized HLA-DQ8/RIP-B7.1 mice. *Clin Immunol*. 139:336-349.

100. Huang, H., W. Dawicki, X. Zhang, J. Town, and J.R. Gordon. 2010. Tolerogenic dendritic cells induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells. *J Immunol*. 185:5003-5010.

101. Sun, W., Q. Wang, L. Zhang, J. Pan, M. Zhang, G. Lu, H. Yao, J. Wang, and X. Cao. 2002. TGF-beta(1) gene modified immature dendritic cells exhibit enhanced tolerogenicity but induce allograft fibrosis in vivo. *J Mol Med (Berl)*. 80:514-523.

102. Bonham, C.A., L. Lu, R.A. Banas, P. Fontes, A.S. Rao, T.E. Starzl, A. Zeevi, and A.W. Thomson. 1996. TGF-beta 1 pretreatment impairs the allostimulatory function of human bone marrow-derived antigen-presenting cells for both naive and primed T cells. *Transpl Immunol*. 4:186-191.

103. Sato, K., N. Yamashita, M. Baba, and T. Matsuyama. 2003. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity*. 18:367-379.

104. Lan, Y.Y., Z. Wang, G. Raimondi, W. Wu, B.L. Colvin, A. de Creus, and A.W. Thomson. 2006. "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol*. 177:5868-5877.

105. Torres-Aguilar, H., C. Sanchez-Torres, L.J. Jara, M. Blank, and Y. Shoenfeld. 2010. IL-10/TGF-beta-treated dendritic cells, pulsed with insulin, specifically reduce the response to insulin of CD4+ effector/memory T cells from type 1 diabetic individuals. *J Clin Immunol*. 30:659-668.

106. Karakhanova, S., T. Bedke, A.H. Enk, and K. Mahnke. 2011. IL-27 renders DC immunosuppressive by induction of B7-H1. *J Leukoc Biol*. 89:837-845.

107. Shinomiya, M., S.M. Fazle Akbar, H. Shinomiya, and M. Onji. 1999. Transfer of dendritic cells (DC) ex vivo stimulated with interferon-gamma (IFN-gamma) down-modulates autoimmune diabetes in non-obese diabetic (NOD) mice. *Clin Exp Immunol*. 117:38-43.

108. Thomson, A.W., H.R. Turnquist, and G. Raimondi. 2009. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol*. 9:324-337.

109. Turnquist, H.R. and A.W. Thomson. 2008. Taming the lions: manipulating dendritic cells for use as negative cellular vaccines in organ transplantation. *Curr Opin Organ Transplant*. 13:350-357.

110. Turnquist, H.R., G. Raimondi, A.F. Zahorchak, R.T. Fischer, Z. Wang, and A.W. Thomson. 2007. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol*. 178:7018-7031.

111. Taner, T., H. Hackstein, Z. Wang, A.E. Morelli, and A.W. Thomson. 2005. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. *Am J Transplant*. 5:228-236.

112. Horibe, E.K., J. Sacks, J. Unadkat, G. Raimondi, Z. Wang, R. Ikeguchi, D. Marsteller, L.M. Ferreira, A.W. Thomson, W.P. Lee, and M. Feili-Hariri. 2008. Rapamycin-conditioned, alloantigen-pulsed dendritic cells promote indefinite survival of vascularized skin allografts in association with T regulatory cell expansion. *Transpl Immunol*. 18:307-318.

113. Ikeguchi, R., J.M. Sacks, J.V. Unadkat, M. Solari, E.K. Horibe, A.W. Thomson, A.W. Lee, and M. Feili-Hariri. 2008. Long-term survival of limb allografts induced by pharmacologically conditioned, donor alloantigen-pulsed dendritic cells without maintenance immunosuppression. *Transplantation*. 85:237-246.

114. Lee, J.I., R.W. Ganster, D.A. Geller, G.J. Burckart, A.W. Thomson, and L. Lu. 1999. Cyclosporine A inhibits the expression of costimulatory molecules on in vitro-generated dendritic cells: association with reduced nuclear translocation of nuclear factor kappa B. *Transplantation*. 68:1255-1263.

115. Szabo, G., C. Gavala, and P. Mandrekar. 2001. Tacrolimus and cyclosporine A inhibit allostimulatory capacity and cytokine production of human myeloid dendritic cells. *J Investig Med*. 49:442-449.

116. Emmer, P.M., J. van der Vlag, G.J. Adema, and L.B. Hilbrands. 2006. Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hyporesponsiveness. *Transplantation*. 81:1451-1459.

117. Piemonti, L., P. Monti, M. Sironi, P. Fraticelli, B.E. Leone, E. Dal Cin, P. Allavena, and V. Di Carlo. 2000. Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol*. 164:4443-4451.

118. Unger, W.W., S. Laban, F.S. Kleijwegt, A.R. van der Slik, and B.O. Roep. 2009. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol*. 39:3147-3159.

119. Hackstein, H., A.E. Morelli, A.T. Larregina, R.W. Ganster, G.D. Papworth, A.J. Logar, S.C. Watkins, L.D. Falo, and A.W. Thomson. 2001. Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells. *J Immunol*. 166:7053-7062.

120. Morelli, A.E. and A.W. Thomson. 2003. Dendritic cells under the spell of prostaglandins. *Trends Immunol*. 24:108-111.

121. Colic, M., Z. Stojic-Vukanic, B. Pavlovic, D. Jandric, and I. Stefanoska. 2003. Mycophenolate mofetil inhibits differentiation, maturation and allostimulatory function of human monocyte-derived dendritic cells. *Clin Exp Immunol*. 134:63-69.

122. Mehling, A., S. Grabbe, M. Voskort, T. Schwarz, T.A. Luger, and S. Beissert. 2000. Mycophenolate mofetil impairs the maturation and function of murine dendritic cells. *J Immunol*. 165:2374-2381.

123. Steinschulte, C., T. Taner, A.W. Thomson, G. Bein, and H. Hackstein. 2003. Cutting edge: sanglifehrin A, a novel cyclophilin-binding immunosuppressant blocks bioactive IL-12 production by human dendritic cells. *J Immunol*. 171:542-546.

124. Immecke, S.N., N. Baal, J. Wilhelm, J. Bechtel, A. Knoche, G. Bein, and H. Hackstein. 2011. The cyclophilin-binding agent Sanglifehrin A is a dendritic cell chemokine and migration inhibitor. *PLoS One*. 6:e18406.

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

126. Onai, N., A. Obata-Onai, M.A. Schmid, T. Ohteki, D. Jarrossay, and M.G. Manz. 2007. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol*. 8:1207-1216.

127. Reizis, B. 2010. Regulation of plasmacytoid dendritic cell development. *Curr Opin Immunol*. 22:206-211.

128. Abe, M., Z. Wang, A. de Creus, and A.W. Thomson. 2005. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. *Am J Transplant*. 5:1808-1819.

129. Fancke, B., M. Suter, H. Hochrein, and M. O'Keeffe. 2008. M-CSF: a novel plasmacytoid and conventional dendritic cell poietin. *Blood*. 111:150-159.

130. Esashi, E. and Y.J. Liu. 2008. E-box protein E2-2 is a crucial regulator of plasmacytoid DC development. *Eur J Immunol*. 38:2386-2388.

131. Cisse, B., M.L. Caton, M. Lehner, T. Maeda, S. Scheu, R. Locksley, D. Holmberg, C. Zweier, N.S. den Hollander, S.G. Kant, W. Holter, A. Rauch, Y. Zhuang, and B. Reizis. 2008. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell*. 135:37-48.

132. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature*. 434:772-777.

133. Schotte, R., M. Nagasawa, K. Weijer, H. Spits, and B. Blom. 2004. The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J Exp Med*. 200:1503-1509.

134. Coates, P.T., S.M. Barratt-Boyes, L. Zhang, V.S. Donnenberg, P.J. O'Connell, A.J. Logar, F.J. Duncan, M. Murphey-Corb, A.D. Donnenberg, A.E. Morelli, C.R. Maliszewski, and A.W. Thomson. 2003. Dendritic cell subsets in blood and lymphoid tissue of rhesus monkeys and their mobilization with Flt3 ligand. *Blood*. 102:2513-2521.

135. Dzionek, A., Y. Sohma, J. Nagafune, M. Cella, M. Colonna, F. Facchetti, G. Gunther, I. Johnston, A. Lanzavecchia, T. Nagasaka, T. Okada, W. Vermi, G. Winkels, T. Yamamoto, M. Zysk, Y. Yamaguchi, and J. Schmitz. 2001. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J Exp Med*. 194:1823-1834.

136. Cao, W., D.B. Rosen, T. Ito, L. Bover, M. Bao, G. Watanabe, Z. Yao, L. Zhang, L.L. Lanier, and Y.J. Liu. 2006. Plasmacytoid dendritic cell-specific receptor ILT7-Fc

epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *J Exp Med*. 203:1399-1405.

137. Bonaccorsi, I., C. Cantoni, P. Carrega, D. Oliveri, G. Lui, R. Conte, M. Navarra, R. Cavaliere, E. Traggiai, M. Gattorno, A. Martini, M.C. Mingari, A. Moretta, and G. Ferlazzo. 2010. The immune inhibitory receptor LAIR-1 is highly expressed by plasmacytoid dendritic cells and acts complementary with NKp44 to control IFNalpha production. *PLoS One*. 5:e15080.

138. Rissoan, M.C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt, and Y.J. Liu. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science*. 283:1183-1186.

139. Grouard, G., M.C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med*. 185:1101-1111.

140. Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med*. 5:919-923.

141. Perussia, B., V. Fanning, and G. Trinchieri. 1985. A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. *Nat Immun Cell Growth Regul*. 4:120-137.

142. Siegal, F.P., N. Kadowaki, M. Shodell, P.A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y.J. Liu. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science*. 284:1835-1837.

143. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol*. 2:1144-1150.

144. Nakano, H., M. Yanagita, and M.D. Gunn. 2001. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med*. 194:1171-1178.

145. Blasius, A.L., W. Barchet, M. Cella, and M. Colonna. 2007. Development and function of murine B220+CD11c+NK1.1+ cells identify them as a subset of NK cells. *J Exp Med*. 204:2561-2568.

146. Asselin-Paturel, C., G. Brizard, J.J. Pin, F. Briere, and G. Trinchieri. 2003. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol*. 171:6466-6477.

147. Blasius, A.L., E. Giurisato, M. Cella, R.D. Schreiber, A.S. Shaw, and M. Colonna. 2006. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J Immunol*. 177:3260-3265.

148. Evans, D.T., R. Serra-Moreno, R.K. Singh, and J.C. Guatelli. 2010. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends Microbiol*. 18:388-396.

149. Loschko, J., A. Schlitzer, D. Dudziak, I. Drexler, N. Sandholzer, C. Bourquin, W. Reindl, and A.B. Krug. 2011. Antigen delivery to plasmacytoid dendritic cells via BST2 induces protective T cell-mediated immunity. *J Immunol*. 186:6718-6725.

150. Blasius, A.L., M. Cella, J. Maldonado, T. Takai, and M. Colonna. 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood*. 107:2474-2476.

151. Zhang, J., A. Raper, N. Sugita, R. Hingorani, M. Salio, M.J. Palmowski, V. Cerundolo, and P.R. Crocker. 2006. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood*. 107:3600-3608.

152. Loschko, J., S. Heink, D. Hackl, D. Dudziak, W. Reindl, T. Korn, and A.B. Krug. 2011. Antigen targeting to plasmacytoid dendritic cells via Siglec-H inhibits Th cell-dependent autoimmunity. *J Immunol*. 187:6346-6356.

153. Triebel, F., S. Jitsukawa, E. Baixeras, S. Roman-Roman, C. Genevee, E. Viegas-Pequignot, and T. Hercend. 1990. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med*. 171:1393-1405.

154. Huard, B., M. Tournier, T. Hercend, F. Triebel, and F. Faure. 1994. Lymphocyte-activation gene 3/major histocompatibility complex class II interaction modulates the antigenic response of CD4+ T lymphocytes. *Eur J Immunol*. 24:3216-3221.

155. Okamura, T., K. Fujio, M. Shibuya, S. Sumitomo, H. Shoda, S. Sakaguchi, and K. Yamamoto. 2009. CD4+CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2. *Proc Natl Acad Sci U S A*. 106:13974-13979.

156. Camisaschi, C., C. Casati, F. Rini, M. Perego, A. De Filippo, F. Triebel, G. Parmiani, F. Belli, L. Rivoltini, and C. Castelli. 2010. LAG-3 expression defines a subset of

CD4(+)CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites. *J Immunol*. 184:6545-6551.

157. Li, N., C.J. Workman, S.M. Martin, and D.A. Vignali. 2004. Biochemical analysis of the regulatory T cell protein lymphocyte activation gene-3 (LAG-3; CD223). *J Immunol*. 173:6806-6812.

158. Workman, C.J., D.S. Rice, K.J. Dugger, C. Kurschner, and D.A. Vignali. 2002. Phenotypic analysis of the murine CD4-related glycoprotein, CD223 (LAG-3). *Eur J Immunol*. 32:2255-2263.

159. Workman, C.J., Y. Wang, K.C. El Kasmi, D.M. Pardoll, P.J. Murray, C.G. Drake, and D.A. Vignali. 2009. LAG-3 regulates plasmacytoid dendritic cell homeostasis. *J Immunol*. 182:1885-1891.

160. Gigley, J.P. and I.A. Khan. 2011. Plasmacytoid DC from aged mice down-regulate CD8 T cell responses by inhibiting cDC maturation after Encephalitozoon cuniculi infection. *PLoS One*. 6:e20838.

161. Asselin-Paturel, C., G. Brizard, K. Chemin, A. Boonstra, A. O'Garra, A. Vicari, and G. Trinchieri. 2005. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J Exp Med*. 201:1157-1167.

162. Seth, S., L. Oberdorfer, R. Hyde, K. Hoff, V. Thies, T. Worbs, S. Schmitz, and R. Forster. 2011. CCR7 essentially contributes to the homing of plasmacytoid dendritic cells to lymph nodes under steady-state as well as inflammatory conditions. *J Immunol*. 186:3364-3372.

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

164. Matsutani, T., T. Tanaka, K. Tohya, K. Otani, M.H. Jang, E. Umemoto, K. Taniguchi, H. Hayasaka, K. Ueda, and M. Miyasaka. 2007. Plasmacytoid dendritic cells employ multiple cell adhesion molecules sequentially to interact with high endothelial venule cells--molecular basis of their trafficking to lymph nodes. *Int Immunol*. 19:1031-1037.

165. Sozzani, S., W. Vermi, A. Del Prete, and F. Facchetti. 2010. Trafficking properties of plasmacytoid dendritic cells in health and disease. *Trends Immunol*. 31:270-277.

166. Penna, G., S. Sozzani, and L. Adorini. 2001. Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells. *J Immunol*. 167:1862-1866.

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

168. Vanbervliet, B., N. Bendriss-Vermare, C. Massacrier, B. Homey, O. de Bouteiller, F. Briere, G. Trinchieri, and C. Caux. 2003. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. *J Exp Med*. 198:823-830.

169. Zaballos, A., J. Gutierrez, R. Varona, C. Ardavin, and G. Marquez. 1999. Cutting edge: identification of the orphan chemokine receptor GPR-9-6 as CCR9, the receptor for the chemokine TECK. *J Immunol*. 162:5671-5675.

170. Wurbel, M.A., J.M. Philippe, C. Nguyen, G. Victorero, T. Freeman, P. Wooding, A. Miazek, M.G. Mattei, M. Malissen, B.R. Jordan, B. Malissen, A. Carrier, and P. Naquet. 2000. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol*. 30:262-271.

171. Kunkel, E.J., J.J. Campbell, G. Haraldsen, J. Pan, J. Boisvert, A.I. Roberts, E.C. Ebert, M.A. Vierra, S.B. Goodman, M.C. Genovese, A.J. Wardlaw, H.B. Greenberg, C.M. Parker, E.C. Butcher, D.P. Andrew, and W.W. Agace. 2000. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J Exp Med*. 192:761-768.

172. Wendland, M., N. Czeloth, N. Mach, B. Malissen, E. Kremmer, O. Pabst, and R. Forster. 2007. CCR9 is a homing receptor for plasmacytoid dendritic cells to the small intestine. *Proc Natl Acad Sci U S A*. 104:6347-6352.

173. Gilliet, M., W. Cao, and Y.J. Liu. 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol*. 8:594-606.

174. Yasuda, K., C. Richez, J.W. Maciaszek, N. Agrawal, S. Akira, A. Marshak-Rothstein, and I.R. Rifkin. 2007. Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J Immunol*. 178:6876-6885.

175. Vollmer, J., R. Weeratna, P. Payette, M. Jurk, C. Schetter, M. Laucht, T. Wader, S. Tluk, M. Liu, H.L. Davis, and A.M. Krieg. 2004. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur J Immunol*. 34:251-262.

176. Sakamoto, E., F. Hato, T. Kato, C. Sakamoto, M. Akahori, M. Hino, and S. Kitagawa. 2005. Type I and type II interferons delay human neutrophil apoptosis via activation of STAT3 and up-regulation of cellular inhibitor of apoptosis 2. *J Leukoc Biol*. 78:301-309.

177. Kropp, K.A., K.A. Robertson, G. Sing, S. Rodriguez-Martin, M. Blanc, P. Lacaze, M.F. Hassim, M.R. Khondoker, A. Busche, P. Dickinson, T. Forster, B. Strobl, M. Mueller, S. Jonjic, A. Angulo, and P. Ghazal. 2011. Reversible inhibition of murine cytomegalovirus replication by gamma interferon (IFN-gamma) in primary macrophages involves a primed type I IFN-signaling subnetwork for full establishment of an immediate-early antiviral state. *J Virol*. 85:10286-10299.

178. Gerosa, F., A. Gobbi, P. Zorzi, S. Burg, F. Briere, G. Carra, and G. Trinchieri. 2005. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J Immunol*. 174:727-734.

179. Martinez, J., X. Huang, and Y. Yang. 2008. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *J Immunol*. 180:1592-1597.

180. Marrack, P., J. Kappler, and T. Mitchell. 1999. Type I interferons keep activated T cells alive. *J Exp Med*. 189:521-530.

181. Jego, G., A.K. Palucka, J.P. Blanck, C. Chalouni, V. Pascual, and J. Banchereau. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity*. 19:225-234.

182. Fonteneau, J.F., M. Larsson, A.S. Beignon, K. McKenna, I. Dasilva, A. Amara, Y.J. Liu, J.D. Lifson, D.R. Littman, and N. Bhardwaj. 2004. Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J Virol*. 78:5223-5232.

183. Simmons, D.P., P.A. Wearsch, D.H. Canaday, H.J. Meyerson, Y.C. Liu, Y. Wang, W.H. Boom, and C.V. Harding. 2012. Type I IFN Drives a Distinctive Dendritic Cell Maturation Phenotype That Allows Continued Class II MHC Synthesis and Antigen Processing. *J Immunol*. 188:3116-3126.

184. Lau, A.H., M. Abe, and A.W. Thomson. 2006. Ethanol affects the generation, cosignaling molecule expression, and function of plasmacytoid and myeloid dendritic cell subsets in vitro and in vivo. *J Leukoc Biol*. 79:941-953.

185. Kang, H.K., M. Liu, and S.K. Datta. 2007. Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific regulatory T cells and contraction of inflammatory Th17 cells. *J Immunol*. 178:7849-7858.

186. Turner, M.S., L.P. Kane, and P.A. Morel. 2009. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. *J Immunol*. 183:4895-4903.

187. Sharma, M.D., B. Baban, P. Chandler, D.Y. Hou, N. Singh, H. Yagita, M. Azuma, B.R. Blazar, A.L. Mellor, and D.H. Munn. 2007. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J Clin Invest*. 117:2570-2582.

188. Ochando, J.C., C. Homma, Y. Yang, A. Hidalgo, A. Garin, F. Tacke, V. Angeli, Y. Li, P. Boros, Y. Ding, R. Jessberger, G. Trinchieri, S.A. Lira, G.J. Randolph, and J.S. Bromberg. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol*. 7:652-662.

189. Ouabed, A., F.X. Hubert, D. Chabannes, L. Gautreau, M. Heslan, and R. Josien. 2008. Differential control of T regulatory cell proliferation and suppressive activity by mature plasmacytoid versus conventional spleen dendritic cells. *J Immunol*. 180:5862-5870.

190. Kitamura, N., H. Yokoyama, T. Yashiro, N. Nakano, M. Nishiyama, S. Kanada, T. Fukai, M. Hara, S. Ikeda, H. Ogawa, K. Okumura, and C. Nishiyama. 2011. Role of PU.1 in MHC class II expression through transcriptional regulation of class II transactivator pI in dendritic cells. *J Allergy Clin Immunol*.

191. Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol*. 4:336-347.

192. Okazaki, T. and T. Honjo. 2006. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol*. 27:195-201.

193. Tokita, D., G.V. Mazariegos, A.F. Zahorchak, N. Chien, M. Abe, G. Raimondi, and A.W. Thomson. 2008. High PD-L1/CD86 ratio on plasmacytoid dendritic cells correlates with elevated T-regulatory cells in liver transplant tolerance. *Transplantation*. 85:369-377.

194. Keir, M.E., M.J. Butte, G.J. Freeman, and A.H. Sharpe. 2008. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 26:677-704.

195. Baumgart, D.C., D. Metzke, O. Guckelberger, A. Pascher, C. Grotzinger, I. Przesdzing, Y. Dorffel, J. Schmitz, and S. Thomas. 2011. Aberrant plasmacytoid dendritic cell distribution and function in patients with Crohn's disease and ulcerative colitis. *Clin Exp Immunol*. 166:46-54.

196. Pepper, M., F. Dzierszinski, E. Wilson, E. Tait, Q. Fang, F. Yarovinsky, T.M. Laufer, D. Roos, and C.A. Hunter. 2008. Plasmacytoid dendritic cells are activated by Toxoplasma gondii to present antigen and produce cytokines. *J Immunol*. 180:6229-6236.

197. Martin-Gayo, E., E. Sierra-Filardi, A.L. Corbi, and M.L. Toribio. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood*. 115:5366-5375.

198. Hanabuchi, S., T. Ito, W.R. Park, N. Watanabe, J.L. Shaw, E. Roman, K. Arima, Y.H. Wang, K.S. Voo, W. Cao, and Y.J. Liu. 2010. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. *J Immunol*. 184:2999-3007.

199. Swiecki, M. and M. Colonna. 2010. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol Rev*. 234:142-162.

200. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature*. 415:389-395.

201. Tian, J., A.M. Avalos, S.Y. Mao, B. Chen, K. Senthil, H. Wu, P. Parroche, S. Drabic, D. Golenbock, C. Sirois, J. Hua, L.L. An, L. Audoly, G. La Rosa, A. Bierhaus, P. Naworth, A. Marshak-Rothstein, M.K. Crow, K.A. Fitzgerald, E. Latz, P.A. Kiener, and A.J. Coyle. 2007. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol*. 8:487-496.

202. Isaksson, M., B. Ardesjo, L. Ronnblom, O. Kampe, H. Lassmann, M.L. Eloranta, and A. Lobell. 2009. Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE. *Eur J Immunol*. 39:2925-2935.

203. Kavousanaki, M., A. Makrigiannakis, D. Boumpas, and P. Verginis. 2010. Novel role of plasmacytoid dendritic cells in humans: induction of interleukin-10-producing Treg cells by plasmacytoid dendritic cells in patients with rheumatoid arthritis responding to therapy. *Arthritis Rheum*. 62:53-63.

204. Jongbloed, S.L., R.A. Benson, M.B. Nickdel, P. Garside, I.B. McInnes, and J.M. Brewer. 2009. Plasmacytoid dendritic cells regulate breach of self-tolerance in autoimmune arthritis. *J Immunol*. 182:963-968.

205. Bailey-Bucktrout, S.L., S.C. Caulkins, G. Goings, J.A. Fischer, A. Dzionek, and S.D. Miller. 2008. Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. *J Immunol*. 180:6457-6461.

206. Irla, M., N. Kupfer, T. Suter, R. Lissilaa, M. Benkhoucha, J. Skupsky, P.H. Lalive, A. Fontana, W. Reith, and S. Hugues. 2010. MHC class II-restricted antigen presentation by plasmacytoid dendritic cells inhibits T cell-mediated autoimmunity. *J Exp Med*. 207:1891-1905.

207. Saxena, V., J.K. Ondr, A.F. Magnusen, D.H. Munn, and J.D. Katz. 2007. The countervailing actions of myeloid and plasmacytoid dendritic cells control autoimmune diabetes in the nonobese diabetic mouse. *J Immunol*. 179:5041-5053.

208. Diana, J., V. Brezar, L. Beaudoin, M. Dalod, A. Mellor, A. Tafuri, M. von Herrath, C. Boitard, R. Mallone, and A. Lehuen. 2011. Viral infection prevents diabetes by inducing regulatory T cells through NKT cell-plasmacytoid dendritic cell interplay. *J Exp Med*. 208:729-745.

209. Goubier, A., B. Dubois, H. Gheit, G. Joubert, F. Villard-Truc, C. Asselin-Paturel, G. Trinchieri, and D. Kaiserlian. 2008. Plasmacytoid dendritic cells mediate oral tolerance. *Immunity*. 29:464-475.

210. Palomares, O., B. Ruckert, T. Jartti, U.C. Kucuksezer, T. Puhakka, E. Gomez, H.B. Fahrner, A. Speiser, A. Jung, W.W. Kwok, L. Kalogjera, M. Akdis, and C.A. Akdis. 2012. Induction and maintenance of allergen-specific FOXP3+ Treg cells in human tonsils as potential first-line organs of oral tolerance. *J Allergy Clin Immunol*. 129:510-520, 520 e511-519.

211. De Heer, H.J., H. Hammad, T. Soullie, D. Hijdra, N. Vos, M.A. Willart, H.C. Hoogsteden, and B.N. Lambrecht. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med*. 200:89-98.

212. Crother, T.R., N.W. Schroder, J. Karlin, S. Chen, K. Shimada, A. Slepenkin, R. Alsabeh, E. Peterson, and M. Arditi. 2011. Chlamydia pneumoniae infection induced allergic airway sensitization is controlled by regulatory T-cells and plasmacytoid dendritic cells. *PLoS One*. 6:e20784.

213. Oriss, T.B., M. Ostroukhova, C. Seguin-Devaux, B. Dixon-McCarthy, D.B. Stolz, S.C. Watkins, B. Pillemer, P. Ray, and A. Ray. 2005. Dynamics of dendritic cell phenotype and interactions with CD4+ T cells in airway inflammation and tolerance. *J Immunol*. 174:854-863.

214. Abe, M., B.L. Colvin, and A.W. Thomson. 2005. Plasmacytoid dendritic cells: in vivo regulators of alloimmune reactivity? *Transplant Proc*. 37:4119-4121.

215. Abe, M., Z. Wang, A. De Creus, and A.W. Thomson. 2005. Plasmacytoid dendritic cell precursors induce allogeneic T cell hyporesponsiveness and prolong heart graft survival. *Am J Transplant*. 5:1808-1819.

216. Arpinati, M., G. Chirumbolo, B. Urbini, G. Perrone, D. Rondelli, and C. Anasetti. 2003. Role of plasmacytoid dendritic cells in immunity and tolerance after allogeneic hematopoietic stem cell transplantation. *Transpl Immunol*. 11:345-356.

217. Fugier-Vivier, I.J., F. Rezzoug, Y. Huang, A.J. Graul-Layman, C.L. Schanie, H. Xu, P.M. Chilton, and S.T. Ildstad. 2005. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exp Med*. 201:373-383.

218. Huang, Y., L.D. Bozulic, T. Miller, H. Xu, L.R. Hussain, and S.T. Ildstad. 2011. CD8α+ plasmacytoid precursor DCs induce antigen-specific regulatory T cells that enhance HSC engraftment in vivo. *Blood*. 117:2494-2505.

219. Leventhal, J., M. Abecassis, J. Miller, L. Gallon, K. Ravindra, D.J. Tollerud, B. King, M.J. Elliott, G. Herzig, R. Herzig, and S.T. Ildstad. 2012. Chimerism and tolerance without GVHD or engraftment syndrome in HLA-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci Transl Med*. 4:124ra128.

220. Lu, Y., C.R. Giver, A. Sharma, J.M. Li, K.A. Darlak, L.M. Owens, J.D. Roback, J. Galipeau, and E.K. Waller. 2012. IFN-gamma and indoleamine 2,3-dioxygenase signaling between donor dendritic cells and T cells regulates graft versus host and graft versus leukemia activity. *Blood*. 119:1075-1085.

221. Ito, T., M. Yang, Y.H. Wang, R. Lande, J. Gregorio, O.A. Perng, X.F. Qin, Y.J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med*. 204:105-115.

222. Colvin, B.L., T.L. Sumpter, D. Tokita, J. Salati, A.L. Mellor, and A.W. Thomson. 2009. Allostimulatory activity of bone marrow-derived plasmacytoid dendritic cells is independent of indoleamine dioxygenase but regulated by inducible costimulator ligand expression. *Hum Immunol*. 70:313-320.

223. Bjorck, P., P.T. Coates, Z. Wang, F.J. Duncan, and A.W. Thomson. 2005. Promotion of long-term heart allograft survival by combination of mobilized donor plasmacytoid dendritic cells and anti-CD154 monoclonal antibody. *J Heart Lung Transplant*. 24:1118-1120.

224. Liu, X., P. Mishra, S. Yu, J. Beckmann, M. Wendland, J. Kocks, S. Seth, K. Hoffmann, M. Hoffmann, E. Kremmer, R. Forster, and T. Worbs. 2011. Tolerance induction towards cardiac allografts under costimulation blockade is impaired in CCR7-deficient animals but can be restored by adoptive transfer of syngeneic plasmacytoid dendritic cells. *Eur J Immunol*. 41:611-623.

225. Li, X.L., S. Menoret, S. Bezie, L. Caron, D. Chabannes, M. Hill, F. Halary, M. Angin, M. Heslan, C. Usal, L. Liang, C. Guillonneau, B. Le Mauff, M.C. Cuturi, R. Josien, and I. Anegon. 2010. Mechanism and localization of CD8 regulatory T cells in a heart transplant model of tolerance. *J Immunol*. 185:823-833.

226. Boor, P.P., H.J. Metselaar, S. Jonge, S. Mancham, L.J. van der Laan, and J. Kwekkeboom. 2011. Human plasmacytoid dendritic cells induce CD8 LAG-3 Foxp3 CTLA-4 regulatory T cells that suppress allo-reactive memory T cells. *Eur J Immunol*. 41:1663-1674.

227. Mazariegos, G.V., A.F. Zahorchak, J. Reyes, L. Ostrowski, B. Flynn, A. Zeevi, and A.W. Thomson. 2003. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. *Am J Transplant*. 3:689-696.

228. Gupta, A., C. Ashokkumar, M. Ningappa, Q. Sun, B.W. Higgs, S. Snyder, M. Johnson, A. Zeevi, K.M. Abu-Elmagd, A.W. Thomson, G.V. Mazariegos, and R. Sindhi. 2010. Elevated myeloid: plasmacytoid dendritic cell ratio associates with early acute cellular rejection in pediatric small bowel transplantation. *Transplantation*. 89:55-60.

229. Hadeiba, H., T. Sato, A. Habtezion, C. Oderup, J. Pan, and E.C. Butcher. 2008. CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. *Nat Immunol*. 9:1253-1260.

230. Segura, E., J. Wong, and J.A. Villadangos. 2009. Cutting edge: B220+CCR9- dendritic cells are not plasmacytoid dendritic cells but are precursors of conventional dendritic cells. *J Immunol*. 183:1514-1517.

231. Jensen, T.O., H. Schmidt, H.J. Moller, F. Donskov, M. Hoyer, P. Sjoegren, I.J. Christensen, and T. Steiniche. 2011. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer*.

232. Zou, W., V. Machelon, A. Coulomb-L'Hermin, J. Borvak, F. Nome, T. Isaeva, S. Wei, R. Krzysiek, I. Durand-Gasselin, A. Gordon, T. Pustilnik, D.T. Curiel, P. Galanaud, F. Capron, D. Emilie, and T.J. Curiel. 2001. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nat Med*. 7:1339-1346.

233. Charles, J., J. Di Domizio, D. Salameire, N. Bendriss-Vermare, C. Aspord, R. Muhammad, C. Lefebvre, J. Plumas, M.T. Leccia, and L. Chaperot. 2010. Characterization of circulating dendritic cells in melanoma: role of CCR6 in plasmacytoid dendritic cell recruitment to the tumor. *J Invest Dermatol*. 130:1646-1656.

234. Hartmann, E., B. Wollenberg, S. Rothenfusser, M. Wagner, D. Wellisch, B. Mack, T. Giese, O. Gires, S. Endres, and G. Hartmann. 2003. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res*. 63:6478-6487.

235. Wei, S., I. Kryczek, L. Zou, B. Daniel, P. Cheng, P. Mottram, T. Curiel, A. Lange, and W. Zou. 2005. Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. *Cancer Res*. 65:5020-5026.

236. Labidi-Galy, S.I., V. Sisirak, P. Meeus, M. Gobert, I. Treilleux, A. Bajard, J.D. Combes, J. Faget, F. Mithieux, A. Cassignol, O. Tredan, I. Durand, C. Menetrier-Caux, C. Caux, J.Y. Blay, I. Ray-Coquard, and N. Bendriss-Vermare. 2011. Quantitative and functional alterations of plasmacytoid dendritic cells contribute to immune tolerance in ovarian cancer. *Cancer Res*. 71:5423-5434.

237. Munn, D.H., M.D. Sharma, D. Hou, B. Baban, J.R. Lee, S.J. Antonia, J.L. Messina, P. Chandler, P.A. Koni, and A.L. Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J Clin Invest*. 114:280-290.

238. Sharma, M.D., D.Y. Hou, Y. Liu, P.A. Koni, R. Metz, P. Chandler, A.L. Mellor, Y. He, and D.H. Munn. 2009. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood*. 113:6102-6111.

239. Manlapat, A.K., D.J. Kahler, P.R. Chandler, D.H. Munn, and A.L. Mellor. 2007. Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory CD19+ dendritic cells. *Eur J Immunol*. 37:1064-1071.

240. Matta, B.M., A. Castellaneta, and A.W. Thomson. 2010. Tolerogenic plasmacytoid DC. *Eur J Immunol*. 40:2667-2676.

241. Naranjo-Gomez, M., N. Climent, J. Cos, H. Oliva, M. Bofill, J.M. Gatell, T. Gallart, R. Pujol-Borrell, and F.E. Borras. 2006. Tacrolimus treatment of plasmacytoid dendritic cells inhibits dinucleotide (CpG-)-induced tumour necrosis factor-alpha secretion. *Immunology*. 119:488-498.

242. Hung, C.H., Y.T. Chu, J.L. Suen, M.S. Lee, H.W. Chang, Y.C. Lo, and Y.J. Jong. 2009. Regulation of cytokine expression in human plasmacytoid dendritic cells by prostaglandin I2 analogues. *Eur Respir J*. 33:405-410.

243. Fabricius, D., M. Neubauer, B. Mandel, C. Schutz, A. Viardot, A. Vollmer, B. Jahrsdorfer, and K.M. Debatin. 2010. Prostaglandin E2 inhibits IFN-alpha secretion and Th1 costimulation by human plasmacytoid dendritic cells via E-prostanoid 2 and E-prostanoid 4 receptor engagement. *J Immunol*. 184:677-684.

244. Awasthi, A., Y. Carrier, J.P. Peron, E. Bettelli, M. Kamanaka, R.A. Flavell, V.K. Kuchroo, M. Oukka, and H.L. Weiner. 2007. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol*. 8:1380-1389.

245. Pallotta, M.T., C. Orabona, C. Volpi, C. Vacca, M.L. Belladonna, R. Bianchi, G. Servillo, C. Brunacci, M. Calvitti, S. Bicciato, E.M. Mazza, L. Boon, F. Grassi, M.C. Fioretti, F. Fallarino, P. Puccetti, and U. Grohmann. 2011. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat Immunol*. 12:870-878.

246. de Siqueira, A.L., M. Russo, A.A. Steil, S. Facincone, M. Mariano, and S. Jancar. 1997. A new murine model of pulmonary eosinophilic hypersensitivity: contribution to experimental asthma. *J Allergy Clin Immunol*. 100:383-388.

247. Morelli, A.E., A.F. Zahorchak, A.T. Larregina, B.L. Colvin, A.J. Logar, T. Takayama, L.D. Falo, and A.W. Thomson. 2001. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood*. 98:1512-1523.

248. Qian, S., A.J. Demetris, N. Murase, A.S. Rao, J.J. Fung, and T.E. Starzl. 1994. Murine liver allograft transplantation: tolerance and donor cell chimerism. *Hepatology*. 19:916-924.

249. Ueki, S., R. Dhupar, J. Cardinal, A. Tsung, J. Yoshida, K.S. Ozaki, J.R. Klune, N. Murase, and D.A. Geller. 2010. Critical role of interferon regulatory factor-1 in murine liver transplant ischemia reperfusion injury. *Hepatology*. 51:1692-1701.

250. John, R., H.A. Rajasinghe, J.M. Chen, A.D. Weinberg, P. Sinha, D.M. Mancini, Y. Naka, M.C. Oz, C.R. Smith, E.A. Rose, and N.M. Edwards. 2001. Long-term outcomes after cardiac transplantation: an experience based on different eras of immunosuppressive therapy. *Ann Thorac Surg*. 72:440-449.

251. Nouri-Shirazi, M. and A.W. Thomson. 2006. Dendritic cells as promoters of transplant tolerance. *Expert Opin Biol Ther*. 6:325-339.

252. Wood, K.J. and R. Goto. 2012. Mechanisms of rejection: current perspectives. *Transplantation*. 93:1-10.

253. Brennan, T.V., A. Jaigirdar, V. Hoang, T. Hayden, F.C. Liu, H. Zaid, C.K. Chang, R.P. Bucy, Q. Tang, and S.M. Kang. 2009. Preferential priming of alloreactive T cells with indirect reactivity. *Am J Transplant*. 9:709-718.

254. Sanchez-Fueyo, A. and T.B. Strom. 2011. Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs. *Gastroenterology*. 140:51-64.

255. Latchman, Y.E., S.C. Liang, Y. Wu, T. Chernova, R.A. Sobel, M. Klemm, V.K. Kuchroo, G.J. Freeman, and A.H. Sharpe. 2004. PD-L1-deficient mice show that PD-L1

on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A*. 101:10691-10696.

256. Jeong, H.Y., Y.J. Lee, S.K. Seo, S.W. Lee, S.J. Park, J.N. Lee, H.S. Sohn, S. Yao, L. Chen, and I. Choi. 2008. Blocking of monocyte-associated B7-H1 (CD274) enhances HCV-specific T cell immunity in chronic hepatitis C infection. *J Leukoc Biol*. 83:755-764.

257. Seo, S.K., H.Y. Jeong, S.G. Park, S.W. Lee, I.W. Choi, L. Chen, and I. Choi. 2008. Blockade of endogenous B7-H1 suppresses antibacterial protection after primary Listeria monocytogenes infection. *Immunology*. 123:90-99.

258. Hirano, F., K. Kaneko, H. Tamura, H. Dong, S. Wang, M. Ichikawa, C. Rietz, D.B. Flies, J.S. Lau, G. Zhu, K. Tamada, and L. Chen. 2005. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res*. 65:1089-1096.

259. Pfistershammer, K., C. Klauser, W.F. Pickl, J. Stockl, J. Leitner, G. Zlabinger, O. Majdic, and P. Steinberger. 2006. No evidence for dualism in function and receptors: PD-L2/B7-DC is an inhibitory regulator of human T cell activation. *Eur J Immunol*. 36:1104-1113.

260. Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med*. 203:883-895.

261. Schreiner, B., S.L. Bailey, T. Shin, L. Chen, and S.D. Miller. 2008. PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. *Eur J Immunol*. 38:2706-2717.

262. Wang, W., K. Carper, F. Malone, Y. Latchman, J. Perkins, Y. Fu, J. Reyes, and W. Li. 2008. PD-L1/PD-1 signal deficiency promotes allogeneic immune responses and accelerates heart allograft rejection. *Transplantation*. 86:836-844.

263. Wang, L., R. Han, and W.W. Hancock. 2007. Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance. *Eur J Immunol*. 37:2983-2990.

264. Grabie, N., I. Gotsman, R. DaCosta, H. Pang, G. Stavrakis, M.J. Butte, M.E. Keir, G.J. Freeman, A.H. Sharpe, and A.H. Lichtman. 2007. Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell mediated injury in the heart. *Circulation*. 116:2062-2071.

265. Hori, J., M. Wang, M. Miyashita, K. Tanemoto, H. Takahashi, T. Takemori, K. Okumura, H. Yagita, and M. Azuma. 2006. B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts. *J Immunol*. 177:5928-5935.

266. Krupnick, A.S., A.E. Gelman, W. Barchet, S. Richardson, F.H. Kreisel, L.A. Turka, M. Colonna, G.A. Patterson, and D. Kreisel. 2005. Murine vascular endothelium activates and induces the generation of allogeneic CD4+25+Foxp3+ regulatory T cells. *J Immunol*. 175:6265-6270.

267. Bjorck, P., P.T. Coates, Z. Wang, F.J. Duncan, and A.W. Thomson. 2005. Promotion of long-term heart allograft survival by combination of mobilized donor plasmacytoid dendritic cells and anti-CD154 monoclonal antibody. *Journal of Heart and Lung Transplantation*. 24:1118-1120.

268. Crispe, I.N. 2009. The liver as a lymphoid organ. *Annu Rev Immunol*. 27:147-163.

269. Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol*. 1:305-310.

270. Chen, W., X. Liang, A.J. Peterson, D.H. Munn, and B.R. Blazar. 2008. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *J Immunol*. 181:5396-5404.

271. Kingham, T.P., U.I. Chaudhry, G. Plitas, S.C. Katz, J. Raab, and R.P. DeMatteo. 2007. Murine liver plasmacytoid dendritic cells become potent immunostimulatory cells after Flt-3 ligand expansion. *Hepatology*. 45:445-454.

272. Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*. 192:1027-1034.

273. Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann. 1991. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J Immunol*. 147:2713-2716.

274. Dong, H., S.E. Strome, D.R. Salomao, H. Tamura, F. Hirano, D.B. Flies, P.C. Roche, J. Lu, G. Zhu, K. Tamada, V.A. Lennon, E. Celis, and L. Chen. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*. 8:793-800.

275. Pflanz, S., J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W.M. Blumenschein, J.D. Mattson, J.L. Wagner, W. To, S. Zurawski, T.K. McClanahan, D.M. Gorman, J.F. Bazan, R. de Waal Malefyt, D. Rennick, and R.A. Kastelein. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity*. 16:779-790.

276. Hall, A.O., J.S. Silver, and C.A. Hunter. 2012. The Immunobiology of IL-27. *Adv Immunol*. 115:1-44.

277. Pflanz, S., L. Hibbert, J. Mattson, R. Rosales, E. Vaisberg, J.F. Bazan, J.H. Phillips, T.K. McClanahan, R. de Waal Malefyt, and R.A. Kastelein. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol*. 172:2225-2231.

278. Collison, L.W. and D.A. Vignali. 2008. Interleukin-35: odd one out or part of the family? *Immunol Rev*. 226:248-262.

279. Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T.W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol*. 170:4886-4890.

280. Stumhofer, J.S., J.S. Silver, A. Laurence, P.M. Porrett, T.H. Harris, L.A. Turka, M. Ernst, C.J. Saris, J.J. O'Shea, and C.A. Hunter. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol*. 8:1363-1371.

281. Murugaiyan, G., A. Mittal, R. Lopez-Diego, L.M. Maier, D.E. Anderson, and H.L. Weiner. 2009. IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J Immunol*. 183:2435-2443.

282. Levings, M.K. and M.G. Roncarolo. 2000. T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties. *J Allergy Clin Immunol*. 106:S109-112.

283. Sasaoka, T., M. Ito, J. Yamashita, K. Nakajima, I. Tanaka, M. Narita, Y. Hara, K. Hada, M. Takahashi, Y. Ohno, T. Matsuo, Y. Kaneshiro, H. Tanaka, and K. Kaneko. 2011. Treatment with IL-27 attenuates experimental colitis through the suppression of the development of IL-17-producing T helper cells. *Am J Physiol Gastrointest Liver Physiol*. 300:G568-576.

284. Liu, H. and C. Rohowsky-Kochan. 2011. Interleukin-27-Mediated Suppression of Human Th17 Cells Is Associated with Activation of STAT1 and Suppressor of Cytokine Signaling Protein 1. *J Interferon Cytokine Res*. 31:459-469.

285. Wang, S., Y. Miyazaki, Y. Shinozaki, and H. Yoshida. 2007. Augmentation of antigen-presenting and Th1-promoting functions of dendritic cells by WSX-1(IL-27R) deficiency. *J Immunol*. 179:6421-6428.

286. Karakhanova, S., T. Bedke, A.H. Enk, and K. Mahnke. 2011. IL-27 renders DC immunosuppressive by induction of B7-H1. *J Leukoc Biol*. 89:837-845.

287. Wirtz, S., C. Becker, M.C. Fantini, E.E. Nieuwenhuis, I. Tubbe, P.R. Galle, H.J. Schild, M. Birkenbach, R.S. Blumberg, and M.F. Neurath. 2005. EBV-induced gene 3 transcription is induced by TLR signaling in primary dendritic cells via NF-kappa B activation. *J Immunol*. 174:2814-2824.

288. Hunter, C.A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol*. 5:521-531.

289. Chen, Y., G. Jiang, H.R. Yang, X. Gu, L. Wang, C.C. Hsieh, H.S. Chou, J.J. Fung, S. Qian, and L. Lu. 2009. Distinct response of liver myeloid dendritic cells to endotoxin is mediated by IL-27. *J Hepatol*. 51:510-519.

290. Rosas, L.E., A.A. Satoskar, K.M. Roth, T.L. Keiser, J. Barbi, C. Hunter, F.J. de Sauvage, and A.R. Satoskar. 2006. Interleukin-27R (WSX-1/T-cell cytokine receptor) gene-deficient mice display enhanced resistance to leishmania donovani infection but develop severe liver immunopathology. *Am J Pathol*. 168:158-169.

291. Findlay, E.G., R. Greig, J.S. Stumhofer, J.C. Hafalla, J.B. de Souza, C.J. Saris, C.A. Hunter, E.M. Riley, and K.N. Couper. 2010. Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection. *J Immunol*. 185:2482-2492.

292. Yamanaka, A., S. Hamano, Y. Miyazaki, K. Ishii, A. Takeda, T.W. Mak, K. Himeno, A. Yoshimura, and H. Yoshida. 2004. Hyperproduction of proinflammatory cytokines by WSX-1-deficient NKT cells in concanavalin A-induced hepatitis. *J Immunol*. 172:3590-3596.

293. Yang, R., Q. Liu, J.L. Grosfeld, and M.D. Pescovitz. 1994. Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. *J Pediatr Surg*. 29:1145-1148.

294. Spahn, T.W., H.L. Weiner, P.D. Rennert, N. Lugering, A. Fontana, W. Domschke, and T. Kucharzik. 2002. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *Eur J Immunol*. 32:1109-1113.

295. Dubois, B., G. Joubert, M. Gomez de Aguero, M. Gouanvic, A. Goubier, and D. Kaiserlian. 2009. Sequential role of plasmacytoid dendritic cells and regulatory T cells in oral tolerance. *Gastroenterology*. 137:1019-1028.

296. Shiokawa, A., K. Tanabe, N.M. Tsuji, R. Sato, and S. Hachimura. 2009. IL-10 and IL-27 producing dendritic cells capable of enhancing IL-10 production of T cells are induced in oral tolerance. *Immunol Lett*. 125:7-14.

297. O'Connell, P.J., A.E. Morelli, A.J. Logar, and A.W. Thomson. 2000. Phenotypic and functional characterization of mouse hepatic CD8 alpha+ lymphoid-related dendritic cells. *J Immunol*. 165:795-803.

298. Morelli, A.E., P.J. O'Connell, A. Khanna, A.J. Logar, L. Lu, and A.W. Thomson. 2000. Preferential induction of Th1 responses by functionally mature hepatic (CD8alpha- and CD8alpha+) dendritic cells: association with conversion from liver transplant tolerance to acute rejection. *Transplantation*. 69:2647-2657.

299. Brown, J.A., D.M. Dorfman, F.R. Ma, E.L. Sullivan, O. Munoz, C.R. Wood, E.A. Greenfield, and G.J. Freeman. 2003. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol*. 170:1257-1266.

300. Diveu, C., M.J. McGeachy, K. Boniface, J.S. Stumhofer, M. Sathe, B. Joyce-Shaikh, Y. Chen, C.M. Tato, T.K. McClanahan, R. de Waal Malefyt, C.A. Hunter, D.J. Cua, and R.A. Kastelein. 2009. IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. *J Immunol*. 182:5748-5756.

301. Yang, J., M. Yang, T.M. Htut, X. Ouyang, A. Hanidu, X. Li, R. Sellati, H. Jiang, S. Zhang, H. Li, J. Zhao, A.T. Ting, L. Mayer, J.C. Unkeless, M.E. Labadia, M. Hodge, J. Li, and H. Xiong. 2008. Epstein-Barr virus-induced gene 3 negatively regulates IL-17, IL-22 and RORgamma t. *Eur J Immunol*. 38:1204-1214.

302. Huber, M., V. Steinwald, A. Guralnik, A. Brustle, P. Kleemann, C. Rosenplanter, T. Decker, and M. Lohoff. 2008. IL-27 inhibits the development of regulatory T cells via STAT3. *Int Immunol*. 20:223-234.

303. Stumhofer, J.S., E.D. Tait, W.J. Quinn, 3rd, N. Hosken, B. Spudy, R. Goenka, C.A. Fielding, A.C. O'Hara, Y. Chen, M.L. Jones, C.J. Saris, S. Rose-John, D.J. Cua, S.A. Jones, M.M. Elloso, J. Grotzinger, M.P. Cancro, S.D. Levin, and C.A. Hunter. 2010. A role for IL-27p28 as an antagonist of gp130-mediated signaling. *Nat Immunol*. 11:1119-1126.

304. Villarino, A.V. and C.A. Hunter. 2004. Biology of recently discovered cytokines: discerning the pro- and anti-inflammatory properties of interleukin-27. *Arthritis Res Ther*. 6:225-233.

305. Collison, L.W., C.J. Workman, T.T. Kuo, K. Boyd, Y. Wang, K.M. Vignali, R. Cross, D. Sehy, R.S. Blumberg, and D.A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 450:566-569.

306. Lunz, J.G., 3rd, S.M. Specht, N. Murase, K. Isse, and A.J. Demetris. 2007. Gut-derived commensal bacterial products inhibit liver dendritic cell maturation by stimulating hepatic interleukin-6/signal transducer and activator of transcription 3 activity. *Hepatology*. 46:1946-1959.

307. Owaki, T., M. Asakawa, N. Morishima, I. Mizoguchi, F. Fukai, K. Takeda, J. Mizuguchi, and T. Yoshimoto. 2008. STAT3 is indispensable to IL-27-mediated cell proliferation but not to IL-27-induced Th1 differentiation and suppression of proinflammatory cytokine production. *J Immunol*. 180:2903-2911.

308. Marzec, M., Q. Zhang, A. Goradia, P.N. Raghunath, X. Liu, M. Paessler, H.Y. Wang, M. Wysocka, M. Cheng, B.A. Ruggeri, and M.A. Wasik. 2008. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proc Natl Acad Sci U S A*. 105:20852-20857.

309. Tong, H., Y. Miyazaki, M. Yamazaki, H. Hara, H. Waldmann, S. Hori, and H. Yoshida. 2010. Exacerbation of delayed-type hypersensitivity responses in EBV-induced gene-3 (EBI-3)-deficient mice. *Immunol Lett*. 128:108-115.

310. Tokita, D., G.V. Mazariegos, A.F. Zahorchak, N. Chien, M. Abe, G. Raimondi, and A.W. Thomson. 2008. High PD-L1/CD86 ratio on plasmacytoid dendritic cells correlates with elevated T-regulatory cells in liver transplant tolerance. *Transplantation*. 85:369-377.

311. Shimozato, O., A. Sato, K. Kawamura, M. Chiyo, G. Ma, Q. Li, and M. Tagawa. 2009. The secreted form of p28 subunit of interleukin (IL)-27 inhibits biological functions of IL-27 and suppresses anti-allogeneic immune responses. *Immunology*. 128:e816-825.

312. Wolfle, S.J., J. Strebovsky, H. Bartz, A. Sahr, C. Arnold, C. Kaiser, A.H. Dalpke, and K. Heeg. 2011. PD-L1 expression on tolerogenic APCs is controlled by STAT-3. *Eur J Immunol*. 41:413-424.

313. Holscher, C., A. Holscher, D. Ruckerl, T. Yoshimoto, H. Yoshida, T. Mak, C. Saris, and S. Ehlers. 2005. The IL-27 receptor chain WSX-1 differentially regulates antibacterial immunity and survival during experimental tuberculosis. *J Immunol*. 174:3534-3544.

314. Drakes, M.L., L. Lu, V.M. Subbotin, and A.W. Thomson. 1997. In vivo administration of flt3 ligand markedly stimulates generation of dendritic cell progenitors from mouse liver. *J Immunol*. 159:4268-4278.

315. Pillarisetty, V.G., S.C. Katz, J.I. Bleier, A.B. Shah, and R.P. Dematteo. 2005. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. *J Immunol*. 174:2612-2618.

316. Thomson, A.W., D.A. Geller, C. Gandhi, N. Murase, A.J. Demetris, and D. Beer-Stolz. 2011. Hepatic antigen-presenting cells and regulation of liver transplant outcome. *Immunol Res*. 50:221-227.

317. Lumsden, A.B., J.M. Henderson, and M.H. Kutner. 1988. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood in patients with cirrhosis. *Hepatology*. 8:232-236.

318. Farges, O., P.J. Morris, and M.J. Dallman. 1994. Spontaneous acceptance of liver allografts in the rat. Analysis of the immune response. *Transplantation*. 57:171-177.

319. Castellaneta, A., A.W. Thomson, N. Nayyar, M. de Vera, and G.V. Mazariegos. 2010. Monitoring the operationally tolerant liver allograft recipient. *Curr Opin Organ Transplant*. 15:28-34.

320. Mazariegos, G.V., J. Reyes, I.R. Marino, A.J. Demetris, B. Flynn, W. Irish, J. McMichael, J.J. Fung, and T.E. Starzl. 1997. Weaning of immunosuppression in liver transplant recipients. *Transplantation*. 63:243-249.

321. Ramos, H.C., J. Reyes, K. Abu-Elmagd, A. Zeevi, N. Reinsmoen, A. Tzakis, A.J. Demetris, J.J. Fung, B. Flynn, J. McMichael, and et al. 1995. Weaning of immunosuppression in long-term liver transplant recipients. *Transplantation*. 59:212-217.

322. Reyes, J., A. Zeevi, H. Ramos, A. Tzakis, S. Todo, A.J. Demetris, B. Nour, M. Nalesnik, M. Trucco, K. Abu-Elmagd, and et al. 1993. Frequent achievement of a drug-free state after orthotopic liver transplantation. *Transplant Proc*. 25:3315-3319.

323. Lu, L., W.A. Rudert, S. Qian, D. McCaslin, F. Fu, A.S. Rao, M. Trucco, J.J. Fung, T.E. Starzl, and A.W. Thomson. 1995. Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 182:379-387.

324. Sumpter, T.L., M. Abe, D. Tokita, and A.W. Thomson. 2007. Dendritic cells, the liver, and transplantation. *Hepatology*. 46:2021-2031.

325. Yoneyama, H., K. Matsuno, Y. Zhang, M. Murai, M. Itakura, S. Ishikawa, G. Hasegawa, M. Naito, H. Asakura, and K. Matsushima. 2001. Regulation by chemokines of circulating dendritic cell precursors, and the formation of portal tract-associated lymphoid tissue, in a granulomatous liver disease. *J Exp Med*. 193:35-49.

326. Abe, M., A.F. Zahorchak, B.L. Colvin, and A.W. Thomson. 2004. Migratory responses of murine hepatic myeloid, lymphoid-related, and plasmacytoid dendritic cells to CC chemokines. *Transplantation*. 78:762-765.

327. Bishop, G.A., J. Sun, D.J. DeCruz, K.L. Rokahr, J.D. Sedgwick, A.G. Sheil, N.D. Gallagher, and G.W. McCaughan. 1996. Tolerance to rat liver allografts. III. Donor cell migration and tolerance-associated cytokine production in peripheral lymphoid tissues. *J Immunol*. 156:4925-4931.

328. Sharland, A., S. Shastry, C. Wang, K. Rokahr, J. Sun, A.G. Sheil, G.W. McCaughan, and G.A. Bishop. 1998. Kinetics of intragraft cytokine expression, cellular infiltration, and cell death in rejection of renal allografts compared with acceptance of liver allografts in a rat model: early activation and apoptosis is associated with liver graft acceptance. *Transplantation*. 65:1370-1377.

329. Bosma, B.M., H.J. Metselaar, J.H. Gerrits, N.M. van Besouw, S. Mancham, Z.M. Groothuismink, P.P. Boor, L.J. van der Laan, H.W. Tilanus, E.J. Kuipers, and J. Kwekkeboom. 2010. Migration of allosensitizing donor myeloid dendritic cells into recipients after liver transplantation. *Liver Transpl*. 16:12-22.

330. Lebedeva, T., M.L. Dustin, and Y. Sykulev. 2005. ICAM-1 co-stimulates target cells to facilitate antigen presentation. *Curr Opin Immunol*. 17:251-258.

331. Qian, S., Z. Wang, Y. Lee, Y. Chiang, C. Bonham, J. Fung, and L. Lu. 2001. Hepatocyte-induced apoptosis of activated T cells, a mechanism of liver transplant tolerance, is related to the expression of ICAM-1 and hepatic lectin. *Transplant Proc*. 33:226.

332. Yin, Z., G. Jiang, J.J. Fung, L. Lu, and S. Qian. 2007. ICAM-1 expressed on hepatic stellate cells plays an important role in immune regulation. *Microsurgery*. 27:328-332.

333. Bertolino, P., A. Schrage, D.G. Bowen, K. Klugewitz, S. Ghani, K. Eulenburg, L. Holz, N. Hogg, G.W. McCaughan, and A. Hamann. 2005. Early intrahepatic antigen-specific retention of naive CD8+ T cells is predominantly ICAM-1/LFA-1 dependent in mice. *Hepatology*. 42:1063-1071.

334. Benichou, G., Y. Yamada, S.H. Yun, C. Lin, M. Fray, and G. Tocco. 2011. Immune recognition and rejection of allogeneic skin grafts. *Immunotherapy*. 3:757-770.

335. Afzali, B., R.I. Lechler, and M.P. Hernandez-Fuentes. 2007. Allorecognition and the alloresponse: clinical implications. *Tissue Antigens*. 69:545-556.

336. Schildberg, F.A., A. Wojtalla, S.V. Siegmund, E. Endl, L. Diehl, Z. Abdullah, C. Kurts, and P.A. Knolle. 2011. Murine hepatic stellate cells veto CD8 T cell activation by a CD54-dependent mechanism. *Hepatology*. 54:262-272.

337. Tran, D.Q., D.D. Glass, G. Uzel, D.A. Darnell, C. Spalding, S.M. Holland, and E.M. Shevach. 2009. Analysis of adhesion molecules, target cells, and role of IL-2 in human FOXP3+ regulatory T cell suppressor function. *J Immunol*. 182:2929-2938.

338. van Kooyk, Y. and T.B. Geijtenbeek. 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol Rev*. 186:47-56.

339. Svajger, U., M. Anderluh, M. Jeras, and N. Obermajer. 2010. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. *Cell Signal*. 22:1397-1405.

340. Ueki, S., A. Castellaneta, O. Yoshida, K. Ozaki, M. Zhang, S. Kimura, K. Isse, M. Ross, L. Shao, D.B. Stolz, A.W. Thomson, A.J. Demetris, D.A. Geller, and N. Murase. 2011. Hepatic B7 homolog 1 expression is essential for controlling cold ischemia/reperfusion injury after mouse liver transplantation. *Hepatology*. 54:216-228.

341. Cardenas, P.A., Y. Huang, and S.T. Ildstad. 2011. The role of pDC, recipient T(reg) and donor T(reg) in HSC engraftment: Mechanisms of facilitation. *Chimerism*. 2:65-70.

342. Fugier-Vivier, I.J., F. Rezzoug, Y. Huang, A.J. Graul-Layman, C.L. Schanie, H. Xu, P.M. Chilton, and S.T. Ildstad. 2005. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *Journal of Experimental Medicine*. 201:373-383.

343. Alegre, M.L., L. Chen, T. Wang, E. Ahmed, C.R. Wang, and A. Chong. 2009. Antagonistic effect of toll-like receptor signaling and bacterial infections on transplantation tolerance. *Transplantation*. 87:S77-79.

344. Moseman, E.A., X. Liang, A.J. Dawson, A. Panoskaltsis-Mortari, A.M. Krieg, Y.J. Liu, B.R. Blazar, and W. Chen. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol*. 173:4433-4442.