

Roles for Pin1 in Modulating Cells of the Innate Immune System

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor of Philosophy  
in the Department of Pharmacology and Cancer Biology  
in the Graduate School of  
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ABSTRACT

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## Abstract

Pin1 is a ubiquitously expressed phosphorylation-specific prolyl isomerase that regulates substrate function by catalyzing the *cis-trans* isomerization of prolyl bonds. Through this modulation, Pin1 has been shown to influence the stability, localization, and/or activity of a diverse set of protein substrates that participate in a variety of cellular responses, such as cell cycle progression, modulation of cell stress, and apoptosis. In addition to extensive studies in non-hematopoietic cells, Pin1 has also been shown to regulate immune cell function. Indeed, Pin1 participates in germinal center B cell development and eosinophil granulocyte survival. It also facilitates cytokine production in T cells, eosinophil granulocytes, and plasmacytoid dendritic cells. Through specific activities such as these, Pin1 has been demonstrated to modulate responses to viral challenge, respiratory allergens, and organ transplantation.

Due to previously described functions of Pin1 in regulating cells of both the innate and adaptive immune system, we predicted that Pin1 would participate in systemic inflammatory responses. Upon inducing systemic inflammation in mice, we observed a profound reduction in circulating cytokine concentrations in Pin1-null mice compared to WT mice. This result prompted further investigations, which are described in chapter 3 and chapter 4 of this dissertation. In chapter 3, we evaluate the potential contribution of macrophages to the defects we observe in LPS-challenged Pin1-null

mice. Using primary macrophages, bone marrow-derived macrophages, and MEF, we ultimately exclude a role for Pin1 in modulating LPS-induced production of pro-inflammatory cytokines in these cells. In chapter 4, we uncover a defect in the accumulation of conventional dendritic cells (cDC) in LPS-challenged Pin1-null mice. Upon more careful examination of spleen cDC subsets in Pin1-null mice, we discovered a defect in the CD8<sup>+</sup> subset. Experiments described in this chapter collectively indicate a role for Pin1 in preferentially modulating late stages of development of the CD8<sup>+</sup> subset of cDC. Consistent with such a defect, the expansion of adoptively transferred WT CD8<sup>+</sup> T cells was less robust in Pin1-null mice than WT mice upon infection with the bacterium *Listeria monocytogenes*. At the end of chapter 4, we provide evidence that Pin1 facilitates the degradation of the hematopoietic transcription factor PU.1, and propose that deregulation of PU.1 expression may be one mechanism by which Pin1 modulates CD8<sup>+</sup> cDC development. The work described in this dissertation began by evaluating a potential role for Pin1 in modulating pro-inflammatory cytokine production in macrophages; ultimately, however, we uncovered a novel role for Pin1 in preferentially modulating the development of the CD8<sup>+</sup> subset of cDC. The results presented herein expand the current understanding of DC development and further implicate Pin1 as an important modulator of both innate and adaptive immune responses.

## **Dedication**

This work is dedicated to my aunt, Jean Sacchi, and my friend, Mandy Lereche.

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## List of Acronyms and Abbreviations

PPIase	Peptidyl prolyl isomerase
MEF	Mouse Embryo Fibroblast
APC	Antigen Presenting Cell
DC	Dendritic Cell
cDC	Conventional Dendritic Cell
pDC	Plasmacytoid Dendritic Cell
MHC	Major Histocompatibility
TLR	Toll-like Receptor
LPS	Lipopolysaccharide
FL	Flt3 Ligand
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
M-CSF	Macrophage Colony Stimulating Factor
BMM	Bone Marrow-derived Macrophage
BMDC	Bone Marrow-derived Dendritic Cell
FL-BMDC	Flt3 Ligand-generated Bone Marrow-derived Dendritic Cell
GM-BMDC	GM-CSF-generated Bone Marrow-derived Dendritic Cell
MCMV	Murine Cytomegalovirus
IRF	Interferon Response Factor

IL-6	Interleukin 6
TNF $\alpha$	Tumor Necrosis Factor alpha
ELISA	Enzyme-linked Immunosorbent Assay
RNAi	RNA interference
DMEM	Dulbecco's Modified Eagle's Medium
hiFBS	heat-inactivated Fetal Bovine Serum

## Acknowledgements

Here begins the daunting task of recognizing those individuals who have supported me, both scientifically and personally, during my tenure in graduate school. I first thank my adviser, Tony Means, whose mentorship has been invaluable. I am incredibly grateful for the opportunity to train in his lab, and for the freedom and encouragement I received in pursuing my own interests. I cannot imagine a better place for growing in both scientific knowledge and character. With regard to members of the Means laboratory, working with such a delightful and talented group of people vastly improved the quality of each day. I particularly want to thank Luigi Racioppi for the gift of his enthusiastic guidance and teaching; without his insight and expertise, this would have been a much more difficult road. I am also grateful to Tom Ribar, who has always been generous with his time and assistance. He and his wife, Denise, have been a constant source of encouragement, and provided me a homey retreat on more than one occasion. I also thank Libby MacDougall, Josep Colomer, Pamy Noldner, Michelle Green, and Katherine Swenson-Fields for countless valuable conversations about both science and life.

I would like to thank my thesis committee for their helpful guidance in the completion of my project. I am particularly appreciative of Donald McDonnell and members of his laboratory for thoughtful discussions and continuous entertainment.

The PCB department, as a whole, has been a wonderful place to grow and learn; I have benefitted from many helpful conversations with members of the York lab, the Counter lab, the Reya lab, and the Rathmell lab. I am also grateful for my classmates CDB, HEW, SRJ, NLM, BAE, and GP. None of us really knew what we were getting ourselves into, but I am glad to have experienced it with such fabulous people.

I am extremely fortunate to have found an amazing group of friends to share these past years with. I will never forget Team Hazmatt, Weekly Dinner Night, Basketball Campout, James Joyce Fridays, I-House Halloween, Hospital Chai, Boxing Boot Camp, Hicks from the Sticks, Team Effervescing Elephant, Habitat for Humanity, Game Night, or Back Porch Music. I am equally grateful for the incredible group of women I met while attending W&M; your continued friendship, encouragement, and humor have helped me keep life in perspective. I also thank Jessie Rossol-Allison, Nick Troester, and John Lupton for their incredible support and unwavering faith in me, both as a person and as a scientist. To the Daly family: your home has been a refuge and I am deeply appreciative of your endless support and encouragement.

Most importantly, I thank my parents Phil and Sharon Barberi. From them I have inherited a stubbornness that lends itself to both scientific discovery and leading a life of purpose. They have taught me the value of working hard and living with integrity. I am forever indebted to them, and to my sister Emily, for their unconditional love and willingness to support me as I continue to pursue my passions.

“The rubber bands are heading in the right direction.”  
- *Fortune Cookie*

# 1. Introduction

## 1.1 Regulation of Protein Function by Prolyl Isomerization

Peptidyl prolyl *cis-trans* isomerases (PPIases) are an evolutionarily conserved class of enzymes that catalyze the conversion between *cis* and *trans* conformations of prolyl bonds. Unlike other residues, which favor the *trans* conformation, proline can exist in both *cis* and *trans*. The intrinsic rate of conversion between these two conformations is relatively slow in terms of cellular processes; although proline spends only 5-10% of its time in *cis*, it is thought that under conditions that deplete a specific isomer, catalyzation of this conversion could become crucial for both replenishing the functional isomer as well as for re-establishing the equilibrium (Gothel & Marahiel, 1999; Lu et al, 2007; Lu et al, 2002). Furthermore, due to the ability of prolines produce turns in the peptide backbone, modulation of *cis-trans* isomerization has the potential to significantly alter protein conformation. As protein folding and conformation are linked to both stability and activity, PPIases have the potential to greatly impact substrate function.

PPIases are grouped into four structurally distinct subfamilies which include PTPA, cyclophilins, FK506-binding proteins (FKBPs), and parvulins (Lu et al, 2007). PTPA is a recently identified PPIase that binds and modifies the activity of the protein phosphatase 2A (PP2A) (Jordens et al, 2006). Cyclophilins and FKBP's, also known as immunophilins, are the cellular targets of the immunosuppressive drugs Cyclosporine

and FK506, respectively (Barik, 2006). Members of both subfamilies have been shown to act in concert with heat shock proteins to regulate the folding of several steroid receptors, and also to modulate the activity of the transcription factor NFAT, although it remains unclear whether these functions require PPIase activity (Barik, 2006; Mueller & Bayer, 2008). Compared to the dozens of cyclophilins and FKBP's present in higher eukaryotes, the parvulin subfamily of PPIases is comprised of relatively few members. Although conserved from bacteria to humans, only two members are present in most metazoans, including humans, which express Pin1 and Par14 (Mueller & Bayer, 2008). Pin1 has received much attention, as it is the only known PPIase that specifically binds phosphorylated substrates, implicating it as a regulator of phosphorylation-dependent signaling events (Lu et al, 2002; Mueller & Bayer, 2008). The functions of the remaining members of the parvulin subfamily are not well-understood, but these enzymes do not appear to interact with phosphorylated substrates (Mueller & Bayer, 2008).

Pin1 is comprised of two distinct domains: an amino-terminal WW binding domain, and a carboxy-terminal PPIase domain. Both the WW domain and the PPIase domain of Pin1 have been demonstrated to bind proline preceded by a phosphorylated serine or phosphorylated threonine, but the exact mechanism regulating the coordinated action of these two domains remains incompletely understood (Lu et al, 2007). Because the conversion between *cis* and *trans* conformations occurs even more slowly in the presence of a phosphate group, the presence of an enzyme that catalyzes this conversion

may be particularly important for facilitating interactions with *trans*-specific molecules (Lu et al, 2002; Schutkowski et al, 1998). Many proline-directed kinases and phosphatases are *trans*-specific, implicating Pin1 as a potential regulator of phosphorylation-dependent signaling (Zhou et al, 2000). Indeed, both Pin1 and the *trans*-specific phosphatase PP2A have been shown to coordinately regulate the dephosphorylation of several substrates, whereby interaction with Pin1 is required to facilitate PP2A-mediated dephosphorylation (Dougherty et al, 2005; Yeh et al, 2004; Yeh et al, 2006; Zhou et al, 2000).

## **1.2 Pin1 Regulates Cell Cycle Progression**

Pin1 was identified in a yeast two-hybrid screen for proteins that interacted with the mitotic kinase NIMA in *Aspergillus nidulans* (Crenshaw et al, 1998; Lu et al, 1996). It was found to be 45% identical to the essential Ess1 protein in the yeast *Saccharomyces cerevisiae*. Depletion of Ess1 in yeast led to mitotic arrest and nuclear fragmentation, and a similar phenotype was observed when Pin1 expression was knocked down in HeLa cells. Overexpression of Pin1 in HeLa cells induced G2 arrest, suggesting a role for Pin1 in modulating the G2/M transition of the cell cycle. Furthermore, co-expression of Pin1 with NIMA attenuated the mitosis-promoting effect of NIMA in both yeast and HeLa cells (Lu et al, 1996). Based on these results, Pin1 was initially described as an inhibitor of mitosis. Subsequent studies conducted in *Xenopus laevis* revealed that Pin1 could interact with phosphorylated mitosis-specific proteins, including Wee1, Plk1 and Cdc25

(Crenshaw et al, 1998; Shen et al, 1998). Through regulation of Cdc25, Pin1 was shown to modulate the replication checkpoint (Winkler et al, 2000).

Due to its emerging role as a regulator of cell cycle, and the observation that the Pin1 was essential in both *A.nidulans* and *S.cerevisiae*, it was expected that Pin1 deletion would cause lethality in higher organisms (Hanes et al, 1989; Joseph et al, 2004). This did not turn out to be the case, however, as deletion of the Pin1 homologue *dodo* in the fruit fly *Drosophila melanogaster* and the deletion of Pin1 in mice had no impact on the viability of these organisms (Fujimori et al, 1999; Maleszka et al, 1996).

Upon careful evaluation, loss of *dodo* was found to impact egg chamber development in *D.melanogaster* via regulation of Cf2 degradation downstream of MAPK signaling (Hsu et al, 2001). Further studies in Pin1-null mice also revealed defects, although differences existed between mice of different genetic backgrounds. Initial reports from Pin1-null mice in a mixed genetic background indicated that loss of Pin1 resulted in impaired mammary development in pregnant females, as well as age-dependent defects such as decreased body weight, testicular atrophy and retinal degeneration. Despite the appearance of testicular atrophy in older males, however, young mice were capable of impregnating females (Liou et al, 2002). When Pin1-null mice were bred into an isogenic C57BL/6 background, the mice exhibited severe fertility defects that appeared early in life. The failure of these mice to produce litters was attributed to decreased proliferation of primordial germ cells in both males and females

(Atchison et al, 2003). Consistent with reports from mice in the mixed genetic background, adult Pin1-null males in the C57BL/6 background also exhibited defects in spermatogenesis. Retinal degeneration, however, was not detected (Atchison et al, 2003; Atchison & Means, 2003).

Studies in cells derived from Pin1-null mice revealed additional roles for Pin1 in cell cycle progression. After noting similar phenotypes in both Pin1-null mice and cyclin D1-null mice, mouse embryo fibroblasts (MEF) were utilized to demonstrate that Pin1 could bind phosphorylated cyclin D1 and increase its stability (Liou et al, 2002). This result was consistent with the previous report that Pin1-null MEF were defective in re-entering the cell cycle after G0 arrest, as cyclin D1 is known to modulate cell cycle entry (Fujimori et al, 1999). Further studies demonstrated that stimulation of MEF with Insulin-like Growth Factor-1 (IGF-1) induced Pin1 expression, which correlated with increased expression of cyclin D1 and phosphorylation of Rb, two events that are crucial for the G0/G1-S transition (You et al, 2002). Examination of MEF derived from C57BL/6 mice revealed that Pin1 could also bind phosphorylated cyclin E and facilitate its degradation. In the absence of Pin1, cyclin E is stabilized and asynchronously growing Pin1-null MEF exhibited a longer doubling time, incorporated less BrdU, and accumulated in both G1 and S phases of the cell cycle (Yeh et al, 2006). These data are consistent with the report that cyclin E overexpression impairs replication and retards S

phase progression (Ekholm-Reed et al, 2004). Collectively, these results demonstrated a role for Pin1 in modulating the G0/G1-S transition during cell cycle progression.

Many proteins that regulate cell cycle progression have established roles in tumorigenesis. It is therefore not surprising that Pin1 has been demonstrated to participate in cancer development through its ability to modulate cell cycle proteins. Indeed, both cyclin D1 and cyclin E have been shown to influence tumor progression. Both Pin1 and cyclin D1 are overexpressed in several human cancers, and regulation of cyclin D1 expression by Pin1 was demonstrated to modulate the transformation of mammary epithelial cells (Ryo et al, 2002). Additionally, our lab demonstrated that the loss of Pin1 stabilizes not only cyclin E, but also the immediate-early gene c-myc. Stabilization of cyclin E induced by Pin1 ablation was shown to contribute to genomic instability in MEF that had been immortalized with a dominant negative p53 (p53DD). Furthermore, deletion of Pin1 sensitized p53DD-immortalized MEF to Ras-dependent transformation and exacerbated tumor formation in mice (Yeh et al, 2006). In the case of c-myc, stabilization was demonstrated to contribute to the transformation of human cell lines, and its accumulation also enhanced tumor formation in mice (Yeh et al, 2004).

Presumably owing to its ability to simultaneously modulate a diverse repertoire of substrates within the same cell, the consequence of Pin1 inactivation or overexpression has proven to be both complex and cell context-dependent. A consensus on the cumulative function of Pin1 is further complicated by the ability of genetic

background to influence the outcome of altered Pin1 expression in cells. This is exemplified by reports that Pin1 is overexpressed in breast tumors and under-expressed in kidney cancer; forced expression of Pin1 enhances transformation of mammary epithelial cells, while re-expression of Pin1 in renal cell carcinoma cells that under-express Pin1 can attenuate cell growth (Ryo et al, 2002; Teng et al, 2011). Thus, in the context of cancer, Pin1 has been described as a “conditional tumor suppressor”, in which the presence of other genetic modifiers influences the ability of Pin1 to enhance or inhibit tumor formation (Yeh et al, 2006; Yeh & Means, 2007). It is expected that the outcome of altered Pin1 expression would be similarly context-dependent in other diseases. Indeed, Pin1 has been demonstrated to bind and regulate the function of several substrates that mediate immune cell function, but the consequence of altered Pin1 expression appears to depend on cell type and stimulus (Esnault et al, 2007a; Lufei et al, 2007; Phan et al, 2007; Ryo et al, 2003; Saitoh et al, 2006; Shen et al, 2008). Despite the existence of reports indicating roles for Pin1 in modulating specific aspects of immune cell function, surprisingly few investigations have employed animal models to further evaluate the consequence of altered Pin1 expression on health and disease. Thus, the role for Pin1 in modulating infection and immunity currently remains unclear and underexplored.

### **1.3 Innate and Adaptive Immunity: The Basics**

The immune system is comprised of two arms, the innate immune system and the adaptive immune system. The innate immune system is comprised of cells that have two primary functions during infection: the immediate clearance of pathogens and the stimulation of the adaptive immune system. Granulocytes, macrophages, and dendritic cells (DC) are all innate phagocytes that express various receptors capable of recognizing and responding to invading pathogens. Upon receptor engagement, macrophages release chemokines and cytokines that recruit granulocytes to the site of infection. Both granulocytes and macrophages then utilize distinct mechanisms to destroy invading pathogens, including the production of microbicidal molecules, reactive oxygen species (ROS), and proteolytic enzymes (Savina & Amigorena, 2007; Soehnlein & Lindbom, 2010). Although both granulocytes and macrophages are capable of secreting cytokines, macrophages produce the vast majority of these inflammatory proteins and are therefore considered to be key regulators of the innate immune response (Castellheim et al, 2009). After activation, granulocytes eventually undergo spontaneous apoptosis. This serves not only to protect the host from aberrant release of toxic molecules, but also serves as a signal to macrophages to begin producing anti-inflammatory mediators that can bring about the resolution of inflammation and initiate tissue repair (Fox et al, 2010; Soehnlein & Lindbom, 2010).

Dendritic cells are also capable of phagocytosing invading pathogens, but this activity serves a different purpose in DC. The phagocytic compartments in DC differ from those of granulocytes and macrophages in that they are less toxic and less efficient at proteolysis. This observed “partial degradation” of phagosome contents is fitting in light of the fact that DC are the most potent antigen presenting cells (APCs) in the immune system. Antigenic peptides produced from the digestion of phagocytosed material are loaded onto major histocompatibility (MHC) class II molecules and displayed on the cell surface in order to facilitate activation of adaptive immune cells. In addition to processing exogenous pathogens, such as bacteria, the innate immune system can also detect altered self-cells, such as those infected with virus or those that have sustained damage or a deleterious mutation. Intracellular antigens are processed and presented on the cell surface in the context of MHC class I molecules. While macrophages are also capable of antigen presentation to adaptive immune cells, they are much less efficient than DC, and thought to primarily function in pathogen clearance and cytokine production (Savina & Amigorena, 2007).

The adaptive immune system is comprised of T cells and B cells that, upon activation, undergo clonal proliferation to mount a specific immune response that can last several days. T cells develop in the thymus while B cells develop in the bone marrow, and upon completion of the developmental program, they migrate to secondary lymphoid organs, such as the spleen and lymph nodes (Goldsby, 2002). Each

T cell possesses a unique T cell receptor (TCR) that is generated by DNA recombination events and ensures a diverse repertoire of T cells with the potential to respond to a wide variety of antigens. T cells recognize antigens only in the context of MHC molecules expressed on the surface of APCs. Activation of naïve T cells requires additional co-stimulation by CD80 and CD86 molecules expressed on the surface of the APC. The absence of co-stimulation can lead to T cell anergy, death, and ultimately an inefficient immune response. As dendritic cells are the most potent stimulators of naïve T cells, they are crucial for initiating adaptive T cell-dependent immune responses (Bonilla & Oettgen, 2010; Mesquita Junior et al, 2010).

Two main subsets of T cells exist: CD4+ helper T cells and CD8+ cytotoxic T cells. CD4+ T cells recognize exogenous antigens presented on MHC class II molecules and secrete cytokines that help instruct and activate other immune cells. CD8+ T cells recognize intracellular antigens presented on MHC class I molecules and utilize proteolytic and cytolytic molecules to facilitate contact-dependent apoptosis of infected cells. (Bonilla & Oettgen, 2010; Mesquita Junior et al, 2010). This classical view of antigen presentation has exceptions, however, as some APCs have been found to possess the ability to cross-present exogenous antigens on MHC class I molecules to stimulate a cytotoxic T cell response (Kurts et al, 2010).

Multiple subsets of DC have also been identified, differing in their tissue distribution, receptor expression, and function. Plasmacytoid DC (pDC) and

conventional DC (cDC) are two types of DC that reside in secondary lymphoid organs, where they are in close proximity to T cells and can initiate antigen-specific adaptive T cell responses (Liu & Nussenzweig, 2010). pDC are specialized to respond to viruses by expressing toll-like receptor (TLR) 7 and TLR9, which recognize viral nucleic acids and initiate a response characterized by massive production of IFN $\alpha/\beta$ . Although activated pDC are capable of processing and presenting antigens to T cells to initiate antigen-specific responses, the importance of this function remains under debate; indeed, several reports suggest that pDC are much less efficient at antigen presentation than cDC and that their primary role may be in immunomodulation through cytokine secretion (Coquerelle & Moser, 2010; Swiecki & Colonna, 2010; Villadangos & Young, 2008). cDC express multiple TLRs, which enable them to sense and respond to a variety of pathogens, including bacteria, fungi, protozoa, and virus. These cells can be further divided into functional subsets based on the expression of CD8 and CD4. CD8<sup>-</sup>CD4<sup>+</sup> cDC are thought to primarily activate CD4<sup>+</sup> T helper cell responses. Those that are CD8<sup>+</sup>CD4<sup>-</sup> are less abundant in the periphery than CD8<sup>-</sup>CD4<sup>+</sup> cDC, and are most efficient and activating CD8<sup>+</sup> T cells. They also possess unique functions, such as the ability to cross-present exogenous antigens to CD8<sup>+</sup> T cells in the context of MHC class I molecules. Owing to this specialized ability, CD8<sup>+</sup>CD4<sup>-</sup> cDC have proven to be crucial modulators of CD8<sup>+</sup> T cell-dependent anti-tumor responses (Hildner et al, 2008; Liu & Nussenzweig, 2010; Savina & Amigorena, 2007; Shortman & Heath, 2010).

B cell activation can occur upon recognition of free antigen by the B cell receptor, which is comprised of both antigen-binding immunoglobulins (Igs) and signal-transducing Igs. Like APCs of the innate immune system, B cells express MHC class II and can similarly present antigens to T cells. Full activation of B cells usually requires additional signals provided by CD4<sup>+</sup> helper T cells. In some cases, however, antigens are able to stimulate B cell activation in the absence of T cell help, but this occurs less frequently (Goldsby, 2002). Upon activation, antigen-specific B cells undergo rapid clonal expansion and can differentiate into plasma cells that secrete antigen-specific antibodies that aid in the recognition and clearance of foreign pathogens by phagocytes of the innate immune system. Within germinal centers in the spleen and lymph nodes, B cells undergo immunoglobulin class switching and somatic hypermutation in order to produce a variety of immunoglobulins with increased affinity for the foreign antigen (Bonilla & Oettgen, 2010; Goldsby, 2002; Mesquita Junior et al, 2010).

Coordinated and controlled activation of cells of both the innate and adaptive immune systems is important for a successful immune response. Impaired function of the immune system can lead to a variety of health problems; acute over-activation of immune effectors can lead to tissue damage and organ failure, while sustained low-level inflammation is associated with autoimmune disorders and tumor initiation resulting from recurring inflammatory insults. Immunosuppression, on the other hand, leaves the host susceptible to a variety of infections, and can also promote tumor formation by

preventing immune detection and elimination (Belz et al, 2002; de Visser et al, 2006; Dunn et al, 2002).

#### **1.4 Described Roles for Pin1 in Immune Modulation**

Similar to the PPIases cyclophilin A and FKBP12, Pin1 has been shown impinge on NFAT activity. Indeed, Pin1 acts as a negative regulator of NFAT activity by binding NFAT and inhibiting its dephosphorylation by calcineurin (Liu et al, 2001). Additional functions for Pin1 in T cells have also been described, such as modulating production of the cytokines GM-CSF, IL-2, and IFN $\gamma$ . Blockade of Pin1 using the small molecule inhibitor Juglone was shown to inhibit cytokine production and to prevent lung transplant rejection in rats (Esnault et al, 2007a; Shen et al, 2005). In light of the fact that regulation of NFAT activity and cytokine production is a major mechanism by which Cyclosporine and FK506 exert their effects, it has been proposed that Pin1 inhibition may be similarly beneficial in the clinic for suppressing deleterious immune responses (Esnault et al, 2007a).

In addition to its roles in T cells, Pin1 has been shown to modulate genotoxic stress in germinal center B cells via its ability to bind the anti-apoptotic protein Bcl6 and facilitate its degradation. It was noted that the Pin1-null mice used in this study did not exhibit any obvious alterations in spleen lymphocyte populations under steady-state conditions, but upon immunization with sheep red blood cells, mice lacking Pin1 exhibited an abnormal extent of germinal center expansion in the spleen (Phan et al,

2007). Pin1 has also been demonstrated to bind and facilitate the turnover of Bruton Tyrosine Kinase (BTK), which is a non-receptor tyrosine kinase involved in B cell development and maturation. The experiments, however, were primarily conducted in immortalized cell lines, with the exception of one experiment in which total spleen lysates from Pin1-null mice were analyzed and found to exhibit elevated BTK expression and phosphorylation compared to WT lysates (Yu et al, 2006). The relevance of Pin1-mediated turnover of BTK in B cell development and function was not examined.

Within the innate immune system, Pin1 has been extensively examined for its roles in regulating activation and survival of eosinophils, a subset of granulocytes that modulate allergic inflammation and asthma (Esnault et al, 2007b; Shen et al, 2005; Shen et al, 2008; Shen et al, 2009). Upon eosinophil activation, Pin1 was demonstrated to augment the stability of both GM-CSF and TGF $\beta$  mRNA, which lead to enhanced survival of eosinophils and increased extracellular remodeling in the lung (Shen et al, 2005; Shen et al, 2008). Furthermore, Pin1 was shown to inhibit Bax activity downstream of cytokine signaling, providing a mechanism for the observed cytokine-induced survival of eosinophils that occurs after allergen challenge (Shen et al, 2009).

Experimental models in rodents demonstrated that either genetic ablation of Pin1 or inhibition of Pin1 with Juglone could inhibit eosinophil survival and lung remodeling, thereby improving lung function after insult (Esnault et al, 2007b; Shen et al, 2008).

In contrast to its role as a modulator of eosinophil function, the ability of Pin1 to influence macrophage function has not been extensively studied and remains unclear. One group utilized Pin1-null mice to determine the role of Pin1 in modulating NF- $\kappa$ B-dependent protection from ischemia/reperfusion injury in the liver. The authors determined that Pin1 was able to modulate NF- $\kappa$ B specifically in hepatocytes, but that Pin1 deficiency in liver macrophages, or Kupffer cells, did not alter their ability to stabilize NF- $\kappa$ B and secrete the cytokines TNF $\alpha$  and MIP-2 (Kuboki et al, 2009). Pin1 has also been shown to bind PPAR $\gamma$  and inhibit its transcriptional activity in the THP-1 macrophage cell line. Although silencing Pin1 expression augmented transcription of PPAR $\gamma$  targets, such as HO-1 and CD36, macrophage activation was never directly assessed (Fujimoto et al, 2010). More recently, Pin1 was demonstrated to negatively regulate IL-6 mRNA accumulation in LPS-stimulated peritoneal macrophages, as indicated by the enhanced production of IL-6 mRNA in cells isolated from Pin1-null mice. Surprisingly, secreted IL-6 protein was never measured, leaving us to merely assume that the difference in mRNA would directly translate into increased IL-6 secretion. This group also showed that Pin1 interacts with the myeloid transcription factor PU.1 in THP-1 macrophages, and the authors speculated that this interaction is important for PU.1-dependent transcription of IL-6; this relationship, however, was never demonstrated (Akiyama et al, 2011). In contrast to these results, loss of Pin1 was recently shown to moderately impair IL-6 protein secretion in LPS-stimulated

macrophages that were generated *ex vivo* from bone marrow cells. Although the authors did not provide statistical analysis, it is clear that the absence of Pin1 did not enhance LPS-stimulated IL-6 production in bone marrow-derived macrophages (Tun-Kyi, 2011). Interestingly, this same group utilized the THP-1 macrophage cell line to show interaction between Pin1 and the enzyme IRAK1 upon activation of TLR7 and TLR9. Pin1-null peritoneal macrophages and Pin1-silenced THP-1 macrophages exhibit impaired phosphorylation of IRAK1 in response to TLR7 and TLR9 activation. This same defect in IRAK1 phosphorylation was also detected downstream of TLR2 and TLR4. Perplexingly, the authors cite these data as evidence that Pin1 modulates pDC function, but fail to comment on the potential impact of deregulated IRAK1 phosphorylation on macrophage function (Tun-Kyi, 2011). Collectively, the current body of evidence implicating Pin1 as a regulator of macrophage function is small, contradictory and unclear. More work is needed to understand how macrophage function is modulated by Pin1.

In the aforementioned publication, Tun-Kyi et al. demonstrate that the loss of Pin1 impairs cytokine production in pDC. This is the first and only report to describe a role for Pin1 in dendritic cells. Specifically, the authors show that Pin1 deletion impairs secretion of IFN $\alpha$  in both spleen pDC and bone marrow-derived pDC upon TLR7 and TLR9 stimulation *ex vivo*. Decreased production of IL-6, IL-12, and TNF $\alpha$  was also observed in Pin1-null myeloid (conventional) DC generated by culturing bone marrow

*ex vivo* with the cytokine GM-CSF, but these results were not investigated further. Various cell types, including THP-1 macrophages and MEF, were then utilized to demonstrate that Pin1 binds phosphorylated, kinase-active IRAK1 downstream of TLR7/9. Genetic ablation or RNAi-mediated knock-down of Pin1 expression impaired IRAK1 phosphorylation and subsequent dissociation from the receptor complex, thus preventing IRF7 transcriptional activity and IFN $\alpha$  production (Tun-Kyi, 2011).

Upon examining spleen and lymph node populations, Tun-Kyi et al. determined that Pin1-null mice do not possess any defects in the frequency of B cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, myeloid (conventional) DC, or pDC. Mice were then utilized to examine the role of Pin1 in anti-viral responses *in vivo*. Murine cytomegalovirus (MCMV) - infected Pin1-null mice produced less serum IFN $\alpha$  and were more susceptible to MCMV infection, as indicated by greater weight loss and increased morbidity compared to WT mice (Tun-Kyi, 2011). While these results certainly indicate a role for Pin1 in mediating viral infection, they do not explicitly demonstrate an entirely pDC-dependent defect; indeed, CD8<sup>+</sup> cDC have also been implicated in modulating MCMV responses *in vivo* (Torti et al, 2011).

Additional studies have been performed that indicate other potential roles for Pin1 in immune modulation. Investigations in 293 cells and MEF indicate that Pin1 promotes the stability of the transcription factor IRF3 downstream of the virus-sensing TLR3. Consistent with this role, Pin1-null mice produced elevated levels of serum IFN $\beta$

when challenged with poly (I:C), which mimics viral infection and activates TLR3 (Saitoh et al, 2006). Pin1 has also been implicated as a modulator of Stat3 activity, as it can bind phosphorylated Stat3 and promote the activation of a Stat3 reporter in both MEF and immortalized cell lines (Lufei et al, 2007). Additionally, Pin1 has been demonstrated to modulate the turnover of the transcription factor NF- $\kappa$ B p65 by interfering with I $\kappa$ B $\alpha$ -dependent degradation. Pin1-null mice were deficient in TNF $\alpha$ -induced p65 accumulation in liver and exhibited an increase in TUNEL positive cells, presumably the result of decreased expression of p65, which promotes survival (Ryo et al, 2003).

It is clear from these reports that Pin1 possesses the ability to regulate multiple aspects of the immune response by various mechanisms. The existing data, however, are riddled with conflicting reports and unanswered questions. In particular, the role of Pin1 in macrophages and conventional DC function has not been thoroughly addressed. Given the importance of these cells in modulating innate and adaptive immune responses in the context of both infection and cancer, it is of great interest to identify modulators of these cell types that could potentially be manipulated to fine-tune and enhance immune responses in patients (Delamarre & Mellman, 2011; Ostrand-Rosenberg, 2010).

## **1.5 Dendritic Cell Development**

Dendritic cells develop from progenitors in the bone marrow. Unlike other hematopoietic lineages, they are unique in their ability to arise from both the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). They share multiple progenitors with macrophages and granulocytes, but eventually these common progenitors give rise to the more restricted common DC progenitor (CDP) which is only capable of producing dendritic cells (Liu et al, 2009). Under conditions of inflammation, it has been demonstrated that monocytes can also give rise to a CD8<sup>-</sup> subset of cDC, but this does not occur during steady-state conditions (Naik et al, 2006). The development of pDC and cDC is somewhat different in that pDC fully develop within the bone marrow, whereas cDC precursors circulate to secondary lymphoid organs, such as the spleen and lymph nodes, where they undergo final stages of development (Liu & Nussenzweig, 2010; Villadangos & Young, 2008).

Due to their ability to stimulate T cell responses, DC have been pursued in the clinic for use as cellular therapy against infections and cancer (Delamarre & Mellman, 2011). Because DC are not an abundant cell type, there is a great deal of interest in identifying growth factors and cytokines that can be utilized to induce expansion of these cells. Early reports indicated that the cytokines GM-CSF and IL-4 could be used to generate large numbers of DC from precursors obtained from the blood of human patients (Markowicz & Engleman, 1990; Romani et al, 1994). Additionally, it was

discovered that the cytokine Flt3 Ligand (FL) has the ability to expand DC *in vivo* when administered to mice (Maraskovsky et al, 1996; O'Keeffe et al, 2002). When subset-specific functions of DC began to be elucidated, efforts became focused on achieving expansion of particular subsets for further examination in the lab, as well as for potential use in the clinic. As a result of this work, it has become clear that not all cytokines are equal; while bone marrow cultured with FL has the ability to produce all subsets of DC found under steady-state conditions *in vivo*, bone marrow cultured with GM-CSF only produces Mac1<sup>+</sup> cDC, which are thought to be synonymous with CD8-CD4<sup>+</sup> cDC generated from monocytes under inflammatory conditions (Naik et al, 2006; Vremec et al, 1997; Xu et al, 2007). The differential requirements for GM-CSF and FL in DC development are further exemplified by studies in mice deficient for these cytokines or their receptors. Mice that lack either Flt3 receptor or FL itself exhibit profound defects in the production of both pDC and cDC (McKenna et al, 2000; Waskow et al, 2008). In contrast, mice lacking GM-CSF or its receptor exhibit very mild decreases in DC numbers, and are still capable of producing pDC and cDC (Vremec et al, 1997).

The identification of DC progenitors in the bone marrow has enabled a more thorough analysis of Flt3 receptor expression. Flt3 is expressed on early hematopoietic stem cells and most progenitors in the bone marrow, but expression is gradually lost in the majority of hematopoietic cells as they reach the final stages of development. DC are the exception, maintaining Flt3 receptor expression and FL-responsiveness even after

reaching the final stages of development (Schmid et al, 2010). Administration of FL *in vivo* induces DC expansion, but interestingly, the extent of the response varies between cDC subsets. Although the CD8<sup>+</sup>CD4<sup>-</sup> subset of cDC are less numerous in the steady-state compared to the CD8<sup>-</sup> subsets, these cells exhibit the greatest degree of expansion in response to *in vivo* FL administration (O'Keeffe et al, 2002).

Efforts aimed at identifying molecular determinants of DC development and subset specification are ongoing. Several transcription factors have been identified that act during early stages of development and direct the generation of multiple subsets of DC. One example is Stat3, which mediates Flt3 receptor signaling and has been shown to be necessary for the production of CDP and all subsets of DC that arise from this progenitor (Laouar et al, 2003). Similarly, Gfi-1 was identified downstream of the Flt3 receptor, and impinges on Stat3 activity, producing defects in multiple DC subsets when ablated in mice (Rathinam et al, 2005). Most recently, the myeloid transcription factor PU.1 was found to bind the promoter of the Flt3 receptor and regulate its transcription. Not surprisingly, progenitor cells lacking PU.1 were found to be deficient in producing both cDC and pDC, presumably owing to a lack of Flt3 signaling (Carotta et al, 2010a).

A number of other transcription factors appear to have more specific roles, but their precise contributions to each subset is difficult to disentangle due to overlapping expression in multiple cell types (Gabriele & Ozato, 2007; Watowich & Liu, 2010; Zenke & Hieronymus, 2006). The transcription factor E2-2 has been shown to be required for

pDC development, and is capable of regulating the expression of Spi-B and IRF8, two additional transcription factors required for pDC development (Cisse et al, 2008). Conversely, Id2 binds and antagonizes E2-2, enabling it to act as a negative regulator of pDC development. Furthermore, Id2 is highly expressed in CD8+ cDC and appears to be important for the specification of CD8+ cDC versus CD4+ cDC, as mice lacking Id2 are skewed towards CD4+ cDC and have fewer CD8+ cDC (Hacker et al, 2003; Jackson et al, 2011; Watowich & Liu, 2010). Multiple interferon regulatory factors (IRFs) have also been shown to participate in DC development (Gabriele & Ozato, 2007; Tamura et al, 2005). IRF4 is highly expressed in CD4+ cDC and, accordingly, IRF4 null mice are defective in producing CD4+ cDC, but produce CD8+ cDC and pDC to a similar extent as WT mice. IRF8 is highly expressed in CD8+ cDC and pDC, and mice lacking IRF8 are specifically impaired in production of these two DC subsets (Aliberti et al, 2003; Tamura et al, 2005). Most recently, the transcription factor NFIL3 was described to act upstream of Batf3, both of which are specifically required for CD8+ cDC development. Loss of either of these transcription factors impairs CD8+ cDC development, and in the case of Batf3, this lack of CD8+ cDC was demonstrated to impair cross-priming of CD8+ T cells in response to both virus and induced syngeneic fibrosarcoma in immunocompetent mice (Hildner et al, 2008; Kashiwada et al, 2011).

Further complicating the picture of DC subset specification is the ability of many of these proteins to interact with each other; indeed, it is believed that the presence or

absence of potential binding partners dictates specific transcriptional activities and developmental outcomes (Carotta et al, 2010b; Gabriele & Ozato, 2007; Watowich & Liu, 2010; Zenke & Hieronymus, 2006). This is perhaps best illustrated by studies of an IRF8 point mutant that harbors a single amino acid substitution in the IRF Association Domain. Unlike IRF8 null mice, which lack both CD8+ cDC and pDC, mice that express the IRF8 R294C mutant are only impaired in CD8+ cDC development. Furthermore, upon analysis of IRF8 binding partners, it was found that the R294C mutant is unable to associate with DNA and fails to dimerize with PU.1. The mRNA expression of the IRF8-PU.1 target Cytostatin C was impaired in CD8+ cDC expressing the IRF8 R294C mutant; further analysis by chromatin immunoprecipitation (ChIP) revealed that, despite the presence of PU.1, the R294C mutant was unable to bind the Cytostatin C promoter (Tailor et al, 2008). These results demonstrate that DC specification is not only determined by the presence or absence of a particular transcription factor, but that it can also be greatly influenced by changes in protein-protein interactions.

## **1.6 Remaining Questions**

With the exception of severe infertility, Pin1-null mice generated in a pure C57BL/6 background appear healthy and lack noticeable abnormalities (Atchison et al, 2003). Pin1 modulates many important cellular processes and is believed to exert its effects by acting as a molecular timer, influencing substrate activity by catalyzing intrinsically slow *cis-trans* prolyl isomerization (Lu et al, 2007). Consistent with this

concept, our lab has demonstrated that Pin1 is a critical player in processes that require precise timing, such as proliferation of primordial germ cells in both male and female mice (Atchison et al, 2003). In the context of the immune system, Pin1 has been shown to regulate the development of germinal center B cells through modulating the degradation of anti-apoptotic Bcl6 (Phan et al, 2007). Pin1 also facilitates activation-induced survival in lung eosinophils, which mediate responses to allergens (Shen et al, 2009). Most recently, Pin1 was shown to modulate IFN $\alpha$  production in activated pDC, and Pin1-null mice were impaired in their response to MCMV infection (Tun-Kyi, 2011). In each of these instances, critical roles for Pin1 have been revealed through the initiation of events that require rapid and/or precisely timed responses.

The existing body of work describing roles for Pin1 in immune modulation presents a picture that is far from complete. Rather, it is a somewhat incongruous collection of reports describing varied contributions of Pin1 in modulating the function of a few cell types under relatively few conditions. Despite the paucity of data, roles for Pin1 have been elucidated in cells of both the innate and adaptive immune system, suggesting to us that Pin1 might function broadly as a modulator of host responses to a variety of immune challenges.

When this work was begun, no roles had yet been described for Pin1 in modulating bacterial infection or systemic inflammation. Furthermore, relatively few of the studies that implicated Pin1 in modulating immune function had been carried out

using Pin1-null mice. Our goal was to utilize Pin1-null mice to investigate potential roles for Pin1 in modulating a systemic inflammatory response.

Chapter 2 describes the materials and methods that were used to investigate novel roles for Pin1 in modulating immune function. Chapter 3 begins with the induction of systemic inflammation in mice via injection of the bacterial cell wall component lipopolysaccharide (LPS). Upon measuring a panel of serum proteins, we found a striking decrease in cytokine production in LPS-challenged Pin1-null mice, which supported our initial hypothesis that Pin1 participates in modulating systemic inflammation. This result prompted us to further investigate the existence of cellular defects in Pin1-null mice that could account for defective serum cytokine production.

In chapter 3, primary macrophages, bone marrow-derived macrophages (BMM), and MEF are utilized to investigate a potential role for Pin1 in modulating LPS-induced pro-inflammatory cytokine production. Our results indicate that Pin1 is dispensable for both the *ex vivo* differentiation of macrophage from bone marrow cells, as well as the production of cytokines in response to LPS stimulation. In chapter 4 we identify a defect in the LPS-induced accumulation of conventional dendritic cells (cDC). We then provide evidence, both *in vivo* and *ex vivo*, that suggests a novel role for Pin1 in modulating the development of the CD8<sup>+</sup> subset of cDC. Furthermore, we demonstrate that Pin1 modulates the turnover of the transcription factor PU.1, and believe that this may be one mechanism by which Pin1 influences CD8<sup>+</sup> cDC development.

## **2. Materials and Methods**

### **2.1 Reagents**

Lipopolysaccharide from *Escherichia coli*, Sigma L2637; Hanks Balanced Salt Solution (HBSS) without calcium, magnesium and phenol red, Mediatech, Inc. 21-022-CV; EDTA-coated Microtainers, BD Biosciences 365974; Serum separator tubes, BD Biosciences 365956; IL-6 ELISA Max kit, BioLegend 431302; TNF $\alpha$  ELISA Max kit, BioLegend 430902; Dulbecco's Modified Eagle's Medium (DMEM), Mediatech, Inc. 17-205-CV; heat-inactivated Fetal Bovine Serum (hiFBS), Gemini BioProducts 900-108; recombinant mouse Flt3 Ligand, R&D 427-FL/CF; recombinant mouse M-CSF, eBioscience 14-8983; 1x RBC Lysis Buffer, eBioscience 00-4333-57; Cycloheximide, Calbiochem 239763.

### **2.2 Cell Culture Methods**

#### **2.2.1 MEFs**

Embryos were obtained from pregnant female Pin1 +/- mice at day E13.5. A single cell suspension was obtained by mechanical dissociation followed by digestion in Trypsin-EDTA for 30 minutes at 37°C. Cells were then expanded by culturing for 5-7 days in DMEM containing phenol red and supplemented with Pen/Strep and 15% hiFBS. Adherent MEF were then collected, aliquotted into cryotubes, and stored in liquid nitrogen until further use. For use in experiments, cells were thawed and maintained in phenol red-free DMEM supplemented with 10% hiFBS.

## **2.2.2 Bone Marrow-Derived Macrophages**

Bone marrow-derived macrophages were generated by culturing red blood cell-depleted bone marrow cells at  $2 \times 10^6$  cells/ml in DMEM-BM (DMEM containing Pen/Strep, 10% hiFBS, 2 mM L-glutamine, 1 mM Sodium Pyruvate, MEM Non-essential Amino Acids, 10 mM HEPES, and 0.1%  $\beta$ -Mercaptoethanol) supplemented with 15 ng/ml M-CSF for 5-7 days. For qRT-PCR analysis, only adherent cells were collected. For staining and flow cytometry analysis, either adherent only, or both adherent and non-adherent cells were collected.

## **2.2.3 Bone Marrow-Derived Dendritic Cells**

Bone marrow-derived dendritic cells were generated by culturing red blood cell-depleted bone marrow cells at  $2 \times 10^6$  cells/ml in DMEM-BM (DMEM containing Pen/Strep, 10% hiFBS, 2 mM L-glutamine, 1 mM Sodium Pyruvate, MEM Non-essential Amino Acids, 10 mM HEPES, and 0.1%  $\beta$ -Mercaptoethanol). To generate FL-BMDC, cells were supplemented with 50 ng/ml Flt3 Ligand for 9 days. On days 3 and 6, 40% of the media was removed and replaced with an equal volume of DMDM-BM containing 50ng/ml Flt3 Ligand. On day 9, non-adherent cells were collected for analysis. To generate GM-BMDC, cells were supplemented with 5 ng/ml GM-CSF for 5 days; on day 5, non-adherent cells were collected for analysis. For stimulation of BMDC with LPS, 100 ng/ml LPS was added to the media for 24 hours on either day 5 (GM-BMDC) or

day 9 (FL-BMDC). Both adherent and non-adherent cells were collected and analyzed by flow cytometry.

## **2.2.4 Peritoneal Cell Culture**

Cells were obtained from the peritoneal cavity by performing a lavage with HBSS containing 5% hiFBS. Briefly, using a syringe, HBSS + hiFBS was dispensed into the peritoneal cavity and then immediately recovered and transferred to a 50 ml conical on ice. Cells were pelleted, and then resuspended and put into culture with DMEM supplemented with Pen/Step and 10% hiFBS. Cultured cells were maintained at 37°C.

## **2.3 Animal Procedures**

### **2.3.1 Primary Cell Isolation**

#### **2.3.1.1 Splenocyte Isolation**

To obtain splenocytes, spleens were removed and placed in a dish on ice containing DMEM-BM (DMEM supplemented with Pen/Strep, 10% hiFBS, 2 mM L-glutamine, 1 mM Sodium Pyruvate, MEM Non-essential Amino Acids, 10 mM HEPES, and 0.1%  $\beta$ -Mercaptoethanol). Splenocytes were expelled into the media by mechanically crushing the spleen between two squares (approximately 1 in<sup>2</sup>) of nylon mesh. The spleen cell suspension was then pipetted into a 15 ml conical and cells were pelleted for 5 minutes at 4°C and 1300 rpm. Media was removed and cells were resuspended in 3 ml of 1x RBC lysis buffer, and then pelleted again for 5 minutes at 4°C and 1300 rpm. RBC lysis buffer

was then removed and cells were washed in 10 ml DMEM-BM, pelleted, and resuspended in fresh DMEM-BM for counting.

#### **2.3.1.2 Bone Marrow Isolation**

Both femurs (2) and tibia (2) were carefully removed, intact, from each animal and placed in a dish on ice containing DMEM-BM (DMEM supplemented with Pen/Strep, 10% hiFBS, 2 mM L-glutamine, 1 mM Sodium Pyruvate, MEM Non-essential Amino Acids, 10 mM HEPES, and 0.1%  $\beta$ -Mercaptoethanol). The heads of the bones were then cut off and bone marrow was flushed from the bones with DMEM-BM into a 50 ml conical on ice using a needle and syringe. Bone marrow was then pipetted up and down to remove large chunks of marrow and to try to achieve a single cell suspension. Cells were then pelleted by centrifuging 5 minutes at 4°C and 1300 rpm. After removing the media, bone marrow cells were resuspended in 3 ml 1x RBC lysis buffer and then pelleted again. The RBC lysis buffer was removed and cells were then washed in 10-20 ml DMEM-BM. Cells were pelleted again, resuspended in fresh DMEM-BM, and passed through a 70 $\mu$ m cell strainer to remove any remaining cell clumps. Cells were then counted and either put into culture or directly stained and analyzed by flow cytometry.

#### **2.3.1.3 Blood Cell Isolation**

Eight to ten drops of blood were collected in tubes containing 500 $\mu$ l of 5mM EDTA in HBSS, and the tubes were inverted to mix. 800 $\mu$ l of 2% Dextran in HBSS was then added to each tube and mixed gently via pipetting. The cell suspension was then

incubated in a 37°C water bath for 45 minutes to allow for the red blood cells to settle to the bottom of the tubes. The top clear fraction of cells were then transferred to a new tube and pelleted for 5 minutes at 4°C and 1500 rpm. Supernatant was removed and the pellet was resuspended in 300µl of 1x RBC lysis buffer for 3 minutes on ice. Lysis was stopped by adding 1 ml of FACS Buffer (HBSS containing 3% hiFBS, 0.1% NaN<sub>3</sub>, and 10 mM EDTA) and then cells were pelleted. Supernatant was removed, and cells were resuspended in FACS Buffer and stained for analysis by flow cytometry.

#### **2.3.1.4 Peritoneal Cell Isolation**

After euthanization, peritoneal cells were obtained by performing peritoneal lavage. A small opening was made in the outer lining of the peritoneal wall. A hose was then inserted into the opening and the opening was sealed with a pair of surgical clamps. The hose was then attached to a syringe containing approximately 10 ml HBSS supplemented with 2% hiFBS. This volume was dispensed into the peritoneal cavity, and then drawn back into the syringe. The syringe was then removed from the hose and the cell suspension dispensed into a 50 ml conical on ice. This was repeated three times for each animal. Cells were then pelleted and resuspended in media for culturing, or resuspended in FACS buffer for staining and analysis by flow cytometry.

### **2.3.2 Lipopolysaccharide Challenge**

WT and Pin1 *-/-* mice were administered either 300 $\mu$ l HBSS or 15mg/kg lipopolysaccharide diluted in 300 $\mu$ l of HBSS by i.p. injection. Mice were euthanized after 3 or 18 hours.

### **2.3.3 Flt3 Ligand Treatment**

WT and Pin1 *-/-* mice were administered 1 $\mu$ g of Flt3 Ligand in 300 $\mu$ l HBSS by i.p. injection for 9 consecutive days. Mice were euthanized on day 10, and splenocytes were stained and analyzed by flow cytometry.

### **2.3.4 *Listeria monocytogenes* Infection**

Ovalbumin(ova)-specific OT1 CD8<sup>+</sup> T cells were obtained from the spleen of an OT1 CD45.1/2 female in a C57BL/6 background using the EasySep CD8<sup>+</sup> T cell Enrichment Kit (StemCell Technologies, 19753) according to the manufacturer's protocol. 100  $\mu$ l of PBS containing 10,000 ova-specific CD45.1/2<sup>+</sup> OT1 CD8<sup>+</sup> T cells was then injected into the tail vein of each CD45.2<sup>+</sup> WT and Pin1 *-/-* mouse. Recombinant *Listeria monocytogenes* (*L.m.*) engineered to secrete chicken ovalbumin was kindly provided by M. Bevan (University of Washington, Seattle, WA) and was prepared as previously described (Zhang et al, 2008a). Twenty-four hours after the injection of OT1 CD8<sup>+</sup> T cells, WT and Pin1 *-/-* mice were either left uninfected (controls) or infected with 10,000 CFU *L.m.*-ova in 100  $\mu$ l PBS by tail vein injection. Seven days after *L.m.*-ova infection,

WT and Pin1 <sup>-/-</sup> mice were euthanized and their spleens removed and analyzed for the presence of CD45.1+ CD8+ T cells by flow cytometry.

### **2.3.5 Bone Marrow Transplant**

Red blood cell-depleted bone marrow was collected from femurs and tibiae of CD45.2+ WT mice Pin1 <sup>-/-</sup> mice. Cells were counted and 12 million cells were pooled from each of 3 mice from each genotype to give 36 million cells. Cells were then washed and resuspended in 1.8 ml of sterile HBSS to give a final concentration of  $2 \times 10^7$  cells/ml. 100  $\mu$ l (containing 2 million cells) was then injected retro-orbitally into each of 11 WT CD45.1+ recipient mice for each donor genotype (2). At weeks 3, 6, and 9 after transplant, blood was collected by cheek bleed. Blood cells were isolated, stained, and analyzed by flow cytometry. At week 12, mice were euthanized and blood was obtained for staining by cardiac puncture. Additionally, spleens were removed for staining and analysis of CD45.2+ donor splenocyte populations.

## **2.4 Cell Staining and Flow Cytometry**

### **2.4.1 Cell Staining Protocol**

Between one and five million cells were washed in FACS Buffer (HBSS containing 3% hiFBS, 0.1% NaN<sub>3</sub>, and 10 mM EDTA) and then pelleted, resuspended in 100ul Antibody Dilution Buffer (FACS Buffer supplemented with 5% Normal Mouse Serum, 5% Normal Rat Serum, and 1% purified anti-CD16/32(Fc $\gamma$ R)), and incubated on ice for 15 minutes. Primary antibodies were then added to cells in Antibody Dilution Buffer (ADB) for 25

minutes on ice. Cells were washed in 1 ml FACS Buffer, resuspended in FACS Buffer, and then analyzed immediately using a BD FACSCanto II analyzer. For the staining of GMPs in the bone marrow, 1% purified anti-CD16/32(Fc $\gamma$ R) was excluded from the ADB and replaced with CD16/32(Fc $\gamma$ R)-PE-Cy7. After 15 min on ice, the remaining primary antibodies were added to the cells for 25 min on ice, and the rest of the staining protocol was carried out as described above.

#### **2.4.2 Antibodies for Flow Cytometry**

F4/80-FITC, CD11c-APC, MHC class II (I-A)-FITC, B220-PE, B220-PE-Cy7, CD69-APC, CD4-PE, CD45.1-FITC, CD3-FITC, CD3-PE, CD8-PerCP-Cy5.5, CD8-PE, GR1-PE, CD115-APC, PDCA1-biotin, PDCA1-APC, CD19-FITC, CD40-PE, c-Kit-FITC, c-Kit-APC, Flt3-PE-Cy5, IL7R $\alpha$ -PE-Cy7, CD34-FITC, Sca-1-PE-Cy5.5, CD16/32(Fc $\gamma$ R)-PE-Cy7, Mac1-PE, Ter119-PE, purified anti-CD16/32, Normal Rat Serum, and Normal Mouse Serum were all purchased from eBioscience. CD86-APC, CD40-PE-Cy7, CD86-PE-Cy7, GR1-APC-Cy7, CD3-PE-Cy7, CD11c-PerCP-Cy5.5, CD11c-PE-Cy7, Mac1-Pacific Blue, CD19-PE-Cy7, and Sca-1-Pacific Blue were all purchased from BioLegend. CD19-PE and CD3-APC were purchased from Pharmingen. Streptavidin-APC-Cy7 was purchased from BD Biosciences.

#### **2.5 Cytokine Measurement by ELISA**

Blood was either obtained by tail vein bleed, or from cardiac puncture after euthanization. Tail vein blood was collected in EDTA-coated microtainers and then

centrifuged at 5,000 rpm and 4°C for 15 minutes to pellet cells. Supernatant was collected (plasma) and cytokines were measured by ELISA. Blood from cardiac puncture was collected in a serum separator tube and centrifuged at 10,000 rpm for 10 minutes at room temperature to separate serum. Serum was removed from top fraction and cytokines were measured by ELISA.

## **2.6 Real Time Quantitative PCR Analysis**

### **2.6.1 mRNA Preparation and Analysis**

RNA was purified from cells using the RNeasy Mini Kit (Qiagen, 74104) following the manufacturer's protocol. Genomic DNA was removed by on-column digestion with DNaseI using the RNase-free DNase Set (Qiagen, 79254). cDNA was generated from 1-3 µg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, 18064022). RNA was then removed by digestion with RNaseH (Invitrogen, 18021-071). Quantitative PCR analysis was performed using SYBR Green (Invitrogen, 4312704) and the Bio-Rad CFX96 real-time PCR detection system. Cyclophilin A or 18S were used as reference transcripts. Fold change was determined using the  $\Delta\Delta C_t$  method.

### **2.6.2 Primers**

IL-6 Forward: 5' -AGTCCGGAGAGGAGACTTCA-3' and

IL-6 Reverse: 5' -TTGCCATTGCACAACCTCTTT-3'

TNF $\alpha$  Forward: 5' -CCAAAGGGATGAGAAGTTCC-3' and

TNF $\alpha$  Reverse: 5' -CTCCACTTGGTGGTTTGCTA-3'

PU.1 Forward: 5'- GAGAAAGCCATAGCGATCACTACTGG -3' and

PU.1 Reverse: 5'- ATGTGGCGATAGAGCTGCTGTAG -3';

Cyclophilin A Forward: 5'- GAGCTGTTTGCAGACAAAGTTC -3' and

Cyclophilin A Reverse: 5'- CCCTGGCACATGAATCCTGG -3';

18S Forward: 5' - AGGGTTCGATTCCGGAGAGG -3' and

18S Reverse: 5' – CAACTTTAATATACGCTATTGG -3'

## **2.7 Immunoblot Analysis**

Cells were washed in HBSS and then lysed in TritonX Lysis Buffer (150 mM NaCl, 10 mM Tris pH7.4, 10 mM EDTA, 50 mM NaF, and 1% TritonX-100) containing 100 µg/ml Pefabloc (Roche, 11429876001), 10 µg/ml Leupeptin, and 100 nM Okadaic Acid. Lysates were then sonicated and clarified by centrifugation at 10,000 rpm for 5 minutes. Lysates were resolved on 10% or 12% SDS-Page gels and transferred to nitrocellulose.

Membranes were incubated in blocking buffer (0.6x PBS containing 0.25% Fish Gelatin, 0.5 mg/ml casein, and 0.02% sodium azide) for ≥ 1 hour at room temperature, or at 4°C overnight, and then incubated with primary antibodies in blocking buffer containing 0.1% Tween-20 at room temperature for ≥ 2 hours. Membranes were washed in TBS containing 0.05% Tween-20, and then incubated with secondary antibodies in blocking buffer containing 0.1% Tween-20 and 0.01% SDS for 1 hour at room temperature.

Membranes were scanned using the Odyssey Infrared Imager by Li-Cor Biosciences and band intensities were quantified using Odyssey software v3.0.

## **2.8 Cycloheximide Analysis**

Primary WT or Pin1 *-/-* MEF were grown to 85% confluency in DMEM-MEF (DMEM containing 2 mM L-glutamine, 10% hiFBS, and Pen/Strep). Media was then removed and replaced with either DMEM-MEF alone or DMEM-MEF containing 150 µg/ml cycloheximide reconstituted in water. Cells were incubated for 2-10 hours in the presence of cycloheximide. Both adherent and non-adherent cells were collected, lysed, and analyzed by immunoblot.

## **2.9 GST Pull Down Assay**

### **2.9.1 Preparation of Glutathione-agarose-bound GST fusion proteins**

The pGEX-2TK vector alone or pGEX-2TK vector containing either WT *Xenopus* Pin1 or a WW domain mutant of *Xenopus* Pin1 (W11A, W34A) were expressed in BL21(DE3) *E. coli* (Stratagene, 200131). Bacteria were lysed in PBS containing 1 mM DTT, 10 mM EDTA, and 100 µg/ml Pefabloc (Roche, 11429876001) and then sonicated. TritonX-100 was added to produce a final concentration of 1% TritonX-100 and lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4°C. GST and GST-Pin1 fusion proteins were then purified by incubating 5 ml of clarified bacterial lysates with 200 µl of a 50/50 slurry of glutathione-agarose beads for 2 hours at 4°C. Beads were washed in Passive Lysis Buffer (PBS containing 1 mM DTT, 10 mM EDTA, and 1% TritonX-100) supplemented with Pefabloc and stored at 4°C.

### **2.9.2 Pull Down Assay**

Primary WT and Pin1<sup>-/-</sup> MEF were grown to 85% confluency in DMEM-MEF (DMEM containing 2 mM L-glutamine, 10% hiFBS, and Pen/Strep) and then lysed in Passive Lysis Buffer (PLB) supplemented with 100 µg/ml Pefabloc, 10 µg/ml Leupeptin, and 100 nM Okadaic Acid. Cell lysates were sonicated and clarified by centrifugation. 1.2 mg of 1 mg/ml lysates were pre-cleared with 20 µg GST beads for 15 minutes at 4°C. 1 mg of pre-cleared lysate was then incubated with 10 µg GST beads, 10 µg GST-Pin1 beads, or 10 µg GST-Pin1WW beads for 2 hours at 4°C. Beads were washed 4 times in 5 ml of PLB, then pelleted, resuspended in SDS-Page Sample Buffer and boiled to elute proteins. Samples were analyzed by immunoblot analysis.

### **2.10 Statistical Analysis**

Data were determined to be statistically significant (\*) if  $p < 0.05$  by student's t-test.

Error bars represent the standard error of the mean.

## **3. Investigating the Role of Pin1 in Macrophage Function**

### **3.1 Preface**

Pin1 has been described to modulate several different cell types that have the potential to impact responses to immune challenges. At the time this work was begun, however, there did not exist any reports indicating a role for Pin1 in modulating systemic inflammation. We therefore made use of Pin1-null mice to investigate the possibility that loss of Pin1 would impair inflammatory responses *in vivo*. In this chapter, we utilize the bacterial cell wall component lipopolysaccharide (LPS) to induce systemic inflammation in mice. We examine serum cytokine production and then make use of both macrophages and MEF to further evaluate a potential role for Pin1 in modulating pro-inflammatory cytokine production associated with inflammation.

### **3.2 Experimental Results**

#### **3.2.1 Pin1-null Mice Exhibit Impaired Serum Cytokine Production in Response to Systemic Inflammation**

Administration of bacterial LPS is commonly used to examine the function of cells that mediate the inflammatory response in the absence of an actual infection. We induced systemic inflammation in WT and Pin1-null mice by administering 15 mg/kg LPS by intraperitoneal (i.p.) injection. After 18 hours, blood was collected from each mouse and samples were pooled together into 4 experimental groups (WT + Vehicle, WT + LPS, Pin1-null + Vehicle, Pin1-null + LPS). These samples were then submitted to Rules-Based Medicine, Inc. for multi-analyte profiling of serum proteins. The results we

received indicated that Pin1-null mice exhibited a profound defect in their response to systemic inflammation; a majority of the serum proteins induced by administration of LPS in WT mice were greatly reduced in Pin1-null mice (Table 1). These proteins included chemokines as well as both pro-inflammatory and anti-inflammatory cytokines. Although there was not enough serum to confirm each of the observed differences, we were able to verify the decrease in serum IL-6 in Pin1-null mice by performing an ELISA on remaining serum from each individual mouse. The results of the IL-6 ELISA were consistent with the results returned by Rules-Based Medicine, Inc, as IL-6 protein was greatly reduced in the serum of each LPS-challenged Pin1-null mouse (Figure 1). The observation that the majority of serum proteins analyzed were decreased in Pin1-null mice suggested that there might exist an early defect in the inflammatory response to LPS in the absence of Pin1. Because macrophages are among the first cells to respond to LPS, and they are known to be the major producers of serum cytokines, their presence and function was examined in Pin1-null mice.

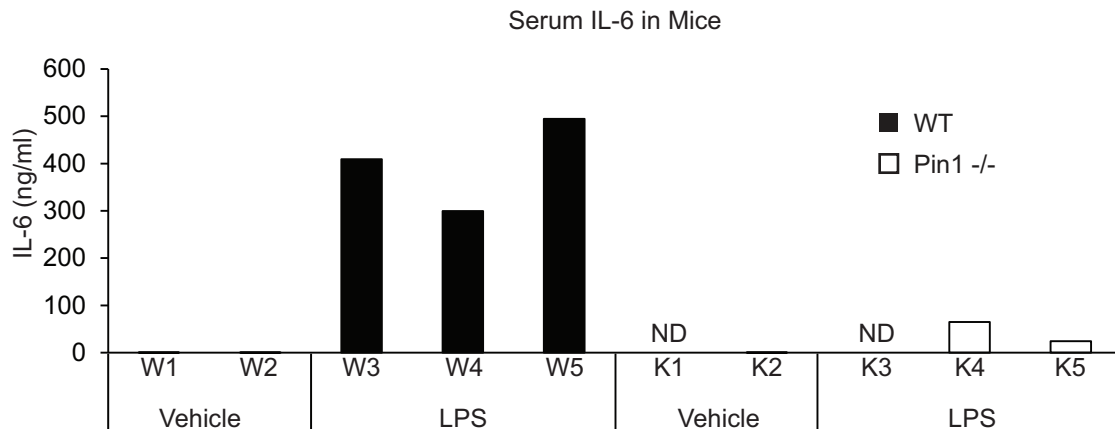
### **3.2.2 Loss of Pin1 Does Not Impact Macrophage Accumulation or Function**

Because LPS was injected intraperitoneally, the first cells to encounter LPS and initiate a response would be peritoneum-resident cells that express TLR4. Both macrophages and granulocytes are present in the peritoneal cavity and are critical early responders to LPS challenge. To determine whether a defect existed in these two cell populations in the absence of Pin1, WT and Pin1-null mice were administered either

vehicle or 15 mg/kg LPS for 3 hours. Mice were then euthanized and peritoneal cells were recovered by peritoneal lavage and stained for markers of both macrophages and granulocytes. Vehicle-treated Pin1-null mice exhibited an elevated frequency of granulocytes and a decreased frequency of macrophages compared to WT mice, but due to the high degree of variability between animals, these differences did not reach statistical significance (Figure 2). Additionally, because these cells are analyzed as a

**Table 1: Serum Protein Panel in WT and Pin1-null Mice**

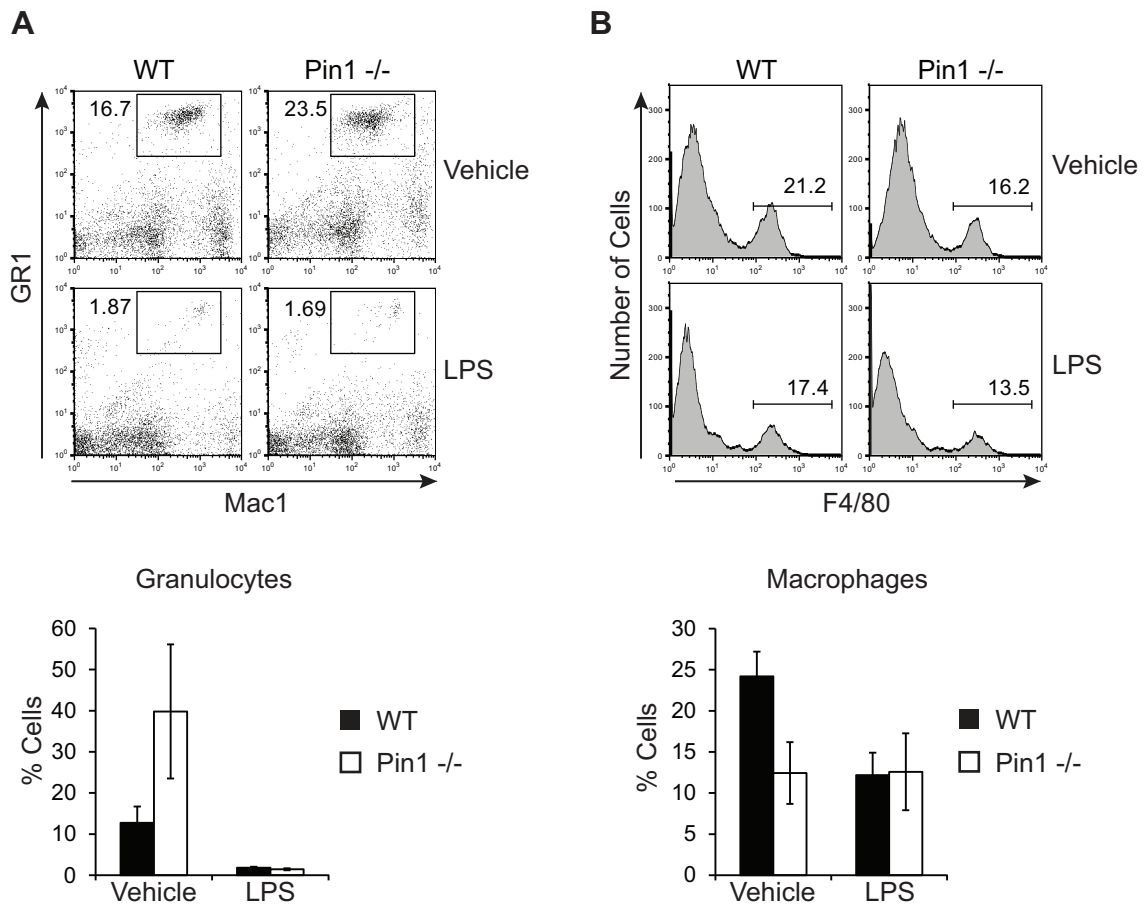
DECREASED	units	Vehicle		18 hr LPS	
		WT	Pin1 -/-	WT	Pin1 -/-
CD40 Ligand	pg/mL	1050	1460	3200	1910
GCP-2 (Granulocyte Chemotactic Protein-2)	ng/mL	46	45	79	55
GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor)	pg/mL	<LOW>	<LOW>	96	9.2
IFN-gamma (Interferon-gamma)	pg/mL	<LOW>	<LOW>	1670	90
IL-10 (Interleukin-10)	pg/mL	199	219	8470	2030
IL-11 (Interleukin-11)	pg/mL	<LOW>	19	563	297
IL-12p70 (Interleukin-12p70)	ng/mL	<LOW>	<LOW>	3.9	1.1
IL-17 (Interleukin-17)	ng/mL	<LOW>	<LOW>	3.0	0.17
IL-18 (Interleukin-18)	ng/mL	4.7	4.9	40	10
IL-1alpha (Interleukin-1alpha)	pg/mL	521	303	1910	612
IL-2 (Interleukin-2)	pg/mL	9.3	<LOW>	580	141
IL-3 (Interleukin-3)	pg/mL	<LOW>	<LOW>	136	21
IL-4 (Interleukin-4)	pg/mL	16	16	354	117
IL-5 (Interleukin-5)	ng/mL	0.74	1.1	3.7	2.2
IL-6 (Interleukin-6)	pg/mL	<LOW>	4.2	>17542	4030
IL-7 (Interleukin-7)	ng/mL	0.055	0.099	1.1	0.52
MCP-5 (Monocyte Chemoattractant Protein-5)	pg/mL	9.6	25	3350	653
MDC (Macrophage-Derived Chemokine)	pg/mL	572	588	3960	1410
MIP-2 (Macrophage Inflammatory Protein-2)	pg/mL	24	21	>7372	750
MIP-3beta (Macrophage Inflammatory Protein-3beta)	ng/mL	1.4	1.5	7.0	3.9
RANTES (Regulation Upon Activation, Normal T-Cell Expressed and Secreted)	pg/mL	0.88	0.52	243	43
TNF-alpha (Tumor Necrosis Factor-alpha)	ng/mL	0.034	<LOW>	1.7	0.72
UNCHANGED	units	Vehicle		18 hr LPS	
IL-1beta (Interleukin-1beta)	ng/mL	8.1	8.9	15	13
MCP-3 (Monocyte Chemoattractant Protein-3)	pg/mL	313	346	5110	4990
M-CSF (Macrophage-Colony Stimulating Factor)	ng/mL	4.8	4.8	10	7.4
MMP-9 (Matrix Metalloproteinase-9)	ng/mL	233	376	374	312
VCAM-1 (Vascular Cell Adhesion Molecule-1)	ng/mL	1580	1940	2990	2420
INCREASED	units	Vehicle		18 hr LPS	
MCP-1 (Monocyte Chemoattractant Protein-1)	pg/mL	166	140	2680	6820



**Figure 1: Serum IL-6 in WT and Pin1-null Mice**

WT and Pin1-null mice were administered either vehicle (HBSS alone) or 15 mg/kg LPS reconstituted in HBSS. After 18 hours, serum was collected and analyzed for the presence of IL-6 protein by ELISA. ND, not detected.

percentage of a total population, it was unclear whether the primary defect was in granulocytes or macrophages; changes in frequency are relative and an increase in the frequency of one population concomitantly decreases the frequency of another population relative to the total cell number. To further determine whether a defect in granulocyte numbers existed, blood was collected from healthy WT and Pin1-null mice and subjected to a Complete Blood Count (CBC) analysis, in which various blood cell populations were quantified. Neutrophils are the most prevalent subset of granulocytes, and we found no indication of altered neutrophil counts in the blood of Pin1-null mice. Additionally, there were no perturbations in lymphocytes, monocytes, red blood cells or platelets (Figure 3). These results suggest that the differences we observe in cytokine



**Figure 2: Frequencies of Peritoneal Granulocytes and Macrophages in WT and Pin1-null Mice**

(A) and (B) WT and Pin1-null Mice were administered vehicle (HBSS alone) or 15 mg/kg LPS reconstituted in HBSS by i.p. injection. After 3 hours, mice were euthanized and peritoneal cells were obtained by performing a peritoneal lavage. Cells were then stained with granulocyte and macrophage markers, fixed, and analyzed by flow cytometry. The upper panels contain representative FACS plots, and the averaged data are graphed directly below in the bottom panels.

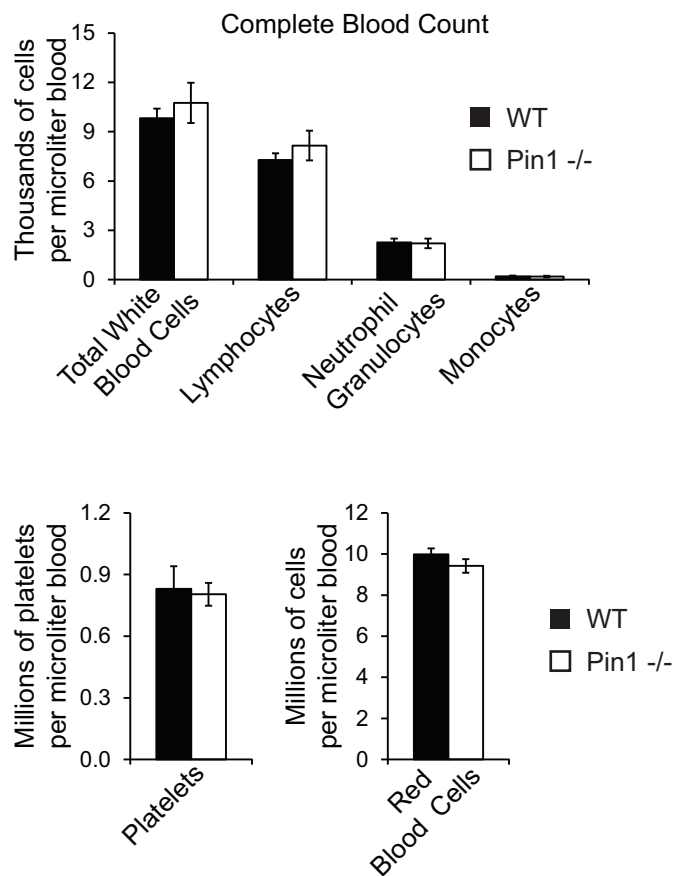
production in LPS-challenged Pin1-null mice do not result from basal defects in

circulating neutrophils, monocytes, or lymphocytes. While activated granulocytes do

produce cytokines upon exposure to LPS, compared to macrophages, their contribution

to circulating cytokine concentrations is minimal. Furthermore, granulocytes cannot

easily be cultured or generated *ex vivo* and frequently undergo degranulation and death when manipulated (Yona et al, 2010). For these reasons, we investigated the possibility that defects existed in Pin1-null macrophages that impaired the immune response in LPS-challenged mice.



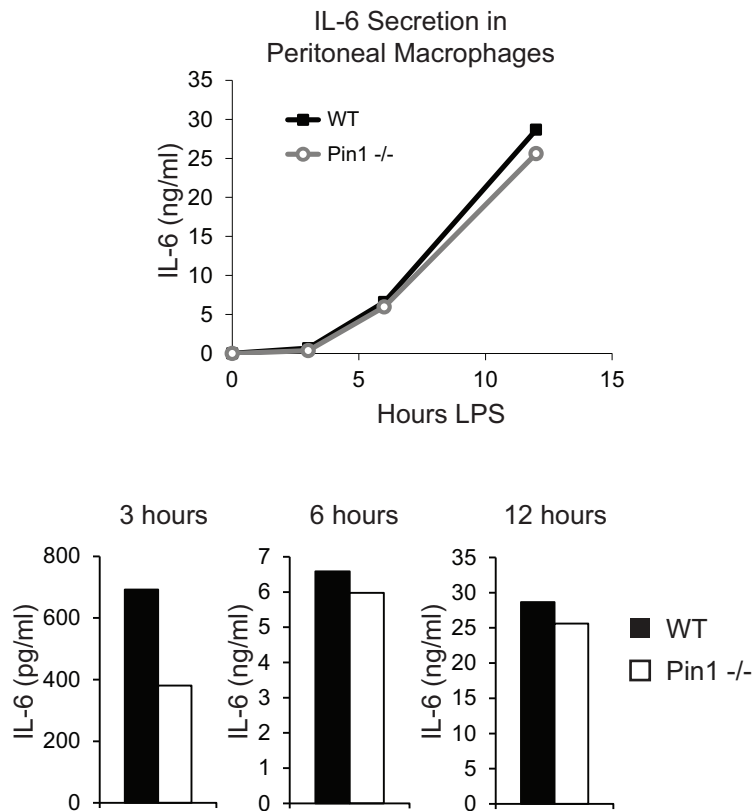
**Figure 3: Complete Blood Count in WT and Pin1 null mice**

Blood was collected from healthy WT and Pin1-null mice in EDTA-coated microtainers and immediately analyzed using a Hemavet 950 FS from Drew Scientific.

LPS-induced activation was first examined in WT and Pin1-null peritoneal macrophages that were obtained by peritoneal lavage and pooled together from 3 WT or 3 Pin1-null mice. After culturing the pooled peritoneal cells for 24 hours to allow macrophages to adhere to the culture dish, non-adherent cells were removed and the remaining adherent macrophages were stimulated with LPS for 3, 6, or 12 hours. Cell culture media was collected at each time-point and assayed by ELISA for the presence of IL-6, as IL-6 is a pro-inflammatory cytokine produced by activated macrophages, and this cytokine was decreased in the serum of LPS-challenged Pin1-null mice. Although Pin1-null peritoneal macrophages exhibited a 50% reduction in IL-6 secretion after 3 hours stimulation with LPS, this reduction was not maintained at 6 and 12 hours (Figure 4). The LPS-induced accumulation of IL-6 mRNA, as well as the mRNA of the pro-inflammatory cytokine TNF $\alpha$ , was also examined. Similar to secreted IL-6 protein, IL-6 mRNA in Pin1-null peritoneal macrophages was only decreased at the early 3 hour time-point, but then recovered to quantities greater than those of WT cells by 6 hours. This result is consistent with recent data reported by Akiyama et al., which demonstrates a similar increase in IL-6 mRNA in LPS-stimulated peritoneal macrophages from Pin1-null mice (Akiyama et al, 2011). In contrast, no difference was detected in TNF $\alpha$  mRNA accumulation between WT and Pin1-null peritoneal macrophages (Figure 5). Because an early defect in IL-6 mRNA and protein was

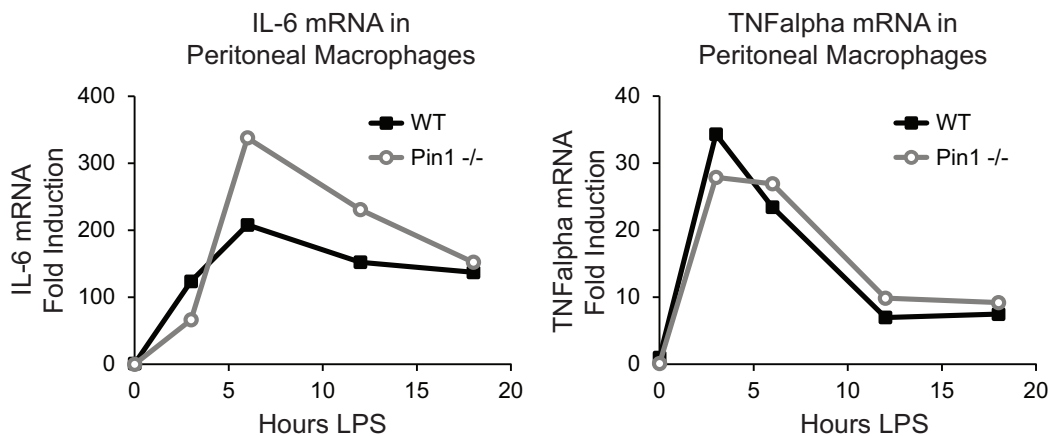
observed in Pin1-null cells, we questioned whether Pin1 might participate in early activation events in macrophages downstream of TLR4.

As peritoneal macrophages are few in number, it is difficult to obtain sufficient numbers of cells from each animal for thorough characterization of macrophage function. To further investigate the role of Pin1 in macrophage production and



**Figure 4: Secreted IL-6 in WT and Pin1-null Peritoneal Macrophages**

Peritoneal cells were obtained from 3 WT mice and 3 Pin1-null mice by peritoneal lavage. Cells from the 3 mice in each genotype group were pooled together and cultured for 24 hours. After 24 hours, non-adherent cells were removed to obtain an enriched population of adherent macrophages. Adherent cells were then stimulated with LPS for 3, 6, or 12 hours and the media was collected for analysis by ELISA at each time.

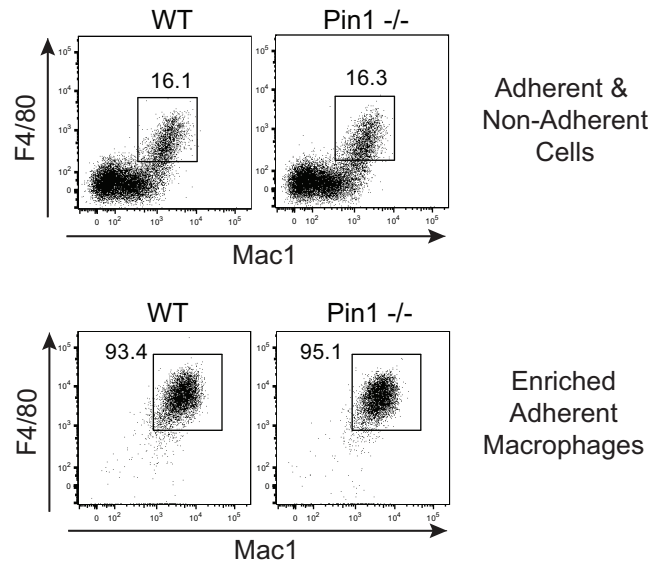


**Figure 5: IL-6 and TNFalpha mRNA in LPS-stimulated WT and Pin1-null Peritoneal Macrophages**

Peritoneal cells were obtained from 3 WT mice and 3 Pin1-null mice by peritoneal lavage. Cells from the 3 mice in each genotype group were pooled together and cultured for 24 hours. After 24 hours, non-adherent cells were removed to obtain an enriched population of adherent macrophages. Adherent cells were then stimulated with LPS for 3, 6, or 12 hours, at which point cells were lysed and RNA was purified and analyzed by RT-qPCR for the expression of IL-6 and TNFalpha.

activation, bone marrow-derived macrophages were generated. It is well-established that culturing bone marrow cells for 5-7 days in the presence of macrophage colony-stimulating factor (M-CSF) will induce the development of macrophages from progenitors present within the bone marrow (Zhang et al, 2008b). Because macrophages are adherent cells, removal of non-adherent cells enables the enrichment of macrophages, which remain attached to the bottom of the culture dish. The ability of Pin1-null bone marrow to give rise to macrophages was examined by collecting both adherent and non-adherent cells after 5-7 days of culture with M-CSF, and then staining cells with the macrophage markers F4/80 and Mac1. Pin1 does not appear to be required for the development of bone marrow-derived macrophages, as the frequencies of WT

and Pin1-null BMM were similar (Figure 6). When non-adherent cells were removed and only adherent cells were stained, macrophages were enriched as expected, comprising over 90% of the total cells recovered (Figure 6).

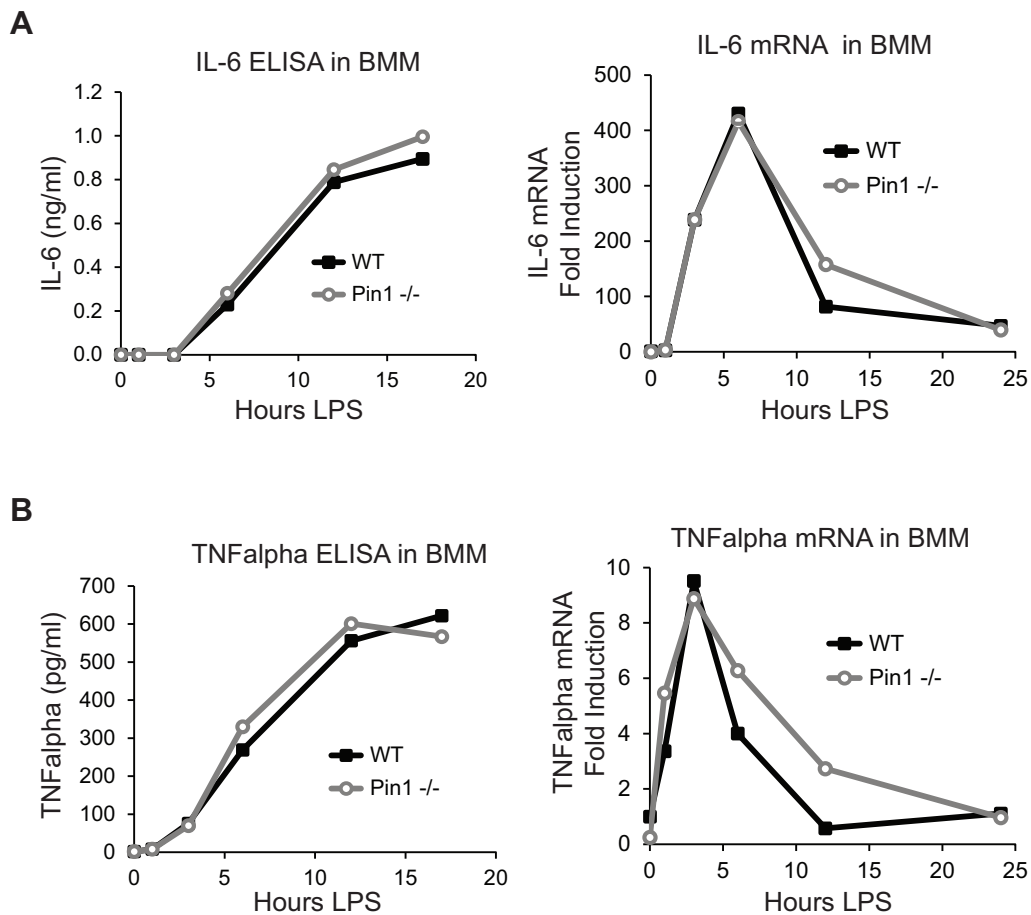


**Figure 6: Pin1 is Dispensable for Generating Bone Marrow Macrophages**

Bone marrow was obtained from WT and Pin1-null mice, depleted of red blood cells, and put into culture with M-CSF to induce macrophage development. After 5-7 days in culture, cells were collected, stained, and analyzed by flow cytometry for the presence of macrophages, which express both F4/80 and Mac1. Representative FACS plots are shown.

BMM were then assessed for the ability to produce the pro-inflammatory cytokines IL-6 and TNF $\alpha$  upon stimulation with LPS. After 5-7 days in culture with M-CSF, non-adherent cells were removed and the macrophage-enriched adherent population was then cultured in the presence of LPS for 3-17 hours. At each time point, media was collected and assayed for IL-6 and TNF $\alpha$  protein content by ELISA. No difference was observed in the ability of WT and Pin1-null BMM to secrete IL-6 or TNF $\alpha$

proteins into the media (Figure 7). To further confirm the absence of a defect cytokine secretion, mRNA was harvested from adherent BMM 3-24 hours after the addition of LPS. Consistent with the results obtained by ELISA, there was no defect in the ability of Pin1-null BMM to produce IL-6 or TNF $\alpha$  mRNA in response to LPS (Figure 7).

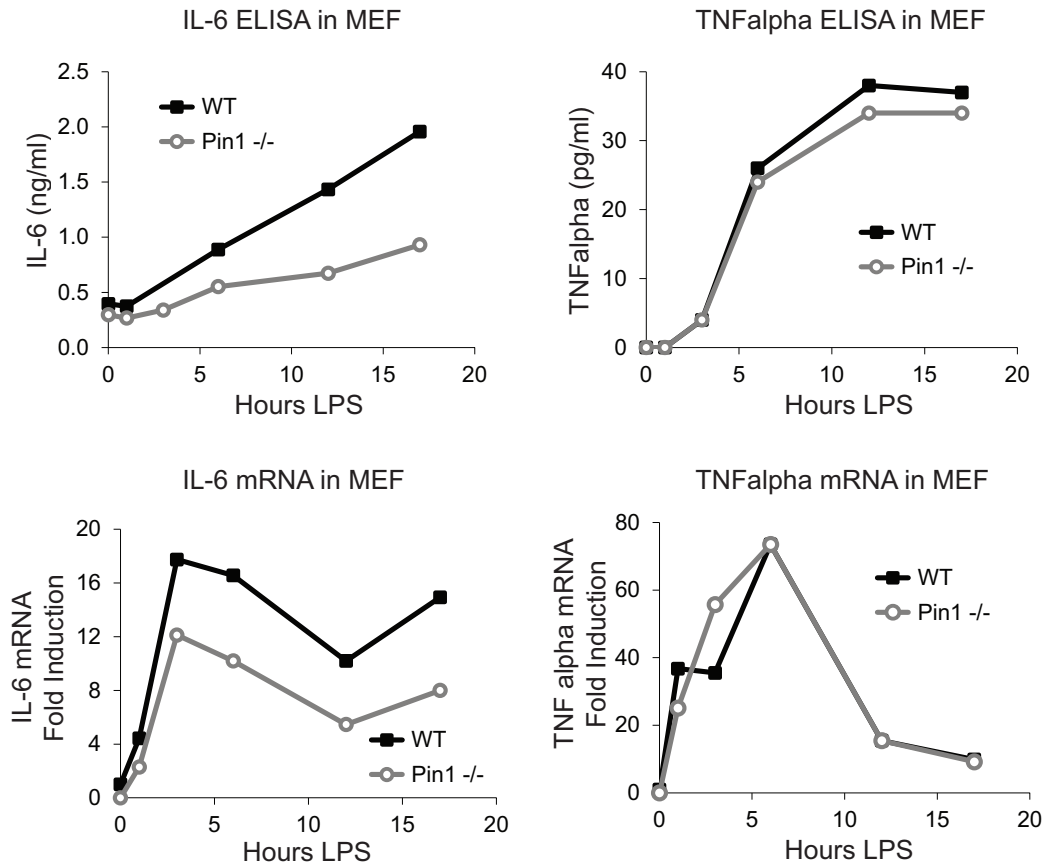


**Figure 7: Loss of Pin1 Does Not Impair IL-6 or TNF $\alpha$  Production in Bone Marrow-Derived Macrophage (BMM)**

(A) and (B) Red blood cell-depleted bone marrow from WT and Pin1-null mice was cultured in the presence of M-CSF for 5-7 days. Adherent macrophages were then stimulated with LPS for 3 to 17 hours. At each time point, media was collected for analysis of secreted IL-6 or TNF $\alpha$  by ELISA, and cells were collected for purification of RNA for RT-qPCR analysis of IL-6 and TNF $\alpha$  mRNA expression.

### 3.2.3 Cytokine Production in Mouse Embryo Fibroblasts

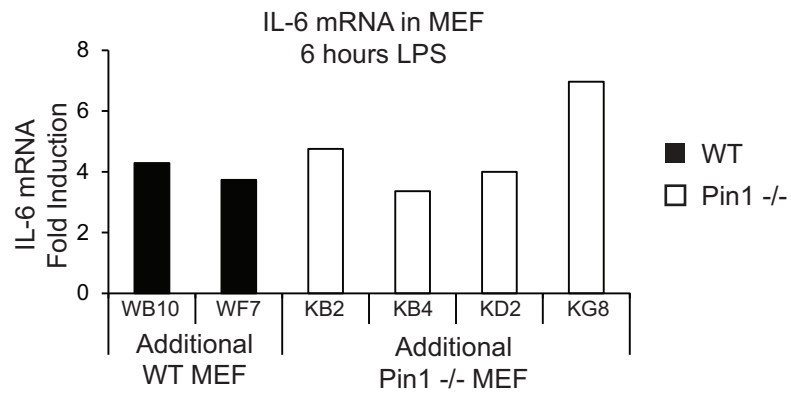
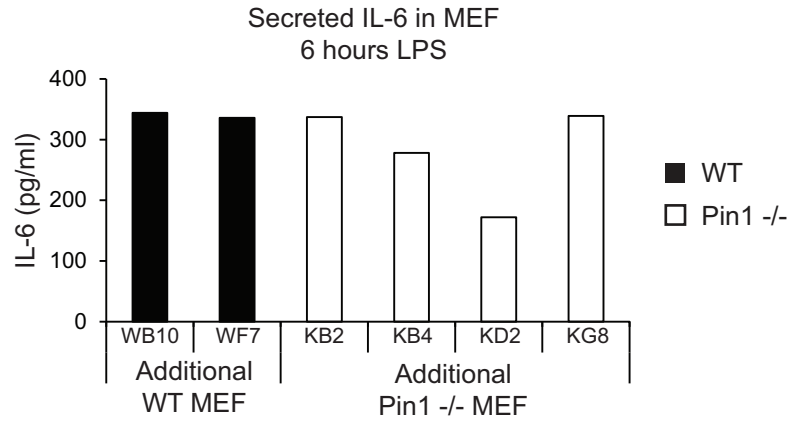
In addition to examining peritoneal macrophages and bone marrow-derived macrophages, primary mouse embryo fibroblasts (MEF) were also utilized to investigate the role of Pin1 in LPS-induced cytokine production. These cells express TLR4 and have been shown to produce IL-6 and TNF $\alpha$  when exposed to LPS (Kurt-Jones et al, 2004). Furthermore, in contrast to macrophages, MEF lack IL-6R $\alpha$ , which prevents further enhancement of IL-6 transcription that occurs downstream of signaling events initiated by IL-6 binding to the IL-6R $\alpha$ /gp130 heterodimeric receptor complex (Vanden Berghe et al, 2000). Thus, MEF provided an opportunity to investigate IL-6 production in a setting in which IL-6R signaling was not a contributing factor. Initial experiments in Pin1-null MEF indicated a specific defect in IL-6 mRNA induction and protein secretion, but not in TNF $\alpha$  mRNA and protein secretion (Figure 8). Subsequent experiments that were performed in MEF derived from additional embryos did not produce the same results; rather, it appears that there exists much heterogeneity in LPS-stimulated production of IL-6 between MEF derived from different embryos, whether they be WT or Pin1-null (Figure 9).



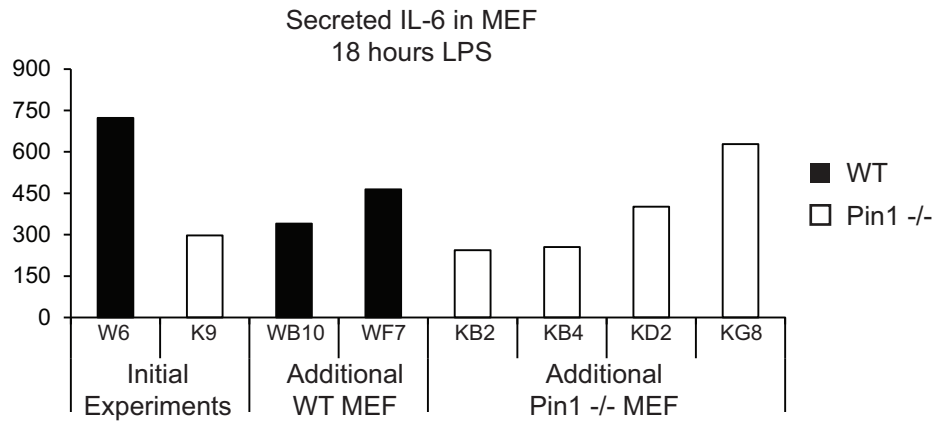
**Figure 8: Initial Results Indicate an Impairment in IL-6 Production in MEF Stimulated with LPS**

Primary WT and Pin1-null MEF were cultured to 85% confluency and then stimulated with 100ng/ml LPS for up to 17 hours. At each time point, media was collected for cytokine analysis via ELISA, and cells were harvested for RNA isolation and subsequent qRT-PCR analysis of cytokine mRNA expression.

**A**



**B**



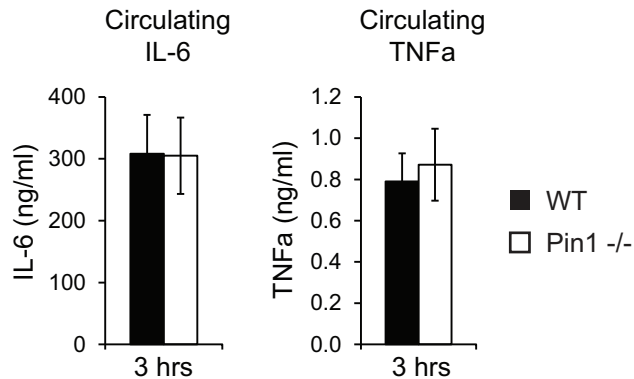
**Figure 9: LPS-Stimulated MEF Produce Variable Amounts of IL-6**

### **Figure 9 (Continued): LPS-Stimulated MEF Produce Variable Amounts of IL-6**

(A) and (B) MEF were generated from several embryos (each denoted below corresponding bar in graph; W denotes WT and K denotes Pin1 <sup>-/-</sup>). Cells were grown to 85% confluency and then stimulated with 100ng/ml LPS for 6 (A) or 18 (B) hours. Media was collected for IL-6 measurement by ELISA, and cells were collected for RNA isolation followed by qRT-PCR analysis of IL-6 mRNA.

### **3.2.4 Loss of Pin1 Does Not Impair Initial Production of Serum IL-6 or TNF $\alpha$ Upon Induction of Systemic Inflammation**

Having not found any indication of a defect in cytokine production in macrophages or MEF, mice were evaluated for the production of serum cytokines during the early response to LPS. Three hours after i.p. administration of LPS, blood was collected either by tail vein bleed or by cardiac puncture after euthanization, and analyzed by ELISA to determine the circulating concentrations of IL-6 and TNF $\alpha$ . In contrast to the decreases that had previously been observed 18 hours after LPS administration, there was no defect in circulating IL-6 or TNF $\alpha$  in Pin1-null mice 3 hours after LPS challenge (Figure 10). These results indicate that the initial cytokine response to LPS is not impaired in Pin1-null mice. The trend for decreased serum IL-6 levels after 18 hours LPS suggests that Pin1-null mice may be impaired in their ability to maintain cytokine levels, or that resolution of inflammation occurs more quickly in these animals. Due to insufficient numbers of similarly aged Pin1-null mice, it was not possible to perform a thorough analysis of the kinetics of serum cytokine production in response to LPS.



**Figure 10: Early Production of Circulating IL-6 and TNFalpha Unaltered in Absence of Pin1**

WT and Pin1-null mice were administered 15 mg/kg LPS reconstituted in HBSS. After 3 hours, blood was either collected by tail vein bleed, or mice were euthanized and blood was recovered by cardiac puncture. Plasma was prepared from tail vein blood, and serum was prepared from cardiac blood. Plasma and serum were analyzed for IL-6 and TNFalpha by ELISA

### 3.3 Discussion

In this chapter, we have investigated a role for Pin1 in modulating LPS-induced cytokine production in macrophages. Our initial observation that Pin1-null mice had decreased circulating cytokine levels relative to WT mice 18 hours after LPS challenge prompted us to search for a cellular defect that could account for such a difference.

When we isolated peritoneal cells from WT and Pin1-null mice, we found that Pin1-null mice exhibited an increase in the frequency of granulocytes, and a corresponding decrease in the frequency of macrophages. Because the frequencies of these populations are relative to each other, we could not distinguish between the existence of a defect in granulocytes versus macrophages. We further performed CBC analysis and found no

indication of a defect in the number of steady-state neutrophils, the main constituent of granulocyte cells. As granulocytes are difficult to manipulate and culture *ex vivo*, and are not thought to contribute greatly to the production of serum cytokines, we turned out attention to macrophages.

Although both IL-6 and TNF $\alpha$  were reduced in the serum of Pin1-null mice 18 hours after LPS challenge, the mRNA transcripts of these two cytokines were produced to similar extents in LPS-stimulated WT and Pin1-null peritoneal macrophages. Consistent with mRNA expression data, secreted IL-6 protein was similar in WT and Pin1-null peritoneal macrophages. These results were further confirmed by generating macrophages from WT and Pin1-null bone marrow cultures and measuring cytokine production after LPS stimulation. Significantly, we determined that Pin1-null bone marrow could produce macrophages to the same extent as WT bone marrow, which had never been described. Very recently, however, another group generated bone marrow macrophages from WT and Pin1-null mice for the purpose of examining their ability to produce cytokines in response to various TLR ligands. The authors did not comment on the ability of Pin1-null bone marrow to produce BMM, nor were the methods used to produce BMM described (Tun-Kyi, 2011).

In agreement with results from peritoneal macrophages, loss of Pin1 did not impact the ability of BMM to produce IL-6 or TNF $\alpha$  in response to LPS stimulation. Collectively, our results indicate that Pin1 is dispensable for the LPS-induced production

of IL-6 and TNF $\alpha$  in primary macrophages. Because MEF express the LPS-responsive TLR4, but lack a complete IL-6R complex, we also investigated cytokine production using these cells. Although we initially observed a defect in IL-6 mRNA and protein expression in Pin1-null MEF, we determined that this was not a true difference, as revealed by the variability that existed between MEF derived from separate embryos. Based on these results, we conclude that Pin1 is not required for LPS-induced secretion of the pro-inflammatory cytokines IL-6 and TNF $\alpha$  in macrophages. Although the recent report by Tun-Kyi et al. indicates a modest decrease in LPS-stimulated IL-6 production in Pin1-null BMM, no statistical analysis was performed and the authors concluded that Pin1-null macrophages were unimpaired. Our results are consistent with this conclusion.

Serum cytokines were initially measured 18 hours after the injection of high dose LPS in mice. This time point, however, is not ideal for measuring cytokines, as the concentrations of many pro-inflammatory mediators are in decline (Srinivasan et al, 2010; Turnbull et al, 2005). To determine whether defects in cytokine production exist at early time points, we collected blood from WT and Pin1-null mice 3 hours after LPS challenge. At this time, we were unable to detect a decrease in either serum IL-6 or TNF $\alpha$ . This result suggests that the initial production of cytokines, which is predominantly dependent on macrophages, remains unimpaired in the absence of Pin1. The inability of Pin1-null mice to maintain circulating cytokine levels is indicative of a

separate defect. In chapter 4, we investigate additional cell populations in both the innate and adaptive immune system that participate in the LPS-induced immune response and could potentially contribute to decrease in additional circulating cytokine levels observed in Pin1-null mice.

## **4. Pin1 Modulates Development of CD8+ Conventional Dendritic Cells**

### **4.1 Preface**

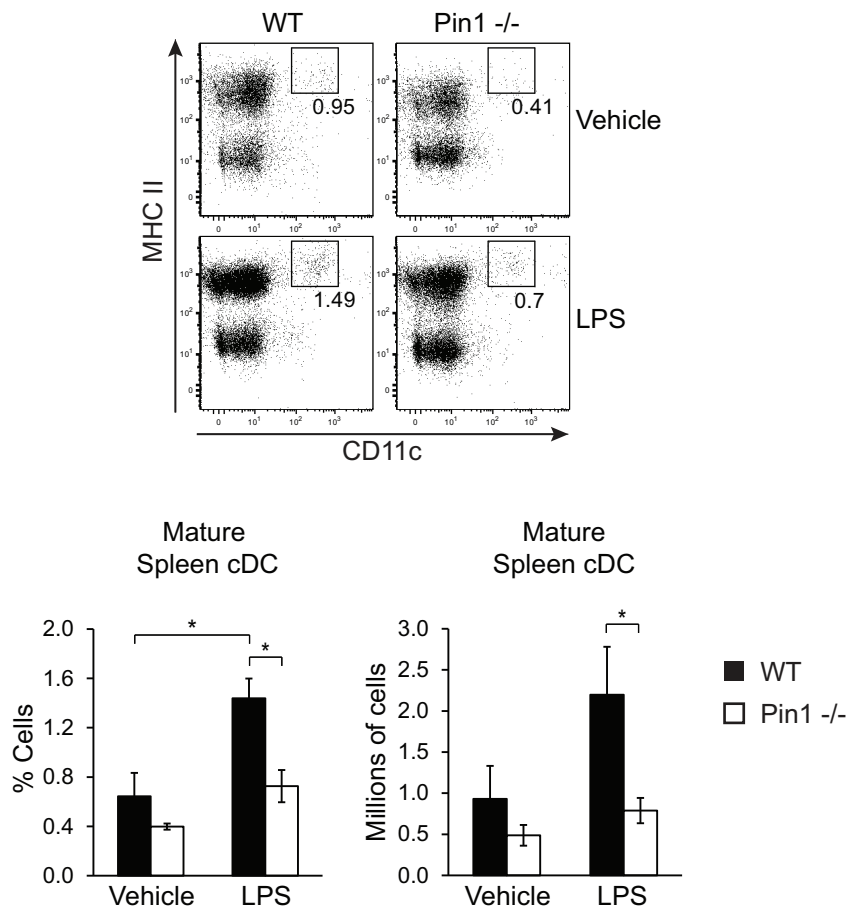
Deregulation of immune responses can result from both impaired cell function as well as impaired cell accumulation. In this chapter, we further investigate cellular defects that have the potential to impact systemic inflammatory responses. Of particular interest were conventional dendritic cells (cDC), which are particularly adept at stimulating adaptive immune responses, and T cells, which are directly activated by cDC. In this chapter, we identify a defect in the accumulation of spleen cDC in Pin1-null mice. We then perform experiments both *in vivo* and *ex vivo* and identify a role for Pin1 in modulating cDC development. To determine whether the observed defects in Pin1-null mice are capable of influencing T cell responses, we infect mice with bacteria and measure T cell proliferation. Finally, we perform biochemical analysis of WT and Pin1-null cells and identify a relevant aberration in the expression of a hematopoietic transcription factor that has been described to modulate DC development.

### **4.2 Experimental Results**

#### **4.2.1 Pin1-null Mice Fail to Accumulate Spleen cDC In Response to LPS Challenge**

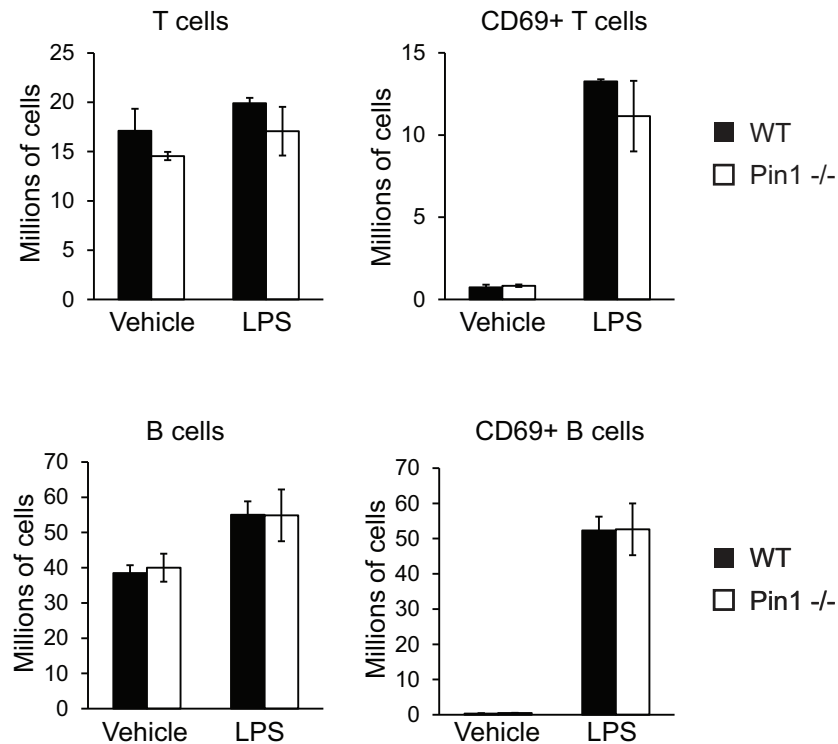
LPS administration induces a variety of cellular responses. In addition to macrophage activation and cytokine secretion, exposure to LPS will activate cDC, which also express TLR4. Because cDC serve as potent antigen presenting cells that induce

activation of naïve T cells (Coquerelle & Moser, 2010; Pulendran et al, 1999), it was of interest to examine the activation of cDC, T cells, and B cells in the spleens of LPS-challenged WT and Pin1-null mice. Eighteen hours after LPS administration, spleens were removed and splenocytes were stained for markers of cDC, T cells, and B cells. Upon analysis, Pin1-null mice were found to be deficient in the accumulation of spleen cDC, which are identified by high expression of both CD11c and MHC class II (Figure 11). We also noted that there was a slight trend for decreased cDC in Pin1-null mice that received vehicle. In contrast to cDC, the frequency of B cells and T cells were comparable between WT and Pin1-null mice. Furthermore, the activation marker CD69 was similarly expressed on B cells and T cells of both WT and Pin1-null mice (Figure 12). Although a role for Pin1 in modulating cDC was recently hinted to by results generated in bone marrow-derived DC (Tun-Kyi, 2011), this possibility remained an open question. Furthermore, detailed analysis of cDC subsets has not been performed in Pin1-null mice. We were therefore interested in examining steady-state production of DC subsets in mice lacking Pin1 to determine whether impaired LPS-stimulated cDC accumulation resulted from a basal defect in DC production.



**Figure 11: Pin1-null Mice Fail To Accumulate Mature Spleen cDC**

WT and Pin1-null mice were administered either HBSS (vehicle) or 15 mg/kg LPS reconstituted in HBSS by i.p. injection. After 18 hours, spleens were removed and splenocytes were stained for markers of mature cDC, which are MHC class II bright and CD11c bright.



**Figure 12: T cells and B cells Accumulate to the Same Extent in WT and Pin1-null Mice After LPS Challenge**

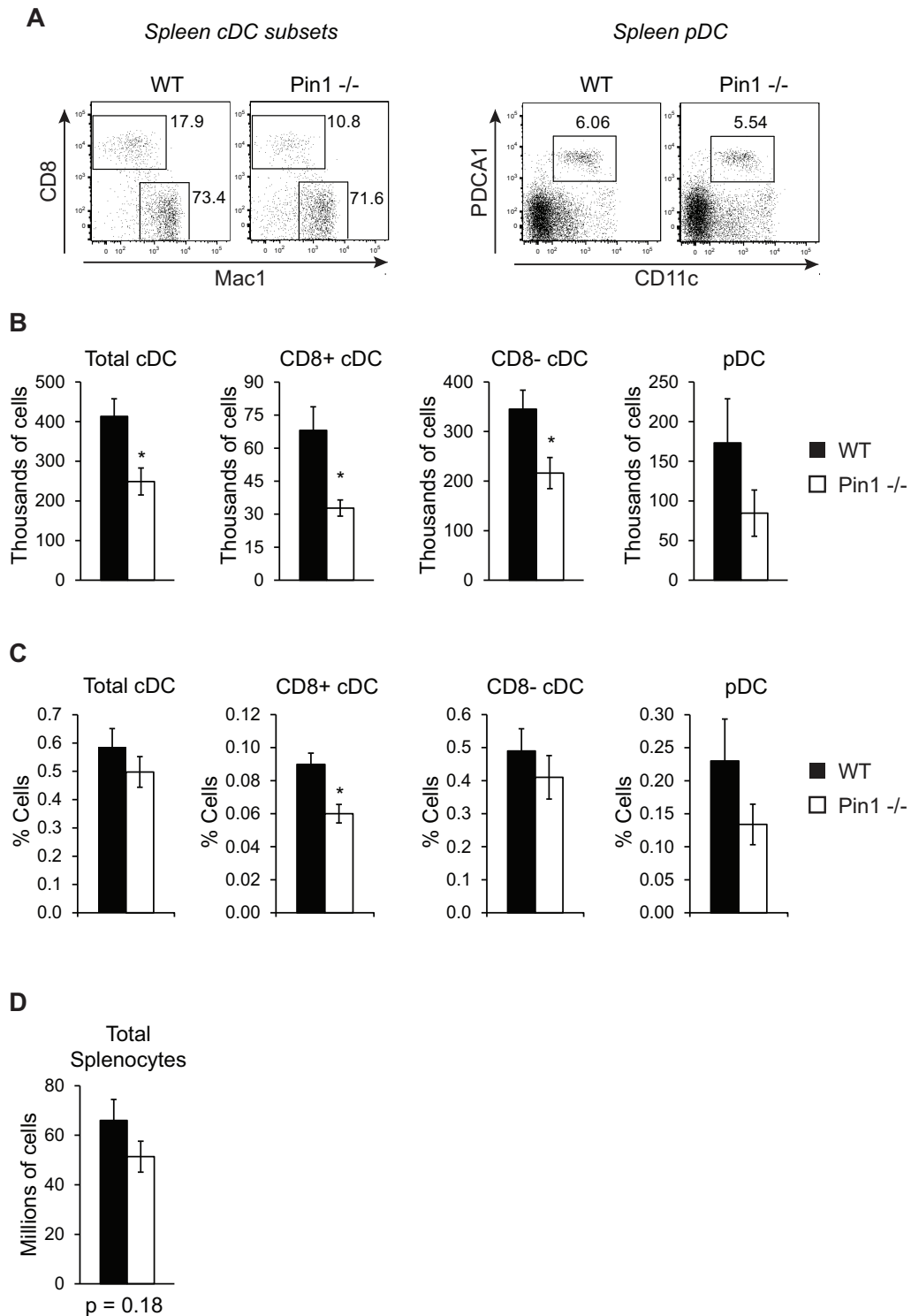
WT and Pin1-null mice were administered HBSS (vehicle) or 15 mg/kg LPS reconstituted in HBSS. After 18 hours, spleens were removed and splenocytes were stained for markers of T cells and B cells.

#### 4.2.2 Impaired Production of Steady-state Spleen cDC in Pin1-null Mice

To determine whether Pin1-null mice possess fewer steady-state spleen cDC, spleens were harvested from healthy WT and Pin1-null mice and stained for multiple DC populations. Pin1-null mice harbored a significant decrease in the number of both the CD8<sup>+</sup> and CD8<sup>-</sup> subsets of spleen cDC, with the greatest defect in the CD8<sup>+</sup> subset, which is decreased 50% compared to WT cells (Figure 13B). Upon examining the

frequency of these populations, however, we encountered a slightly different result. While the frequency of Pin1-null CD8<sup>+</sup> cDC remained significantly decreased compared to WT cells, there was not a significant decrease in the frequency of Pin1-null CD8<sup>-</sup> cDC (Figure 13A, Figure 13C). The discrepancy between total number and frequency of CD8<sup>-</sup> cDC may be explained by the observation that Pin1-null mice tend to have fewer splenocytes than WT mice (Figure 13D). Although this trend does not reach statistical significance, when coupled to a trend for decreased frequency, it produces a significantly different total number. Pin1-null mice also exhibited a decrease in both the number and frequency pDC but neither of these differences was statistically significant (Figure 13A-C). Despite our uncertainty regarding the existence of a defect in Pin1-null CD8<sup>-</sup> cDC, the data clearly indicated that the absence of Pin1 disrupts the ability of CD8<sup>+</sup> cDC to populate the spleen under steady-state conditions.

We next examined a potential role for Pin1 in cDC development by injecting mice with FL and measuring the resulting expansion of DC subsets. Mice were injected with 1 $\mu$ g of FL for 9 consecutive days, as has previously been described (Rathinam et al, 2005). On day 10, splenocytes were stained and DC populations were quantified. Pin1-null mice were unable to expand the CD8<sup>+</sup> subset of cDC to the same extent as WT mice. The FL-induced accumulation of CD8<sup>-</sup> cDC, however, was comparable between WT and Pin1-null mice (Figure 14). This result is consistent with the absence of a decrease in the frequency of steady-state CD8<sup>-</sup> cDC in Pin1-null mice (Figure 13C). Similar to what was

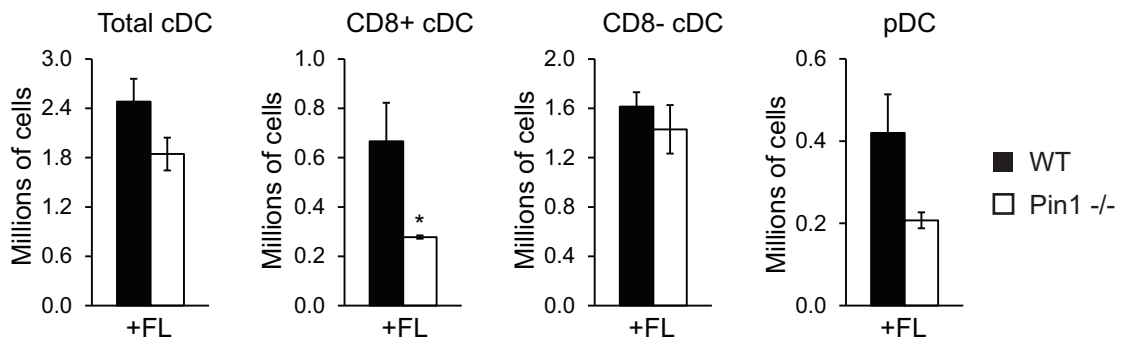


**Figure 13: Pin1-null Mice Are Deficient in Production of Steady-State cDC**

### Figure 13 (Continued): Pin1-null Mice Are Deficient in Production of Steady-State cDC

Splenocytes from healthy WT and Pin1-null mice were stained and analyzed for the presence of dendritic cell populations. (A) Representative FACS plots showing gating of cDC and pDC. (B) Quantitation of the numbers of spleen DC subsets. Cell population numbers were determined by multiplying the frequency of the cell population by the total number of splenocytes obtained from each mouse (n=6). (C) Frequency of dendritic cell populations as a percentage of total spleen cells. (D) Graph showing the total number of splenocytes.

observed in the steady-state, there was also a tendency for FL-treated Pin1-null mice to accumulate fewer pDC, but again this difference does not reach statistical significance (Figure 14). Taken together, these results indicate that Pin1 modulates the accumulation of CD8<sup>+</sup> cDC both in the steady-state and under conditions that induce massive DC expansion *in vivo*.

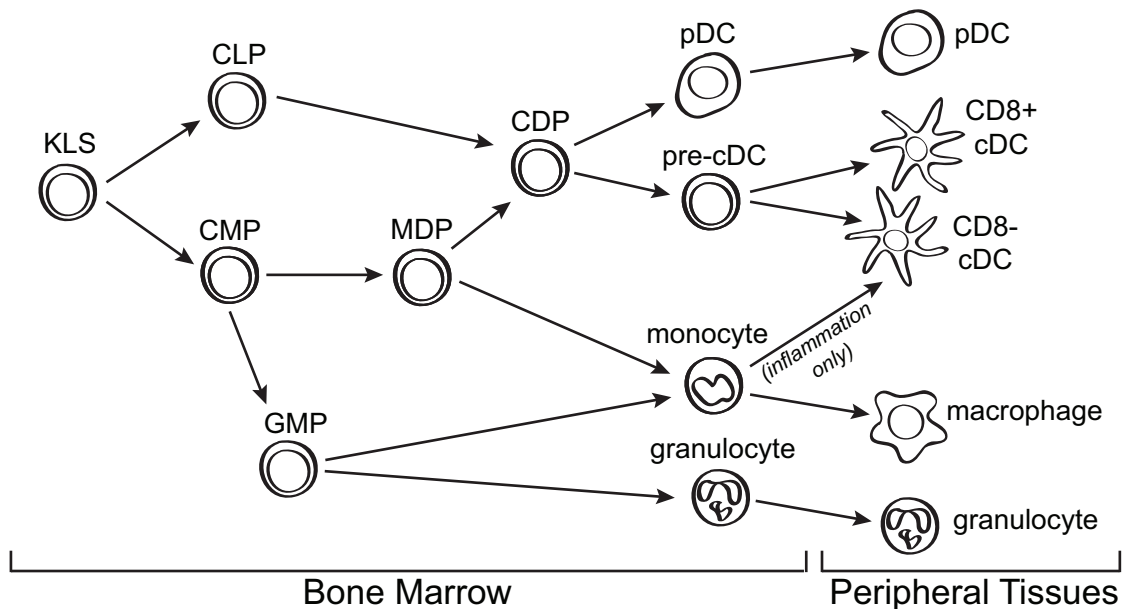


### Figure 14: Pin1-null Mice Are Deficient in Flt3 Ligand-induced Expansion of CD8<sup>+</sup> cDC

WT and Pin<sup>-</sup> null mice were administered 1  $\mu$ g of Flt3 Ligand (FL) for 9 consecutive days. On day 10, spleens were collected and splenocytes were stained and analyzed for the presence of DC subsets. Total cell numbers were calculated by multiplying the frequency of each cell population by the total number of splenocytes obtained from each mouse.

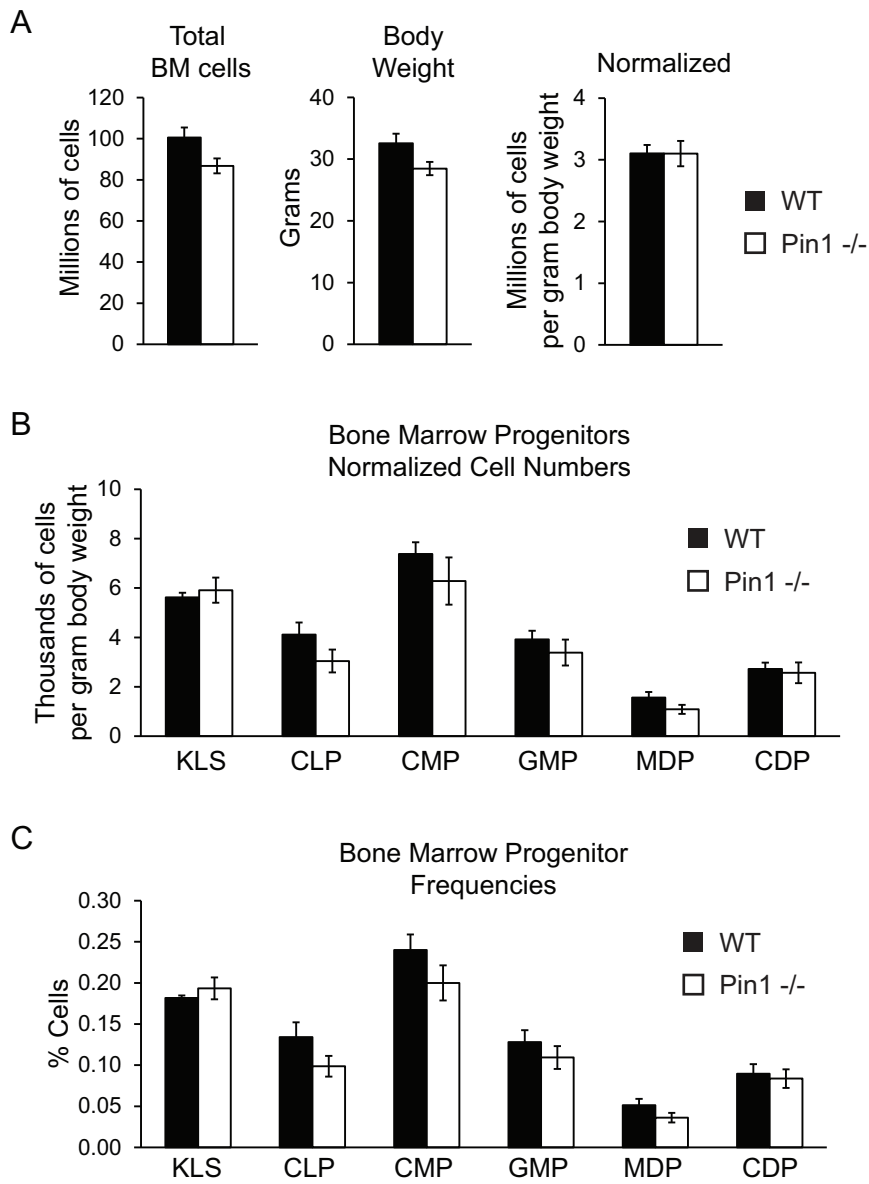
### 4.2.3 Bone Marrow Progenitors are Unaltered in Pin1-null Mice

DC develop from hematopoietic progenitors in the bone marrow that transition through several stages of development, becoming increasingly committed to one particular fate with each subsequent step (Figure 14). To address whether defects existed in bone marrow progenitors of Pin1-null mice that could account for the changes observed in the spleen, bone marrow cells from WT and Pin1-null mice were stained



**Figure 15: Schematic of Dendritic Cell Development**

KLS, ckit+Lin-Sca1+ stem cells; CLP, Common Lymphoid Progenitor; CMP, Common Myeloid Progenitor; GMP, Granulocyte-Monocyte Progenitor; MDP, Monocyte-DC Progenitor; CDP, Common DC Progenitor.



**Figure 16: Bone Marrow Progenitors in Pin1-null Mice**

(A-C) Red blood cell-depleted bone marrow from WT and Pin1-null mice was counted, stained, and analyzed for markers of multiple cell populations. (A) Bone marrow cells were counted and normalized to total body weight of each animal. (B) Total numbers were calculated by multiplying total bone marrow cells by the frequency of each population. (C) Frequencies of each bone marrow population.

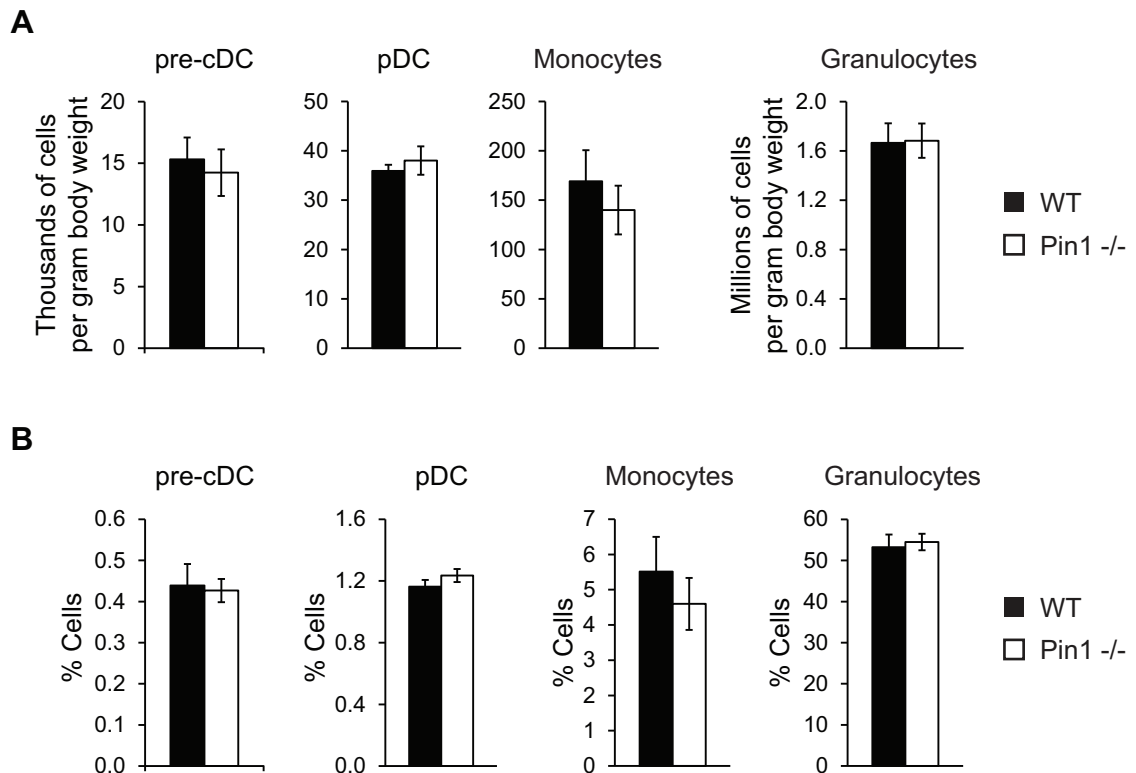
and analyzed for the presence of progenitor populations. As noted with the number of splenocytes, Pin1-null mice exhibited reduced numbers of bone marrow cells. When corrected for differences in total body weight, however, these differences no longer existed (Figure 16A). Upon normalizing by body weight, no defects in the number of Pin1-null bone marrow progenitors were detected (Figure 16B). These results are consistent with the frequencies of bone marrow progenitors, which are also unaltered in Pin1-null mice (Figure 16C).

pDC fully develop within the bone marrow, while pre-cDC leave the bone marrow and travel to peripheral tissues where they undergo the final steps of development to give rise to CD8<sup>-</sup> or CD8<sup>+</sup> cDC (Figure 15). To determine whether defects existed in these two populations, bone marrow cells were also stained with markers of pre-cDC and pDC. Consistent with an absence of defects in bone marrow progenitors, neither of these populations was perturbed in Pin1-null mice, either in number or frequency (Figure 17). The absence of a defect in Pin1-null bone marrow pDC is interesting in light of the trend to have fewer spleen pDC, and suggests that changes in spleen pDC number are not the result of impaired development, but may instead arise from a separate defect.

Granulocytes and monocytes share common progenitors and developmental patterns with DC. Granulocytes, like pDC, fully develop within the bone marrow compartment. Monocytes, like pre-cDC, arise in the bone marrow, but undergo the final

steps of development in peripheral tissues. For these reasons, we examined the presence of both granulocytes and monocytes in the bone marrow. We found no evidence for a defect in either of these bone marrow populations in Pin1-null mice (Figure 17).

These data indicate that the loss of Pin1 is inconsequential to stages of DC development that take place in the bone marrow. This is further supported by the lack



**Figure 17: Bone Marrow Cell Populations in Pin1-null Mice**

(A) and (B) Red blood cell-depleted bone marrow from WT and Pin1-null mice was counted, stained, and analyzed for markers of multiple cell populations. (A) Total numbers were calculated by multiplying total bone marrow cells by the frequency of each population. (B) Frequencies of each bone marrow population.

of defects in cells with shared common progenitors, such as granulocytes and monocytes. Collectively, these results indicate that the decreased numbers of spleen DC observed in Pin1-null mice are not the result of early defects in bone marrow progenitors, but instead must result from defects that occur in the periphery.

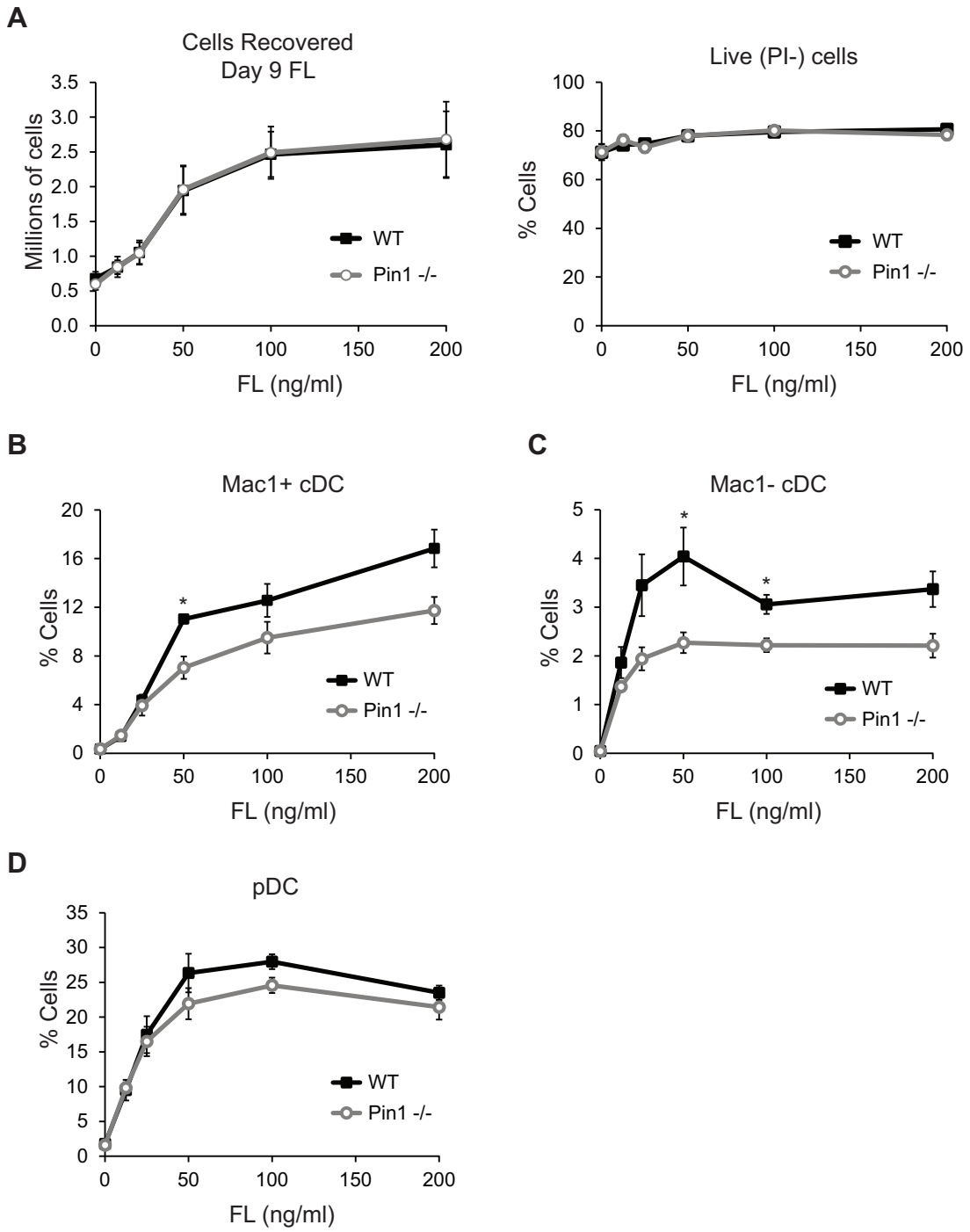
#### **4.2.4 Loss of Pin1 Impairs the Development of Steady-state cDC in *ex vivo* Cultures of Bone Marrow Cells**

It is well-established that bone marrow cells from mice can be cultured *ex vivo* in the presence of FL to generate bone marrow-derived dendritic cells (FL-BMDC) that are functionally equivalent to *in vivo* steady-state DC populations. Despite their similarities, however, bone marrow-derived cDC equivalents do not express CD8, and must instead be distinguished from each other by differential expression of the myeloid marker Mac1. Thus, the FL-BMDC equivalent of the CD8<sup>+</sup> cDC subset is Mac1<sup>-</sup> and the FL-BMDC equivalent of CD8<sup>-</sup> cDC subset is Mac1<sup>+</sup> (Naik et al, 2005). Because we observed differences in both steady-state and FL-expanded spleen cDC populations in Pin1-null mice, and these defects did not appear to be the consequence of reduced DC bone marrow progenitors, we utilized the *ex vivo* bone marrow culture system to confirm a role for Pin1 in DC development.

FL is most commonly used at a concentration of 200 ng/ml to generate FL-BMDC, but the purpose is often to generate the greatest number of FL-BMDC possible for use in other assays. Since we were interested in examining the developmental process itself,

we began our investigation by culturing bone marrow cells with multiple concentrations of FL, ranging from 12.5 ng/ml to 200 ng/ml. After 9 days, we recovered the same number of cells from both WT and Pin1-null cultures, and found that the frequency of live cells, which was determined by exclusion of propidium iodide, was identical (Figure 18A). Staining the cells with markers of DC subsets revealed that Pin1-null bone marrow is less efficient at generating Mac1<sup>+</sup> and Mac1<sup>-</sup> cDC than WT bone marrow, but is fully capable of producing pDC (Figure 18B-D). These defects were detectable at multiple concentrations of FL. As differences in both Mac1<sup>+</sup> and Mac1<sup>-</sup> cDC could be detected at 50 ng/ml and because 50 ng/ml is a previously published concentration of FL that has been used to investigate DC development (Kashiwada et al, 2011), all subsequent FL-BMDC were generated using 50 ng/ml FL.

Upon repeating bone marrow culture experiments using 50 ng/ml FL for 9 days, we confirmed previously observed defects in the production of Mac1<sup>+</sup> and Mac1<sup>-</sup> subsets of Pin1-null cDC. Indeed, Pin1-null bone marrow exhibited a 50% reduction in the generation of Mac1<sup>-</sup> (CD8<sup>+</sup> equivalent) cDC, which mirrored what had been observed *in vivo* (Figure 19A, Figure 19D). The Mac1<sup>+</sup> (CD8<sup>-</sup> equivalent) subset, however, exhibited a more complex phenotype in the absence of Pin1. Rather than exhibiting a straight-forward difference in number, Pin1-null Mac1<sup>+</sup> cDC appear to express less CD11c than WT Mac1<sup>+</sup> cDC (Figure 19B). When gated on the brightest CD11c<sup>+</sup> cells, a significant decrease in bone marrow-derived Mac1<sup>+</sup> cDC can be



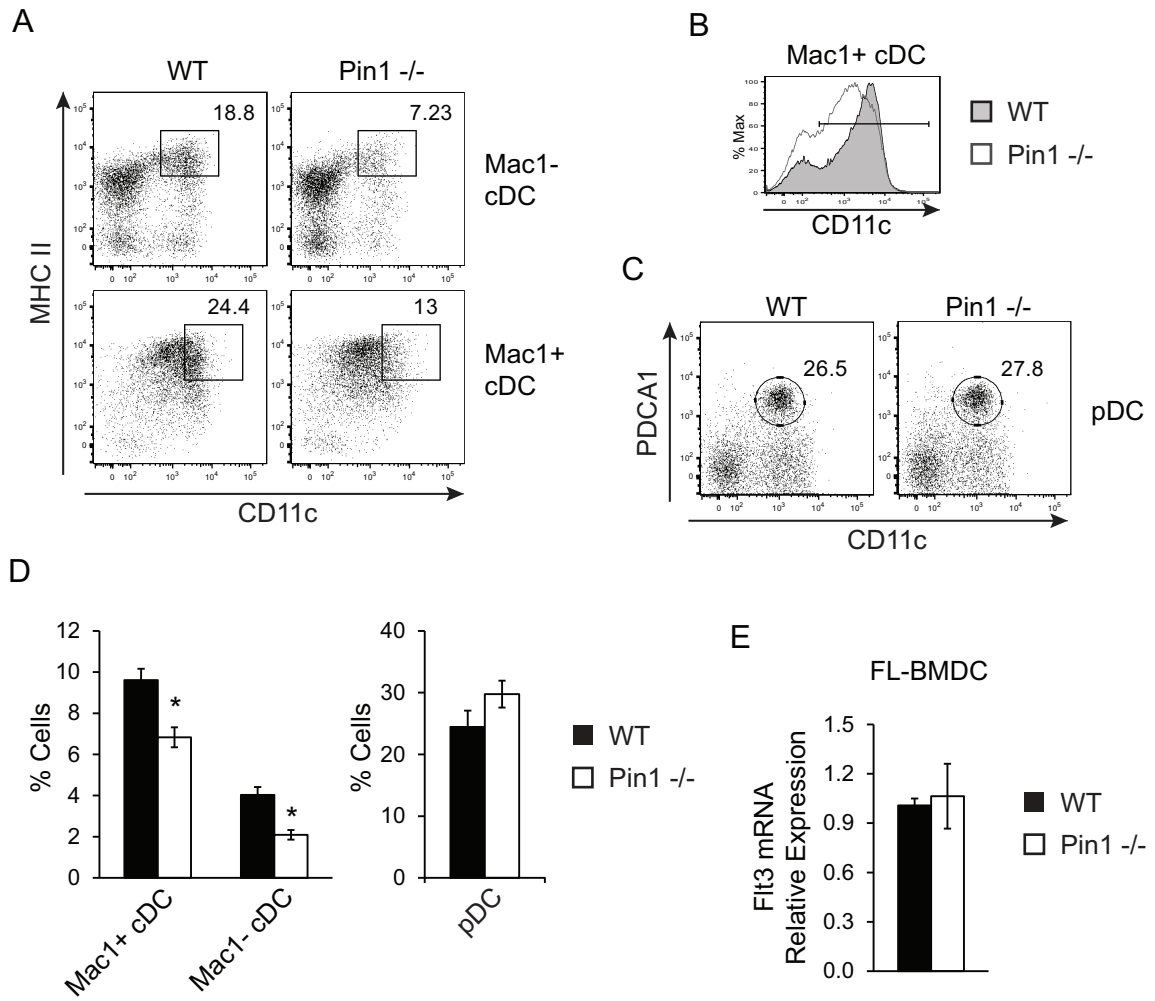
**Figure 18: FL Dose Response in Bone Marrow-Derived DC**

### Figure 18 (Continued): FL Dose Response in Bone Marrow-Derived DC

(A-D) Red blood cell-depleted bone marrow cells from WT and Pin1-null mice were cultured with increasing concentrations of FL for 9 days. On day 9, non-adherent cells were removed, counted, stained and analyzed for multiple DC populations. (A) Non-adherent cells were counted and stained with propidium iodide (PI), a marker of dead cells. (B-D) Frequencies of cDC and pDC populations generated by culturing bone marrow with increasing concentrations of FL.

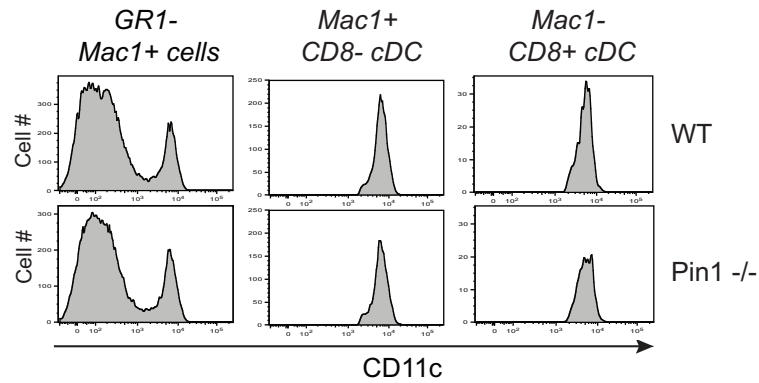
quantified (Figure 19A, Figure 19D). Because a similar decrease in CD11c expression was not observed in CD8<sup>-</sup> cDC *in vivo*, the significance of this observation remained uncertain (Figure 20). In contrast to cDC, the generation of pDC was unaltered in Pin1-null bone marrow cultures, indicating that Pin1 is not required for pDC development (Figure 19C, Figure 19D). To determine whether decreased production of cDC in Pin1-null bone marrow resulted from defects in the expression of Flt3, the receptor that binds FL, RT-qPCR was performed on FL-BMDC to quantify Flt3 mRNA. No differences were detected in Flt3 mRNA in the absence of Pin1, indicating that the observed defects in Pin1-null cDC are unlikely to result from decreases in Flt3 expression (Figure 19E).

cDC can also be produced by culturing bone marrow cells in the presence of the cytokine GM-CSF. Unlike FL, however, GM-CSF only generates the Mac1<sup>+</sup> subset of cDC. Furthermore, GM-CSF produces cDC with a more inflammatory phenotype, similar to monocyte-derived cDC that only arise under conditions of inflammation *in vivo* (Xu et al, 2007). Because we had observed a decrease in mature cDC in LPS-challenged Pin1-null mice, many of which would have been monocyte-derived inflammatory cDC, we investigated the ability of Pin1-null bone marrow to develop into



**Figure 19: Impaired cDC Generation in Pin1-null Bone Marrow Cultured with FL**

(A) and (C) Red blood cell-depleted bone marrow from WT and Pin1-null mice was cultured *ex vivo* with FL for 9 days. On day 9, non-adherent cells were removed, stained, and analyzed for multiple DC populations. Representative FACS plots are shown. (B) Representative histogram of CD11c expression in Mac1+ cDC from WT and Pin1-null mice. (D) Graphs of the frequencies of each bone marrow-derived DC population. (E) After 9 days in culture with FL, RNA was isolated from non-adherent bone marrow-derived DC. Fit3 mRNA expression was determined by RT-qPCR.



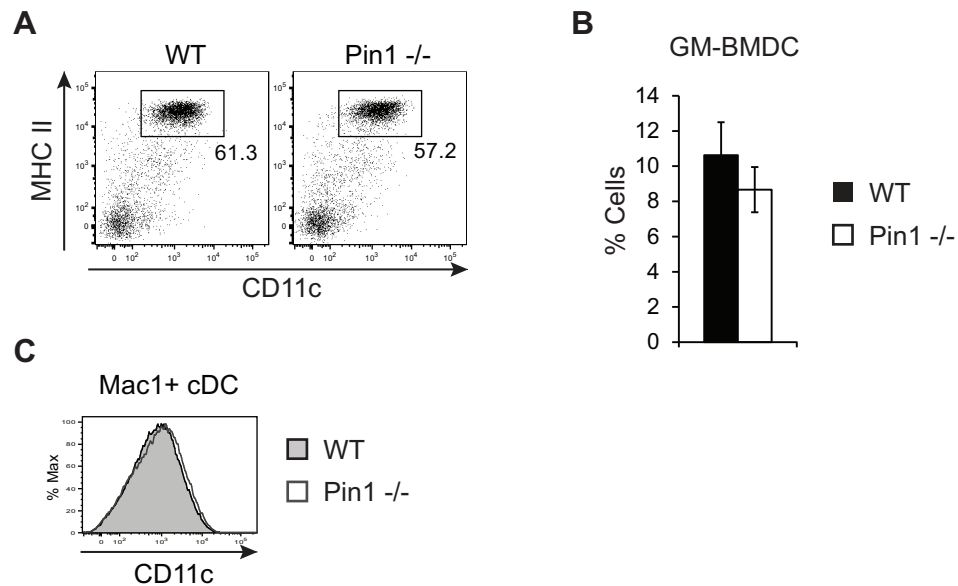
**Figure 20: CD11c Expression is Unaltered in Pin1-null Spleen cDC Subsets**

Spleens were removed from WT and Pin1-null mice. Splenocytes were stained and analyzed for various DC subsets. GR1-Mac1<sup>+</sup> cells were analyzed for CD11c expression for comparison to CD11c expression in bone marrow-derived Mac<sup>+</sup> cDC. Both CD8<sup>-</sup> and CD8<sup>+</sup> subsets of spleen cDC were also analyzed for CD11c expression.

Mac1<sup>+</sup> cDC when cultured *ex vivo* with GM-CSF. In contrast to what we observed upon culturing Pin1-null bone marrow with FL, we did not observe a difference in the ability of these cells to generate Mac1<sup>+</sup> cDC in the presence of GM-CSF (Figure 21A, Figure 21B). Furthermore, the absence of Pin1 did not alter the expression of CD11c in GM-CSF-generated BMDC (GM-BMDC) (Figure 21C). This result is consistent with our observations *in vivo* in which Pin1-null Mac1<sup>+</sup> cDC are not diminished in their expression of CD11c.

Collectively, results from BMDC support a role for Pin1 in modulating the production of Mac1<sup>-</sup> (CD8<sup>+</sup> equivalent) cDC. The development of pDC, however, is unperturbed in the absence of Pin1, indicating a specific role for Pin1 in cDC development. The decrease in the expression of CD11c observed in FL-generated Pin1-null Mac1<sup>+</sup> cDC is not consistent with the expression of CD11c in steady-state Mac1<sup>+</sup>

cDC *in vivo*. Additionally, when Pin1-null bone marrow was cultured with GM-CSF to produce Mac1+ cDC, this decrease in CD11c expression was not present. As FL produces DC that are equivalent to those found in the steady-state *in vivo*, results obtained from BMDC are consistent with a role for Pin1 in modulating steady-state CD8+ cDC development *in vivo*.



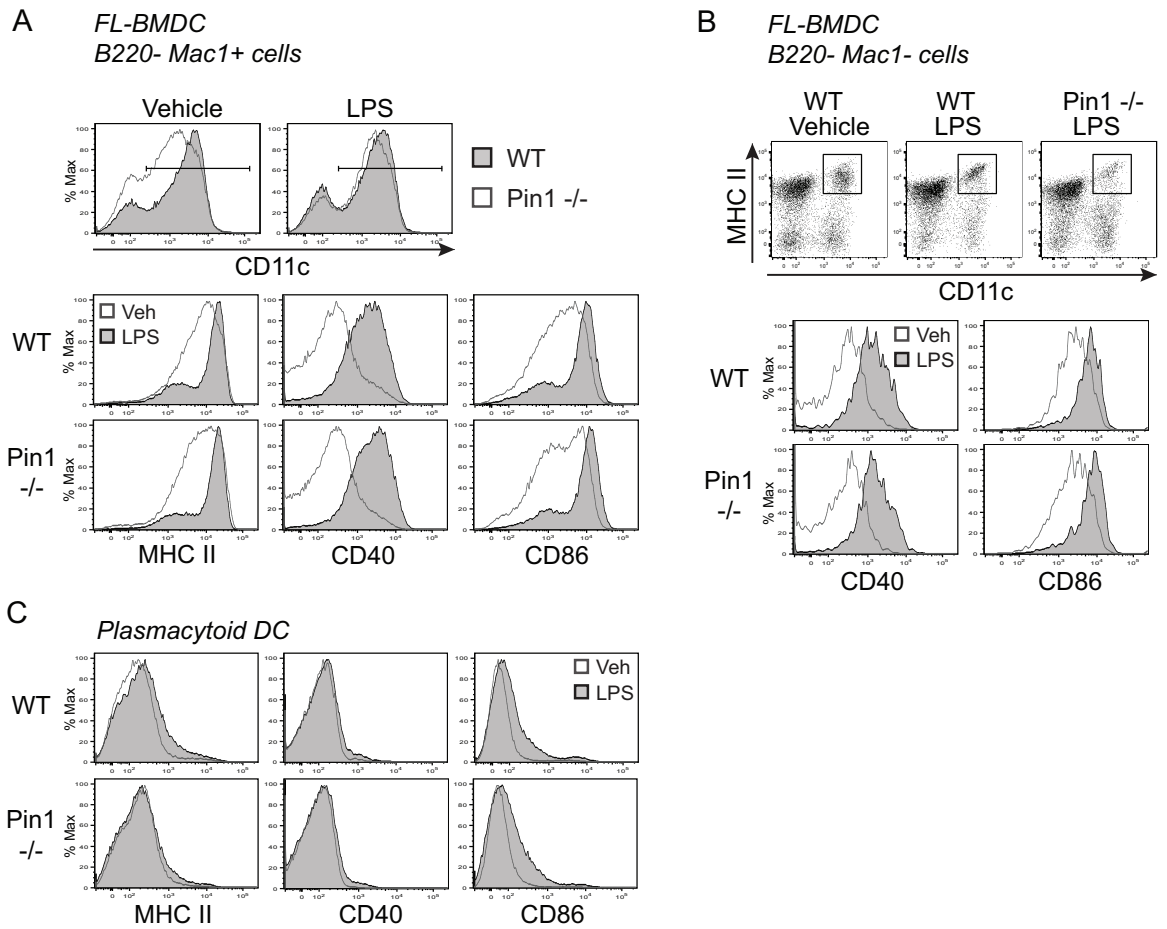
**Figure 21: Lack of Defect in Pin1-null Bone Marrow-Derived Mac1+ cDC Generated with GM-CSF**

(A-C) Red blood cell-depleted WT and Pin1-null bone marrow cells were cultured for 6 days with GM-CSF. On day 6, non-adherent cells were collected, stained, and analyzed for Mac1+ cDC. (A) Representative histogram of CD11c expression in Mac1+ cells. (B) Representative FACS plots of Mac1+ cDC. (C) Graph of the frequency of Mac1+ cDC in WT and Pin1-null bone marrow cultures.

#### **4.2.5 Pin1 is dispensable for LPS-induced MHC class II and co-stimulatory molecule expression**

Our data indicate that the loss of Pin1 deregulates CD8+ cDC development. Because activation-induced maturation of DC is necessary for stimulating T cells *in vivo*, FL-BMDC were assessed for their ability to up-regulate expression of co-stimulatory molecules, which is one indicator of DC maturation. After incubation with LPS for 24 hours, FL- BMDC were stained and analyzed for the expression of MHC class II and the co-stimulatory molecules CD40 and CD86, which are up-regulated upon maturation. Upon LPS stimulation, the defect in CD11c expression present in unstimulated Pin1-null Mac1+ cDC became undetectable; both WT and Pin1-null cells expressed CD11c to the same extent (Figure 22A). While the reason for decreased CD11c expression in FL cultures remains unclear, it does not appear that this defect extends to activated cDC, and is therefore unlikely to directly impact the ability of DC to stimulate adaptive immune responses.

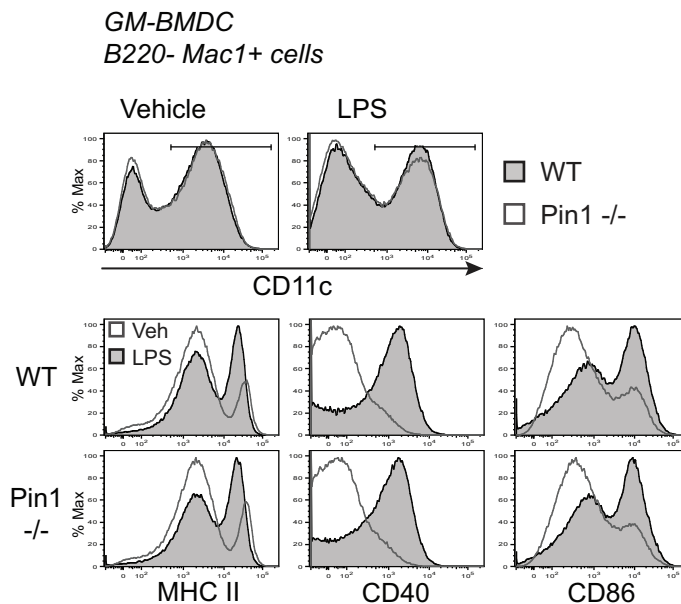
When analyzed for the expression of MHC class II, CD40 and CD86, both Mac1- and Mac1+ subsets of Pin1-null cDC expressed each of these molecules to the same extent as WT cells when stimulated with LPS (Figure 22A, Figure 22B). Neither WT nor Pin1-null pDC increased expression of these proteins, as was expected since pDC lack TLR4 and are unable to respond to LPS (Figure 22C). Thus, although there are fewer Pin1-null Mac1- cDC, those cells that develop are capable of up-regulating MHC class II and co-stimulatory molecule expression in response to LPS.



**Figure 22: Pin1 is Dispensable for Co-stimulatory Molecule Expression in Bone Marrow-derived DC Generated with FL**

(A-C) Red blood cell-depleted WT and Pin1-null bone marrow cells were cultured with FL for 9 days. On day 9, cells were stimulated with 100 ng/ml LPS for 24 hours, and then collected, stained, and analyzed by flow cytometry. (A) Top panel shows CD11c gates used to generate the histograms below; bottom panel includes representative histograms of MHC class II, CD40, and CD86 in Mac1+ cDC. (B) Top panel shows gate that was used to generate the histograms below; bottom panel includes representative histograms of CD40 and CD86 expression in Mac1- cDC. (C) Representative histograms of pDC that were previously gated as CD11c<sup>int</sup>PDCA1<sup>+</sup>

In addition to examining co-stimulatory molecule induction in FL-BMDC, we also examined co-stimulatory molecule expression in LPS-stimulated GM-BMDC. Consistent with results from FL-BMDC, the loss of Pin1 did not impact that ability of GM-BMDC to increase expression of MHC class II, CD40 or CD86 in the presence of LPS (Figure 23).



**Figure 23: Pin1 is Dispensible for Induction of Co-stimulatory Molecule Expression in Bone Marrow-Derived DC Generated with GM-CSF**

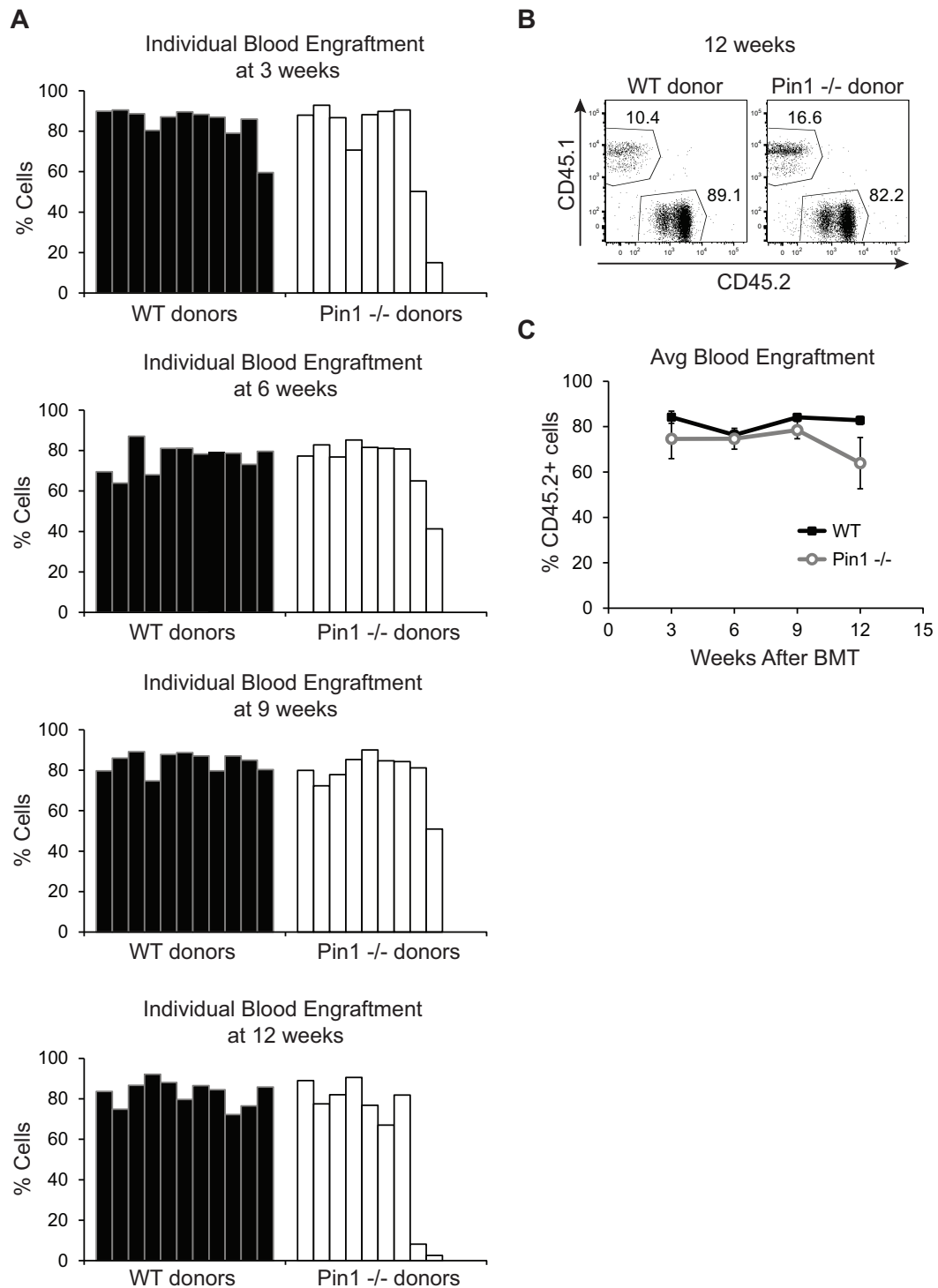
Red blood cell-depleted WT and Pin1-null bone marrow cells were cultured with FL for 9 days. On day 9, cells were stimulated with 100 ng/ml LPS for 24 hours, and then collected, stained, and analyzed by flow cytometry. Top panel shows CD11c gates used to generate the histograms below; bottom panel includes representative histograms of MHC class II, CD40, and CD86 in Mac1+ cDC.

## 4.2.6 Transplantation of WT and Pin1-null Bone Marrow Cells

Results from *ex vivo* bone marrow cultures pointed to a cell-intrinsic defect in cDC development in the absence of Pin1, as culture conditions were identical for both WT and Pin1-null cells. Furthermore, having analyzed bone marrow progenitors and pre-DC, to the best of our knowledge, the bone marrow cells put into culture were similar in population composition. To provide additional evidence of a cell-intrinsic defect in Pin1-null CD8<sup>+</sup> cDC, we performed a bone marrow transplant experiment in mice. WT recipients were lethally irradiated and were then reconstituted by injecting red blood cell-depleted bone marrow pooled from either 3 WT donors or 3 Pin1-null donors. Since we had no evidence that Pin1-null mice were deficient in producing bone marrow progenitors, whole bone marrow was injected into recipient mice; furthermore, bone marrow contains cDC precursors, and very few fully differentiated cDC, ensuring that a majority of the cDC detected in the periphery would arise from progenitors and precursors in the bone marrow. After 48 hours, 2 recipients of WT bone marrow and 2 recipients of Pin1-null bone marrow were sacrificed and bone marrow was collected and stained. Since donor mice expressed the CD45 allelic variant CD45.2, transplanted cells were easily distinguished from host-derived cells, which expressed CD45.1. Detection of CD45.2<sup>+</sup> cells in the bone marrow of recipients confirmed that transplanted cells derived from both WT and Pin1-null mice were able to home properly to the bone marrow (data not shown).

Mice were bled at 3, 6, 9, and 12 weeks post-transplant and then euthanized following the final bleed at week 12. Blood cells were stained and analyzed for the presence of CD45.2+ lymphocytes, pDC, and pre-cDC populations. Interestingly, Pin1-null bone marrow ultimately failed to engraft in two of the WT recipient mice, which exhibited less than 10% chimerism at the 12 week bleed. In contrast, none of the mice that received WT bone marrow exhibited defects in engraftment (Figure 24). While we cannot exclude the possibility that there was an error during the injection of Pin1-null bone marrow cells, it is tempting to speculate that this 20% failure rate is indicative of some defect present in Pin1-null bone marrow. The remaining 80% of mice that received Pin1-null bone marrow exhibited a similar frequency of CD45.2+ donor cells to those mice that received WT bone marrow.

When blood was analyzed for the presence of donor cell populations, we found that at 3 weeks, there was a significant decrease in the frequency of circulating pre-cDC derived from Pin1-null bone marrow (Figure 25). This decrease was not detected in blood collected at week 6, but at week 9, a small but statistically significant decrease in Pin1-null pre-cDC reappeared. At the 12 week bleed, this decrease in pre-cDC was again undetectable (Figure 25). Collectively, these data suggest that Pin1-null bone marrow may be delayed in its ability to reconstitute circulating pre-cDCs, as there were approximately 30% fewer Pin1-null cells than WT cells detected at the early 3 week bleed, but similar frequencies at the 6 week bleed.



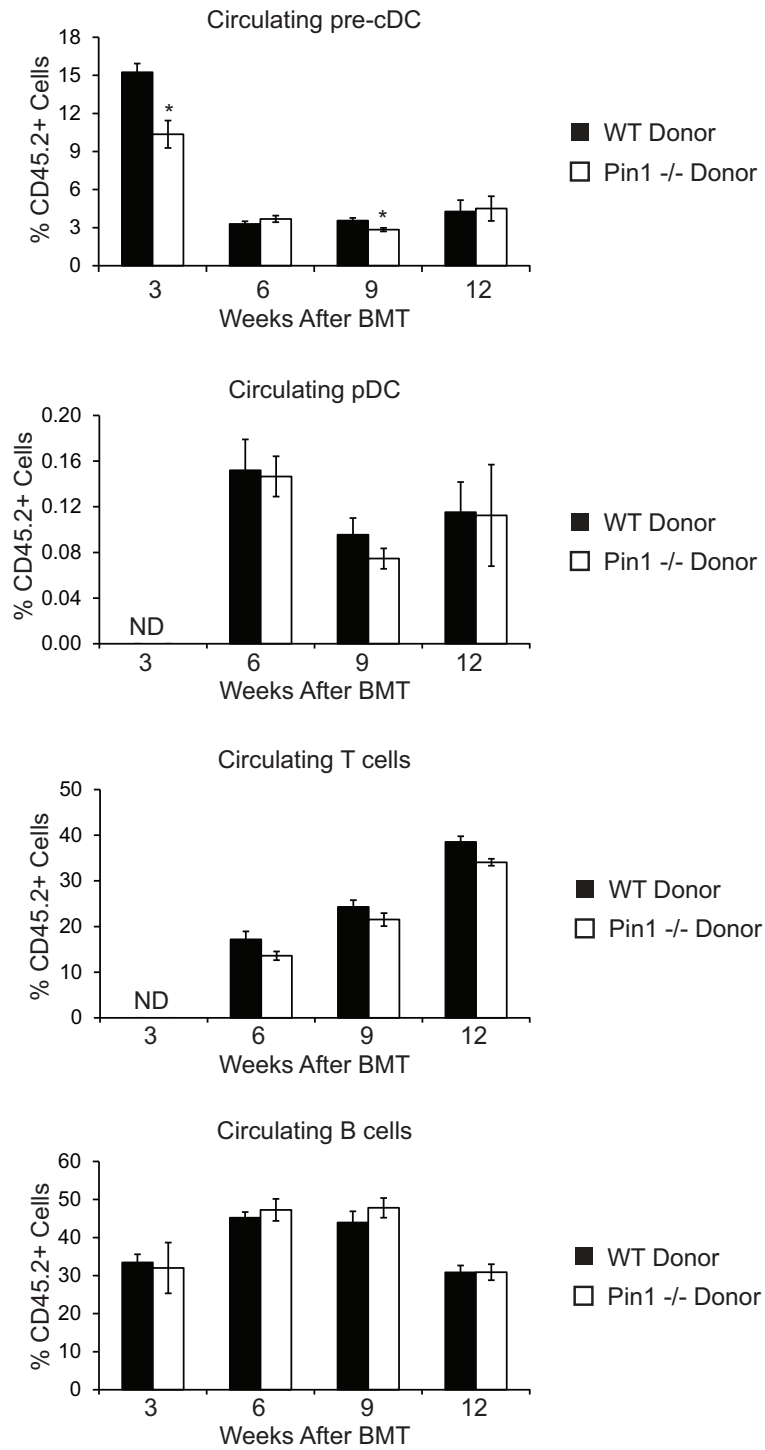
**Figure 24: Blood Engraftment in Recipients of WT and Pin1-null Bone Marrow**

**Figure 24 (Continued): Blood Engraftment in Recipients of WT and Pin1-null Bone Marrow**

(A) Blood was collected from the cheek of WT CD45.1+ recipient mice at 3, 6, 9, and 12 weeks after bone marrow transplant of either WT or Pin1-null CD45.2+ red blood cell-depleted bone marrow. Graphs show the engraftment of each individual recipient of WT or Pin1-null bone marrow at each week following transplant. (B) Representative FACS plot of blood engraftment at week 12. (C) Line graph showing average engraftment over 12 weeks.

Although circulating pDC were not detectable at 3 weeks post-transplant, after 6 weeks they were present at similar frequencies in both mice that received WT bone marrow and mice that received Pin1-null bone marrow (Figure 25). Similarly, T cells were first detected 6 weeks after transplant and continued to increase in frequency over time, but did not exhibit any defects in the absence of Pin1. B cells were present in all four bleeds and were also unaffected by the absence of Pin1 (Figure 25). The delayed appearance of circulating Pin1-null pre-cDC is consistent with a role for Pin1 in modulating cDC development. Because pre-cDC are a heterogeneous population of cells that can give rise to both CD8+ and CD8- cDC, it is tempting to speculate that the modest decrease in the frequency of Pin1-null pre-cDC may be indicative of a defect in a fraction of pre-cDC that gives rise to a specific subset of cDC.

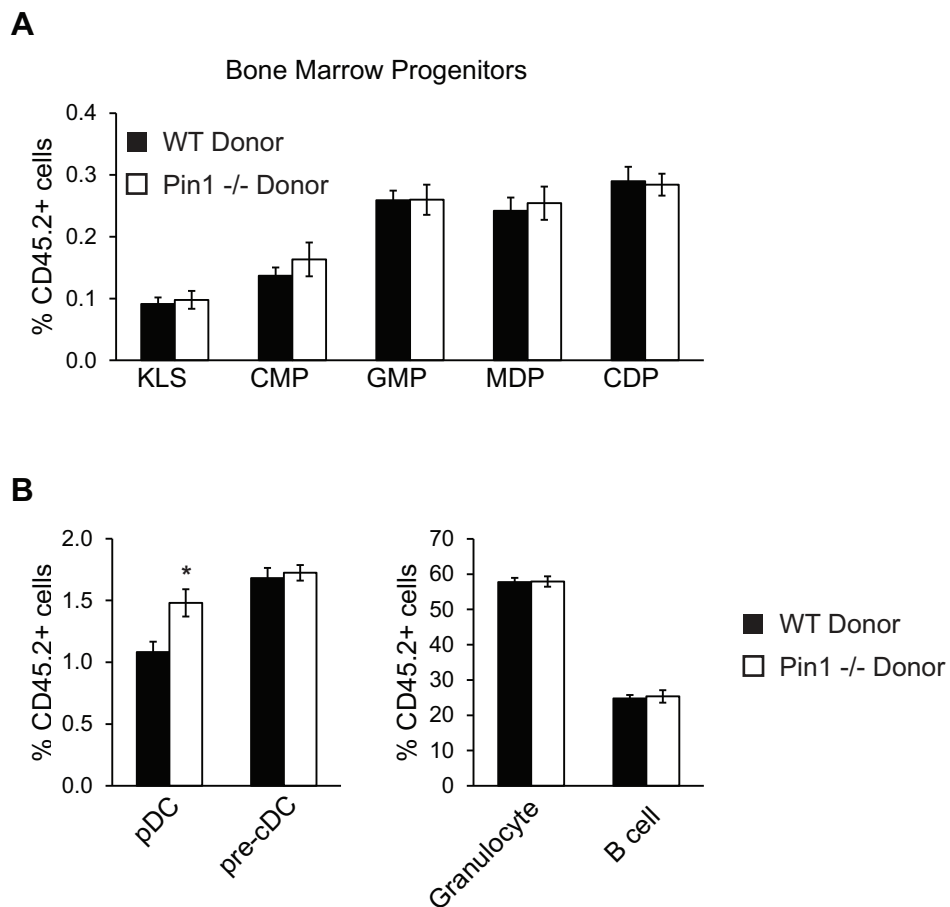
Twelve weeks after transplant, mice were sacrificed and both bone marrow and spleens were harvested, stained, and analyzed for the presence of multiple cell populations. In the bone marrow, no differences were detected between WT and Pin1-null progenitors, granulocytes, or B cells (Figure 26). Consistent with results from the 12 week bleed, there was no difference in the frequency of Pin1-null pre-cDC in the bone marrow. Pin1-null pDC, however, were significantly increased compared to WT pDC,



**Figure 25: Recipients of Pin1-null Bone Marrow Exhibit Delayed Reconstitution of Blood Pre-cDC**

**Figure 25 (Continued): Recipients of Pin1-null Bone Marrow Exhibit Delayed Reconstitution of Blood Pre-cDC**

WT CD45.1+ recipient mice received CD45.2+ bone marrow from WT or Pin1-null mice. Blood was collected at 3 week intervals for 12 weeks and stained for multiple CD45.2+ donor cell populations. Frequency of each population is shown in the graphs. ND, Not Detected.



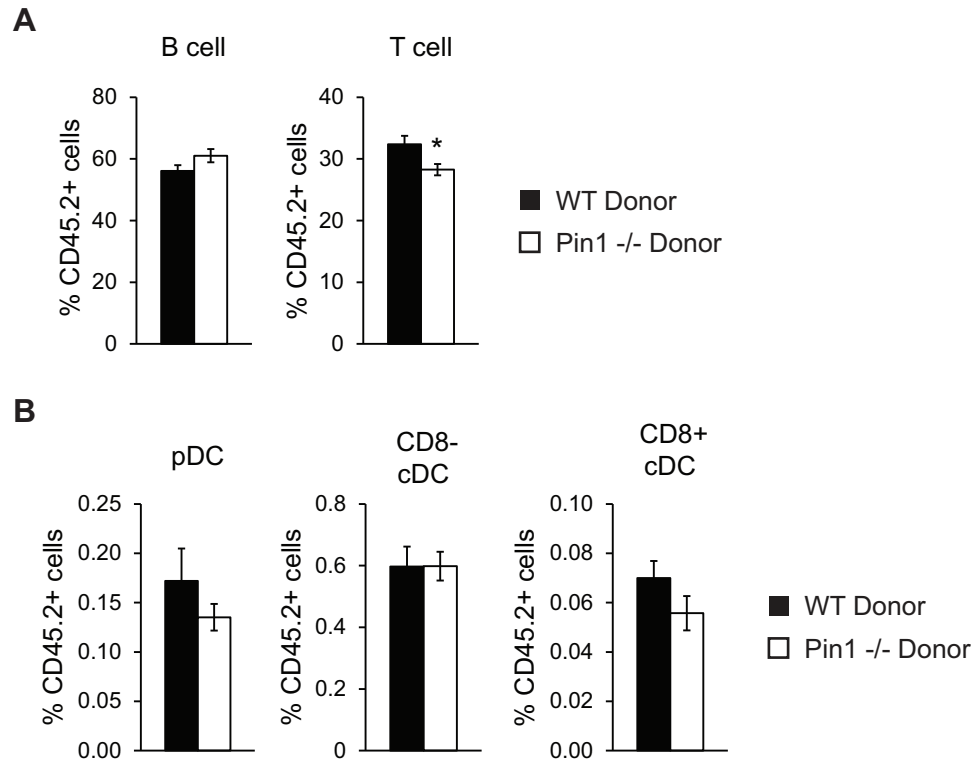
**Figure 26: Recipients of Pin1-null Bone Marrow Exhibit Increased Frequency of pDC in Bone Marrow Compartment**

(A) and (B) Twelve weeks after transplant, recipient mice were euthanized. Bone marrow was collected, stained, and analyzed by flow cytometry for the frequency of various CD45.2+ donor progenitor populations (A) as well as for other bone marrow cell populations (B).

further indicating that Pin1 is not required for pDC development (Figure 26). This result is consistent with the lack of defects observed in bone marrow pDC in Pin1-null mice, and with the ability of Pin1-null bone marrow to give rise to pDC when cultured *ex vivo* in the presence of FL.

Upon staining splenocytes for lymphoid markers, we did not observe any defects in B cells derived from Pin1-null donors. In contrast, we found a small but significant decrease in the frequency of Pin1-null T cells compared to WT T cells, indicating that Pin1 may play a role in T cell development or survival (Figure 27A). When the frequency of donor DC subsets was plotted as a percentage of CD45.2+ donor cells, we were unable to detect any significant differences in pDC, CD8- cDC, or CD8+ cDC (Figure 27B). There was variability in the frequency of donor CD8+ cDC in both groups of mice, however, and although there existed a trend for fewer Pin1-null CD8+ cDC, this trend did not reach statistical significance when values were plotted as a percentage of total CD45.2+ cells (Figure 27B). We did, however, note that about half of mice that received Pin1-null bone marrow (3 of 7) exhibited skewing towards CD8- cDC (Figure 27A, Figure 27B). When values were plotted as frequency of total cDC, we were able to detect a small but significant decrease in Pin1-null CD8+ cDC (Figure 27B).

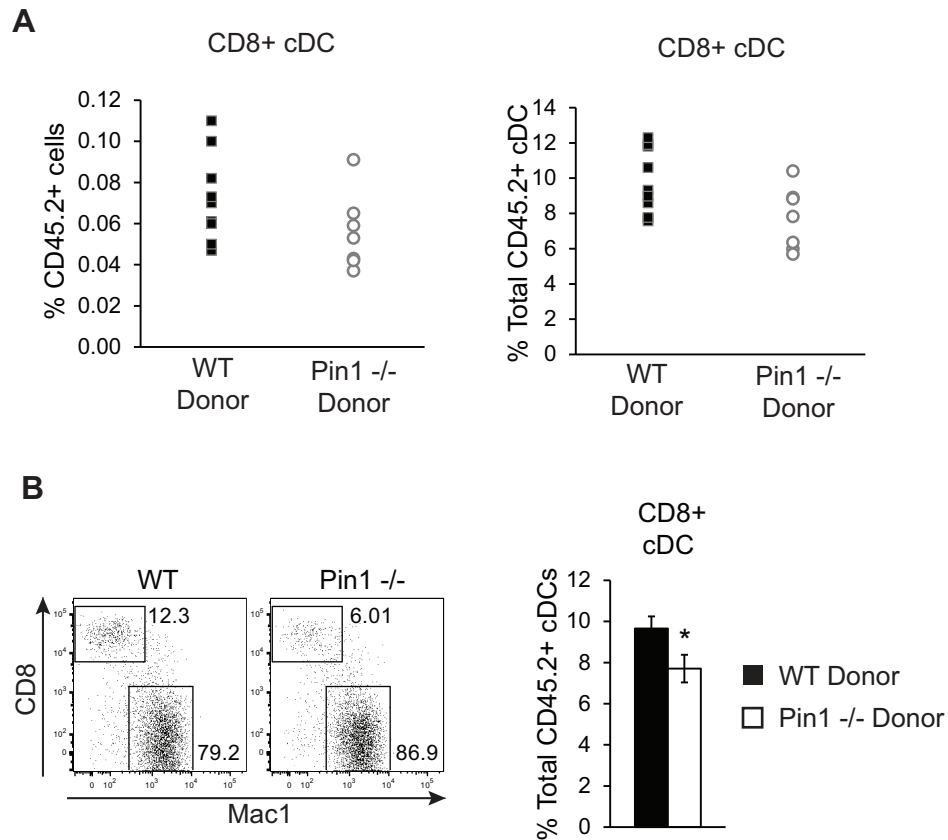
The results from spleen CD8+ cDC are difficult to interpret, and speak to the fact that there exists much variability between animals. It is unclear how the same mixture of bone marrow cells could produce defects in the generation of CD8+ cDC in only



**Figure 27: Spleen Cell Populations in Bone Marrow Transplant at 12 Weeks**

(A) and (B) Twelve weeks after transplant, CD45.1+ WT recipient mice were euthanized and spleens were removed. Splenocytes were stained and analyzed for multiple CD45.2+ donor cell populations. The frequency of each populations is shown as percentage of CD45.2+ donor cells.

a subset of recipients. To more accurately determine whether there exists a true defect in the ability of Pin1-null bone marrow to give rise to CD8+ cDC, this experiment should be repeated with a larger number of mice. Furthermore, despite beginning with 11 recipients of Pin1-null bone marrow, one recipient did not survive the first week after transplant and two recipients ultimately failed to achieve robust engraftment of Pin1-null bone marrow by week 12 after transplantation. These losses prevented us from



**Figure 28: Recipients of Pin1-null Bone Marrow Exhibit a Modest Defect in the Ratio Between CD8- and CD8+ spleen cDC**

(A) The frequency of donor CD8+ spleen cDC from each individual recipient is plotted as a percentage of CD45.2+ donor cells (left panel) and again as a percentage of total donor cDC (right panel). (B) Left panel contains representative FACS plots showing the decrease in Pin1-null CD8+ cDC observed in a small subset of recipient mice. Right panel contains a graph of CD8+ cDC averaged from all recipients and plotted as percentage of donor cDC rather than percentage of total CD45.2+ donor cells.

evaluating a greater number of recipients of Pin1-null bone marrow. Additionally, these results may be indicative of a more fundamental defect in the ability of Pin1-null bone marrow to fully engraft in WT recipients. It is tempting to speculate that some sort of compensation, either in the donor cells or the recipient, has occurred to facilitate robust

engraftment in 7 of 10 mice that received Pin1-null bone marrow. We cannot exclude the possibility that these results are the consequence of an error in the injection of Pin1-null bone marrow cells into a subset of recipients; the absence of this same phenomenon in recipients of WT bone marrow, however, does not support such a scenario, as an equal amount of variability would be expected in both groups.

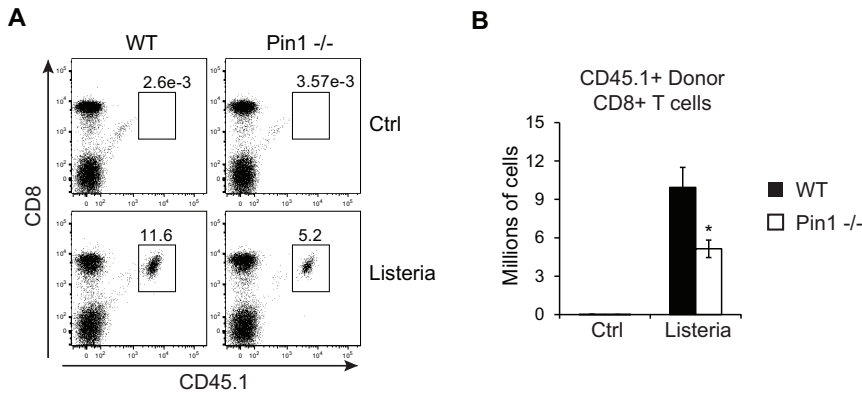
#### **4.2.7 Proliferation of CD8<sup>+</sup> T cells in Pin1-null mice infected with *Listeria monocytogenes***

Results from transplanted bone marrow indicated delayed engraftment of circulating pre-cDC in the absence of Pin1. Additionally, we observed consistent and robust defects in the production of CD8<sup>+</sup> cDC in both Pin1-null mice and in Pin1-null BMDC. Much attention has been focused on these cells in recent years, as they are important modulators of CD8<sup>+</sup> T cell responses to both infections and tumors (Hildner et al, 2008; Shortman & Heath, 2010). To test the hypothesis that decreased CD8<sup>+</sup> cDC in Pin1-null mice would impact the proliferation of CD8<sup>+</sup> T cells *in vivo*, we selected a pathogen that is known to both activate CD8<sup>+</sup> cDC and induce proliferation of CD8<sup>+</sup> T cells. Acknowledging that Pin1 regulates pDC function, and that Pin1 has previously been shown to regulate IFN $\beta$  production in mice injected with poly (I:C), a simulator of viral infection, we did not infect mice with virus (Saitoh et al, 2006; Tun-Kyi, 2011). Instead, we selected *Listeria monocytogenes* (*L.m.*), an intracellular bacterium that has been demonstrated to elicit CD8<sup>+</sup> T cell proliferation (Belz et al, 2005). Several reports suggest that CD8<sup>+</sup> cDC are crucial for inducing CD8<sup>+</sup> T cell expansion upon infection

with *L.m.*; furthermore, pDC are not thought to greatly contribute to this response (Belz et al, 2005; Mitchell et al, 2011; Solodova et al, 2011).

Pin1 has been reported to modulate cytokine production in activated T cells, which could potentially impact proliferation and/or survival (Esnault et al, 2007a; Esnault et al, 2006). It has also been shown that Pin1 can bind and regulate the activity of the transcription factor NFAT, which is an important modulator of T cell activation (Liu et al, 2001). Furthermore, our bone marrow transplant data suggest that Pin1 may play some role in T cell development. To assess proliferation of T cells and to exclude the possibility that Pin1 might regulate T cell activation directly, we injected both WT and Pin1-null mice with WT transgenic CD8<sup>+</sup> T cells from OTI mice, which express an ovalbumin (ova)-specific T cell receptor. The OT1 cells also expressed CD45.1, and were therefore easily distinguished from endogenous CD8<sup>+</sup> T cells, which expressed CD45.2. Twenty-four hours after injecting both WT and Pin1-null mice with 10,000 OT1 CD8<sup>+</sup> T cells, mice were administered  $1 \times 10^4$  CFU of live *L.m.* engineered to express ovalbumin, thereby enabling the induction of a strong response from the ova-specific OT1 CD8<sup>+</sup> T cells. Seven days after infection, mice were euthanized and splenocytes were stained for CD45.1<sup>+</sup> CD8<sup>+</sup> T cells. We found a 50% reduction in the numbers of CD45.1<sup>+</sup> CD8<sup>+</sup> T cells that accumulated in Pin1-null mice compared to WT mice (Figure 29). Although we cannot exclude the contribution of other unidentified defects in Pin1-null mice, these

results are consistent with the hypothesis that a reduced number of steady-state CD8+ cDC can result in a measurable impact on CD8+ T cell proliferation *in vivo*

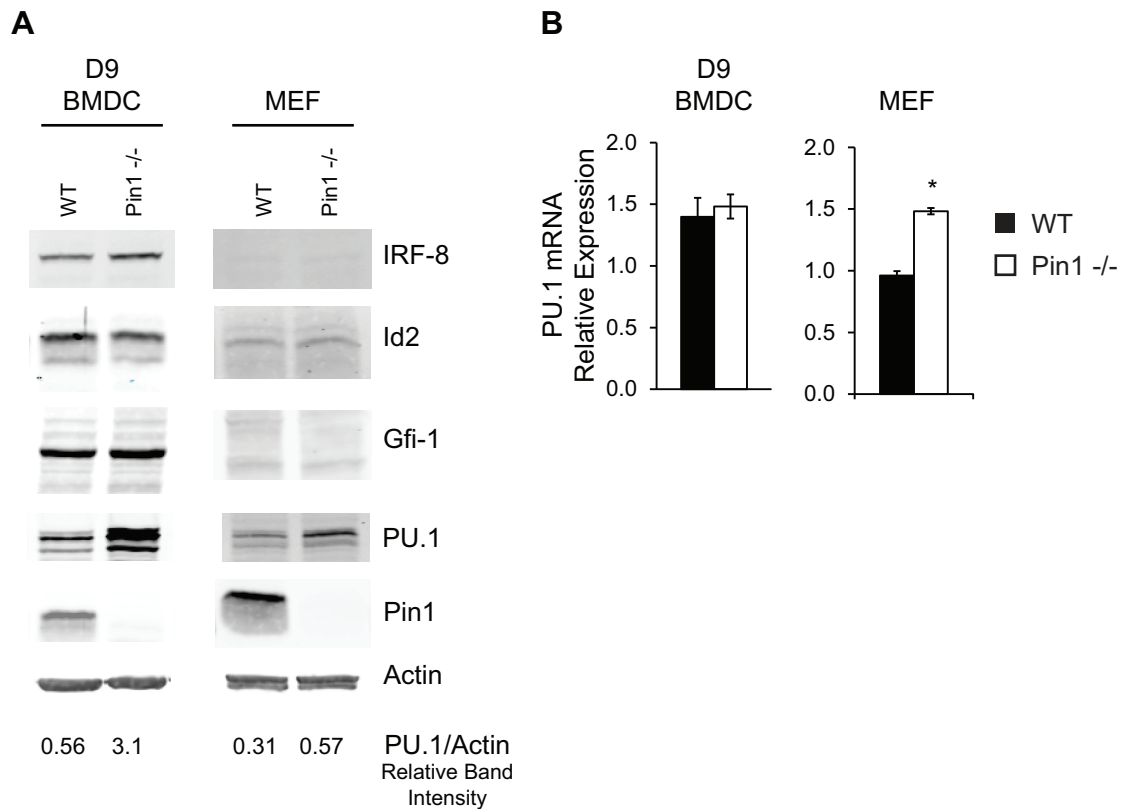


**Figure 29: Pin1-null Mice Exhibit Impaired CD8+ T cell Proliferation Upon Infection with *Listeria monocytogenes*.**

(A) and (B) CD45.2+ WT and Pin1-null mice were injected with CD45.1+ OT1 CD8+ T cells. Twenty-four hours later, mice were infected with *Listeria monocytogenes*. After 7 days, mice were euthanized. Splenocytes were analyzed for the presence of CD45.1+ CD8+ T cells derived from donor OT1 cells. Representative FACS plots from WT and Pin1-null mice are shown in (A), and numbers of donor CD45.1+CD8+ T cells are shown in the graph in (B).

#### 4.2.8 Pin1 Regulates Expression of PU.1

To further understand how Pin1 modulates cDC development, immunoblot analysis was performed on lysates from WT and Pin1-null BMDC. The expression of several different proteins previously identified as regulators of cDC development was measured, including IRF8, Id2, Gfi-1, and PU.1 (Carotta et al, 2010b; Rathinam et al, 2005; Watowich & Liu, 2010). Although the expression of the other proteins appeared to be unaltered (Figure 30A), there was a marked increase in PU.1 protein in Pin1-null BMDC. This same deregulation was confirmed in Pin1-null MEF, which also express



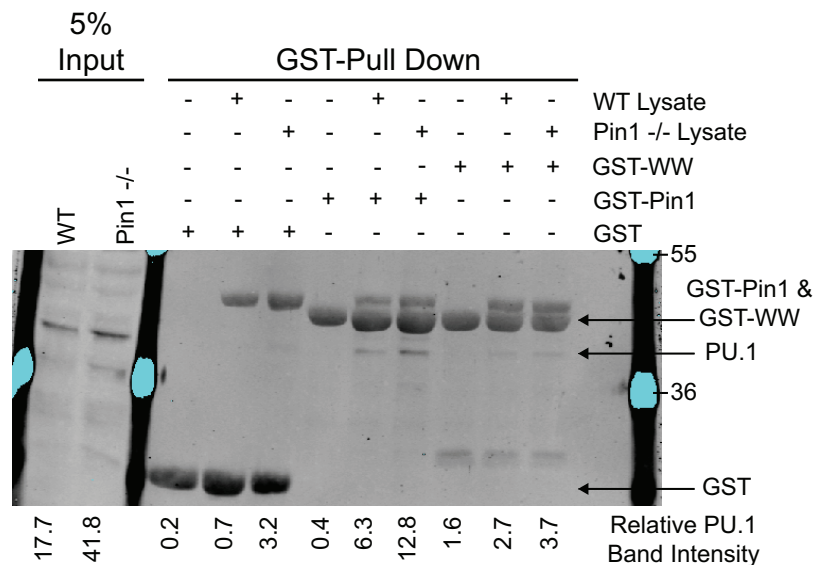
**Figure 30: Pin1-null Cells Exhibit Elevated PU.1 Protein Levels**

(A) and (B) Non-adherent bone marrow-derived DC generated with FL were collected after 9 days in culture. (A) Cells were lysed and analyzed for proteins implicated in DC development by immunoblot. Relative band intensity for PU.1 normalized to Actin are indicated at bottom. (B) Cells were collected and quantitation of PU.1 mRNA was determined by RT-qPCR.

elevated levels of PU.1 protein (Figure 30A). To determine whether increased PU.1 protein was the result of elevated PU.1 mRNA, RT-qPCR analysis was performed on both BMDC and MEF. No change in PU.1 mRNA was detected in Pin1-null BMDC. RT-qPCR analysis in MEF, however, revealed a modest 1.5-fold increase in PU.1 mRNA in the absence of Pin1 (Figure 30B). As PU.1 is known to bind and regulate its own promoter, we speculate that increased PU.1 mRNA in Pin1-null MEF might result from

enhancement of this feed-forward loop. The absence of the same increase in PU.1 mRNA in BMDC, however, may be indicative of additional defects in PU.1 transcriptional activity. Indeed, PU.1 is known to interact with multiple proteins that co-regulate transcription of target genes (Gangenahalli et al, 2005). Because two of these PU.1-binding proteins (IRF-8 and Gfi-1) are absent in MEF (Figure 30A), it is possible that PU.1 transcriptional activity is not regulated in the same manner in MEF as in BMDC.

It was recently published that GST-Pin1 binds endogenous PU.1 in PMA-stimulated THP-1 human macrophages (Akiyama et al, 2011). We confirmed this

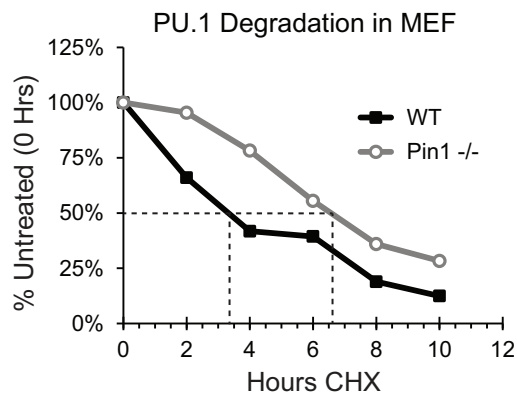
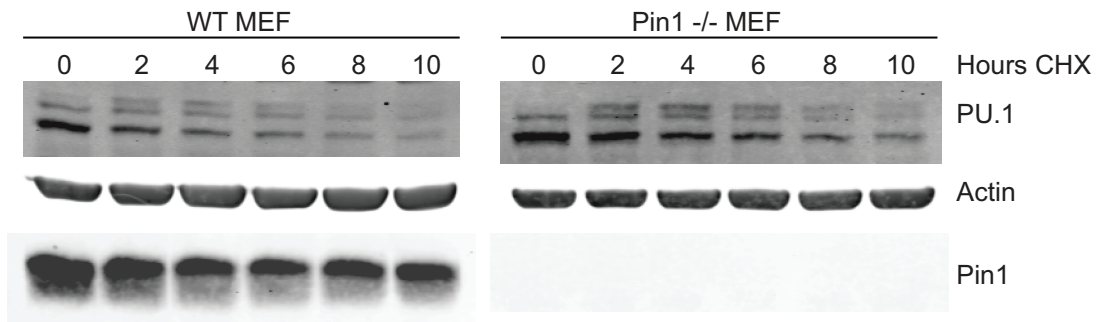


**Figure 31: GST-Pin1 Binds PU.1 and Requires the WW Binding Domain of Pin1**

1mg of total lysate was incubated with GST alone, WT GST-Pin1, or WW GST-Pin1 for 2 hours. After binding, beads were washed, resuspended in SDS-Page sample buffer, boiled, and then analyzed by immunoblot. Membranes were probed for expression of PU.1.

interaction by performing a GST pull-down assay in both WT and Pin1-null MEF and demonstrating that endogenous PU.1 is able to bind WT GST-Pin1. The inability of a WW binding domain mutant of GST-Pin1 (W11A, W34A) to bind PU.1 indicated that this domain of Pin1 is necessary to maintain the interaction with PU.1 (Figure 31), as was previously reported in COS-7 cells (Akiyama et al, 2011).

Three putative Pin1 binding sites exist in the PU.1 protein, two of which are located in the PEST domain. Because PEST domains have been shown to modulate protein degradation, the ability of Pin1 to regulate PU.1 protein turnover was examined (Rechsteiner & Rogers, 1996). Both WT and Pin1-null MEF were incubated with the protein synthesis inhibitor cycloheximide and then the rate of PU.1 degradation was assessed by immunoblot analysis. The absence of Pin1 was found to promote PU.1 stability, nearly doubling its half-life (Figure 32). These data further expand on the recently-described interaction between Pin1 and PU.1 by providing new evidence that Pin1 facilitates PU.1 protein degradation.



**Figure 32: Pin1 Regulates PU.1 Protein Turnover**

PU.1 protein expression in WT and Pin1-null MEFs after being treated with 150  $\mu$ g/ml cycloheximide (CHX) to inhibit protein synthesis for 2, 4, 6, 8, or 10 hours. The immunoblot shown is representative of two independent experiments. PU.1 protein expression is plotted in the bottom graph as a percentage of total PU.1 protein at time zero, and reflects the values obtained from immunoblots shown directly above.

### 4.3 Discussion

Results from chapter 3 demonstrated that Pin1-null macrophages are able to produce pro-inflammatory cytokines to the same extent as WT cells up on LPS stimulation. These data indicated to us that it was unlikely that macrophages were responsible for the inability of Pin1-null mice to maintain circulating cytokine levels. In chapter 4, we investigated the possibility that the loss of Pin1 resulted in impaired

accumulation of additional cell populations that participate in the response to LPS challenge. Although we did not detect any defects in spleen T cells or B cells in Pin1-null mice, we did observe a defect in the LPS-stimulated accumulation of cDC. Due to the importance of cDC in initiating adaptive (T cell-mediated) immunity, we were interested in determining how Pin1 modulated cDC accumulation, prompting us to carefully examine each of the various DC subsets in Pin1-null mice.

Collectively, the results presented in this chapter describe a novel role for Pin1 in modulating late stages of CD8<sup>+</sup> cDC development. Not only are CD8<sup>+</sup> cDC decreased in the spleens of Pin1-null mice, but they fail to expand in response to FL ligand injections. Furthermore, defective development was recapitulated by culturing bone marrow *ex vivo* in the presence of FL. The lack of defects in cDC progenitors in the bone marrow indicate that the developmental defect most likely occurs at later stages of development that occur in the periphery.

In the case of the CD8<sup>-</sup> cDC subset, our results were somewhat less definitive *in vivo*. Despite possessing a decrease in total numbers (which most likely reflects a decrease in total spleen cell numbers in Pin1-null mice), the same significant defect was not apparent when data was presented as frequency of total cells. When mice were injected with FL, however, the modest impairment seen under basal conditions was no longer present; indeed, we observed the same number of FL-expanded CD8<sup>-</sup> cDC in both WT and Pin1-null mice. Bone marrow cultured with FL *ex vivo* also provided some

complex results in that Pin1-null Mac1<sup>+</sup> cells appear, as a whole, to express less CD11c. As bright expression of CD11c is a defining marker for cDC, it is difficult to interpret this result. On the one hand, it may reflect a true decrease in Pin1-null cDC, as indicated by the accumulation of cells that do not express CD11c brightly. On the other hand, these cells could functionally be Mac1<sup>+</sup> cDC that, under these particular culture conditions, are deficient in up-regulating CD11c expression. This result is interesting in light of the fact that B220-Mac1<sup>-</sup> cells (which include the CD8<sup>+</sup> cDC equivalent), are not impaired in CD11c expression. Possible explanations include a subset specific impairment in CD11c expression in Mac1<sup>+</sup> cDC only, or alternatively, that these Mac1<sup>+</sup> CD11c<sup>int</sup> cells may represent a population of pre-cDC that remains in the Pin1-null cultures due to defective development. Indeed, such a pre-CD8<sup>+</sup> cDC subset has been described previously (Jackson et al, 2011; Naik et al, 2006; Wang et al, 2002). This explanation seems plausible in light of the fact that we do not observe similar defects in CD11c expression when Pin1-null bone marrow is cultured in the presence of GM-CSF, indicating that impaired CD11c expression is not common to all cultured Pin1-null Mac1<sup>+</sup> cDC.

A decrease in spleen pDC was detected in Pin1-null mice compared to WT mice, but this difference was not statistically significant. When pDC were quantified in the bone marrow, however, we found no such decrease in Pin1-null cells. Since pDC undergo the terminal stages of their developmental program within the bone marrow, this result indicates that development of pDC is not impaired in Pin1-null mice.

Additionally, when bone marrow was cultured *ex vivo* in the presence of FL, pDC were produced to similar extents in both WT and Pin1-null cultures. And finally, upon staining bone marrow cells from WT mice that had been irradiated and reconstituted with either WT or Pin1-null bone marrow cells, Pin1-null pDC were elevated compared to WT pDC. Thus, we conclude that Pin1 is unlikely to be required for pDC development. The decrease observed in the spleen may reflect separate defects that impact pDC migration, homing, or survival. The conclusion that Pin1 is dispensable for pDC development is supported by the recent report examining the role of Pin1 in pDC function. The authors did not observe differences in the frequency of Pin1-null spleen pDC, nor in the ability of bone marrow from Pin1-null mice to give rise to pDC when cultured *ex vivo* with FL (Tun-Kyi, 2011).

In chapter 3 it was demonstrated that Pin1-null bone marrow cultured *ex vivo* with M-CSF could give rise to macrophages to the same extent as WT bone marrow. While pDC fully develop within the bone marrow, macrophages share similarities with cDC in that they leave the bone marrow as monocyte precursors and undergo final stages of development in peripheral tissues. The absence of defects in generating both macrophage and pDC in Pin1-null bone marrow cultures further supports the notion that Pin1 preferentially regulates cDC development.

In addition to evaluating DC development, we also utilized the bone marrow culture system to determine if the absence of Pin1 impaired the ability of cDC to

upregulate co-stimulatory molecules required for the stimulation of naïve T cells. Upon addition of LPS to bone marrow cultures, we did not observe any differences in the ability of WT and Pin1-null cDC subsets to up-regulate MHC class II, CD40, or CD86 expression. This was true in both FL cultures and GM-CSF cultures. Thus, we conclude that Pin1 is not required for the induction of co-stimulatory molecules in cDC that occurs upon activation with LPS.

While results from *ex vivo* bone marrow cultures were indicative of a cell-intrinsic defect in Pin1-null CD8<sup>+</sup> cDC development, we sought to confirm this by performing a bone marrow transplant in mice. Upon examining circulating populations in the blood after transplant, we found a significant decrease in Pin1-null pre-cDC at 3 weeks that was absent at 6 weeks post-transplant, indicating that there exists a delay in pre-cDC reconstitution when Pin1 is absent. Interestingly, it has previously been shown that at 2 weeks post-bone marrow transplant, the CD8<sup>+</sup> cDC subset peaks in numbers and is the predominant cDC subset present in the spleen (Kamath et al, 2000). Although the pre-cDC we measured in the blood are comprised of cells that presumably give rise to both CD8<sup>+</sup> and CD8<sup>-</sup> cDC, it is tempting to speculate that the decrease in Pin1 -null pre-cDC observed in the 3 week bleed may reflect a deficiency in pre-cDC that preferentially give rise to CD8<sup>+</sup> cDC. Regardless of subset-specificity, this result suggests that the loss of Pin1 results in delayed cDC reconstitution, which is consistent with previously described roles for Pin1 in which it acts as a “molecular timer”; indeed,

in the context of germ cell development, Pin1 has been found to modulate the timing of proliferation, and as a consequence, mice lacking Pin1 exhibit profound defects in fertility owing to reduced numbers of primordial germ cells (Atchison et al, 2003).

When we examined spleen DC populations 12 weeks after transplant, we found that about half of the recipients of Pin1-null bone marrow exhibited a substantial decrease in donor CD8<sup>+</sup> spleen cDC. Although a robust defect was not present in every recipient in which Pin1-null bone marrow engrafted, we were still able to quantify a statistically significant difference when we specifically examined the relationship between CD8<sup>+</sup> and CD8<sup>-</sup> spleen cDC. Indeed, it appeared that of the Pin1-null cDC present in the spleen, a majority of cells were CD8<sup>-</sup> rather than CD8<sup>+</sup>, which is consistent with results from Pin1-null mice and Pin1-null bone marrow-derived DC cultures. The existence of a less severe defect in transplanted cells may be indicative of there being additional cell-extrinsic factors in Pin1-null mice that contribute to a more pronounced reduction in spleen CD8<sup>+</sup> cDC. Furthermore, although we tried to address the existence of potential differences in the starting bone marrow populations put into culture with FL, we cannot rule out the possibility that there remain undetected differences in the cell populations in bone marrow cultures that might also contribute to differences in Mac1-cDC (CD8<sup>+</sup> cDC equivalent) development. We conclude that although the defects observed in Pin1-null spleen CD8<sup>+</sup> cDC in transplant mice were not as severe as those observed in Pin1-null mice, they still support a role for Pin1 in CD8<sup>+</sup> cDC development.

Our results also suggest that additional defects may exist in Pin1-null mice that contribute to a more severe decrease in CD8<sup>+</sup> spleen cDC.

After having established that there are fewer CD8<sup>+</sup> cDC present in Pin1-null mice, and that this is likely to result from impaired CD8<sup>+</sup> cDC development, we provide evidence suggesting that decreased CD8<sup>+</sup> spleen cDC can have a corresponding impact on the proliferation of CD8<sup>+</sup> T cells *in vivo* in the context of infection. Because Pin1 was recently described to modulate cytokine secretion in pDC in response to TLR7/9 ligands (Tun-Kyi, 2011), we infected mice with *Listeria monocytogenes*, a non-viral pathogen that similarly induces CD8<sup>+</sup> T cell activation. We observed that, upon infection, adoptively transferred WT CD8<sup>+</sup> T cells increased in number in both WT and Pin1-null mice. The extent of the expansion, however, was decreased by 50% in Pin1-null mice compared to WT mice, perfectly mirroring the reduced numbers of CD8<sup>+</sup> spleen cDC in Pin1-null mice. This result suggests that the number of CD8<sup>+</sup> spleen cDC may be rate-limiting in terms of stimulating CD8<sup>+</sup> T cell proliferation *in vivo* in response to immune challenge. Furthermore, the lack of a more severe defect in T cell proliferation suggests to us that the primary impairment in Pin1-null CD8<sup>+</sup> cDC lies in their development and accumulation, not their function.

At the end of this chapter, we turned our focus to trying to identify possible molecular mechanisms by which Pin1 could regulate CD8<sup>+</sup> cDC development. Western blot analysis of both FL-BMDC and MEF revealed an increase in the expression of PU.1

protein in the absence of Pin1. PU.1 is a member of the ETS family of transcription factors, and has been shown to be required for the development of multiple hematopoietic lineages (Gangenahalli et al, 2005). The abundance of PU.1 protein varies between different lineages and developmental stages, indicating that regulated changes in expression may be important, and perhaps instructive, for lineage-specific development (DeKoter et al, 2007; Nutt et al, 2005). PU.1 has been described as a regulator of DC development (Anderson et al, 2000; Carotta et al, 2010a; DeKoter et al, 2007). Carotta et al. recently demonstrated that PU.1 can bind the Flt3 promoter and modulate its expression, thereby positively influencing DC development. Interestingly, defects in DC development could not be rescued by re-expressing Flt3 in PU.1-deficient bone marrow cultures, and the authors concluded that PU.1 is likely regulating DC development via multiple mechanisms (Carotta et al, 2010a). Our results indicate that aberrant overexpression of PU.1 may also deregulate DC development. It is interesting to note that we do not detect an increase in Flt3 mRNA in our Pin1-null BMDC, which would have been the predicted result of elevated PU.1 expression. This indicates that Pin1 may be capable of modulating other aspects of PU.1 biology, in addition to its rate of degradation. Given the complexity of PU.1-dependent transcriptional regulation, this is not unlikely. Indeed, PU.1 can both positively and negatively regulate gene transcription, and its activity is influenced by interaction with other proteins as well as phosphorylation (Marecki & Fenton, 2000).

Two putative Pin1 binding sites are located within the PEST domain of PU.1, a region that has been shown to mediate interactions between PU.1 and other proteins (Marecki & Fenton, 2000). Our results confirm the recent report that Pin1 binds to PU.1, and that this interaction is abolished upon mutation of the Pin1 WW domain (Akiyama et al, 2011). Adding to the understanding of this relationship, we determined that Pin1 regulates PU.1 protein turnover, as indicated by the doubling of PU.1 protein half-life in the absence of Pin1. Modulating protein degradation is a common mechanism by which Pin1 regulates the activity of its substrates. Indeed, Pin1 has also been shown to regulate the stability and turnover of other hematopoietic transcription factors, including NF- $\kappa$ B p65, IRF3, and Bcl6 (Phan et al, 2007; Ryo et al, 2003; Saitoh et al, 2006). Although we do not provide direct evidence, it is tempting to speculate that Pin1 might regulate CD8<sup>+</sup> cDC development through cell-specific modulation of PU.1 activity, which could be achieved by regulating PU.1 degradation rate, interactions with binding partners, and perhaps dephosphorylation, as has been shown for other Pin1 substrates (Dougherty et al, 2005; Lu & Zhou, 2007; Yeh et al, 2004). Further work is required to understand how Pin1 binding to PU.1 is regulated, and how this interaction might impact PU.1 function.

The work described in this chapter expands the current understanding of DC development by identifying a novel role for Pin1 in regulating the steady-state production of CD8<sup>+</sup> subset of cDC. The absence of Pin1 specifically impairs the FL-induced expansion of CD8<sup>+</sup> cDC and also impairs proliferation of adoptively transferred

WT CD8+ T cells following bacterial infection in mice, a response that is modulated, at least in part, by the presence of functional CD8+ cDC. Furthermore, we provide evidence that Pin1 regulates expression of the transcription factor PU.1. Because PU.1 is a tightly controlled master regulator of multiple myeloid lineages, including dendritic cells, we believe deregulated expression in the absence of Pin1 is likely a relevant contributor to the defect we observe in cDC development. Collectively, the data provided in this chapter implicate Pin1 as a regulator of CD8+ cDC development, possibly through modulation of PU.1 protein turnover. Our results suggest that Pin1 is important for the stimulation of the adaptive immune response and establish Pin1 as a modulator of innate immunity.

## 5. Conclusions and Future Directions

### 5.1 Conclusions

Pin1 modulates a diverse repertoire of protein substrates that participate in numerous cellular signaling pathways. Despite such variety, the effect of Pin1 on its many substrates is similar in that it fine tunes processes that occur more slowly in its absence, such as dephosphorylation, protein degradation, and protein binding (Lu & Zhou, 2007; Yeh & Means, 2007). Such effects are consistent with its activity as a catalyst of intermolecular conversions between *cis* and *trans* conformations of prolyl bonds, which occur much more slowly in its absence. Thus, because of its ability to enhance processes that still occur to some extent in its absence, loss of Pin1 rarely has profound effects on cellular function. Exceptions exist in processes that require precisely timed events, such as primordial germ cell proliferation, which only occurs within a 5 day window and which is severely disrupted in the absence of Pin1, leading to severe infertility (Atchison et al, 2003).

Pin1 has been shown to modulate the function of many proteins that participate in the immune response. Indeed, Pin1 has been shown to be an important regulator of eosinophil activation and survival (Shen et al, 2008), cytokine production in activated T cells and pDC (Esnault et al, 2007a; Tun-Kyi, 2011), and B cell germinal center formation (Phan et al, 2007). When we began this work, it was evident that Pin1 possessed numerous roles in modulating cells of both the innate and adaptive immune responses,

yet its ability to impact bacterial infection and systemic inflammation was largely unexplored. Additionally, despite the existence of reports indicating potential roles for Pin1 in the modulation of macrophage function, the results are contradictory and unclear.

The work presented in this dissertation expands upon the roles for Pin1 in immune modulation. In chapter 3, we provide evidence that Pin1-null mice are impaired in their response to systemic inflammation induced by injection of LPS. In the absence of Pin1, mice failed to maintain circulating cytokine levels to the same extent as WT mice. Examination of both peritoneal macrophages and bone marrow-derived macrophages revealed that Pin1 was dispensable for the LPS-induced production of IL-6 and TNF $\alpha$ . This result is in agreement with data derived from Kupffer macrophages in liver (Kuboki et al, 2009), but appears to contradict other results generated in peritoneal macrophages (Akiyama et al, 2011) and bone marrow-derived macrophages (Tun-Kyi, 2011). The reason for the observed discrepancies is unknown, but could potentially arise from differences in the quality of commercially purchased LPS as well as culture conditions. Additionally, we have determined that the absence of Pin1 does not impair the development of bone marrow-derived macrophages. Thus, our results lead us to conclude that Pin1 is dispensable for production of bone marrow-derived macrophages and for the LPS-induced secretion of IL-6 and TNF $\alpha$  in macrophages.

In chapter 4, we further investigated potential defects in Pin1-null mice that can account for the impaired inflammatory response observed upon LPS administration. We confirmed other reports that steady-state spleen B cells and T cells are not impaired by the absence of Pin1 (Esnault et al, 2007a; Phan et al, 2007; Tun-Kyi, 2011). Additionally, we identified a decrease in spleen cDC in Pin1-null mice that prompted us to further investigate a role for Pin1 in modulating DC accumulation. Staining of steady-state cDC populations *in vivo*, FL-induced expansion of DC subsets *in vivo*, and *ex vivo* cultures of bone marrow cells all support a role for Pin1 in modulating CD8+ cDC development. Although defects in the CD8- subset of cDC were rather modest and not observed consistently, we cannot rule out the possibility that the loss of Pin1 has a more subtle effect on the development of CD8- cDC. In agreement with the recent report by Tun-Kyi et al., loss of Pin1 did not alter pDC development. Thus, we conclude that Pin1 is required for efficient development of CD8+ cDC in mice.

LPS stimulation of bone marrow-derived cDC subsets indicated that Pin1-null cDC were able to induce CD40 and CD86 expression to the same extent as WT cDC. This was true for both FL cultures and GM-CSF cultures. These results indicate that, despite being fewer in number, those cells that do develop in the absence of Pin1 respond to LPS stimulation to the same extent as WT cells. To determine whether a 50% decrease in the numbers of CD8+ cDC had consequences *in vivo*, expansion of adoptively transferred WT CD8+ T cells was measured in WT and Pin1-null mice that were infected

with *Listeria monocytogenes*. Although we cannot rule out possible contributions of other cells, the 50% decrease in WT CD8<sup>+</sup> T cell expansion in Pin1-null mice suggests that decreased CD8<sup>+</sup> cDC can impact the stimulation of CD8<sup>+</sup> T cell expansion *in vivo*.

Examination of both FL-generated BMDC and MEF revealed that PU.1 protein expression was elevated in the absence of Pin1. MEF were utilized to demonstrate that Pin1 interacts with PU.1. Although an interaction between Pin1 and PU.1 has been shown previously, we expanded on this relationship by demonstrating that Pin1 modulates PU.1 protein half-life. Because PU.1 has been implicated as a modulator of DC development, we believe that the regulation of PU.1 by Pin1 is a relevant contributor to the defects we observe *in vivo*. Further work is required to fully elucidate the mechanism by which Pin1 regulates PU.1 activity, and to determine how deregulated PU.1 activity might preferentially impact CD8<sup>+</sup> cDC development.

Collectively, the work described herein demonstrates a previously unknown role for Pin1 in preferentially modulating CD8<sup>+</sup> cDC development. Because CD8<sup>+</sup> cDC are crucial for eliciting CD8<sup>+</sup> T cell responses, modulation of Pin1 may be relevant for fine-tuning immune responses to a variety of insults, as is the case for the PPIases cyclophilin A and FKBP12 (Cardenas et al, 1994; Fruman et al, 1994). Due to the wealth of literature implicating Pin1 in tumor progression, it has been suggested that Pin1 inhibitors may be beneficial for treating cancer patients (Finn & Lu, 2008; Xu & Etzkorn, 2009). However, based on the results described in this dissertation and elsewhere, the consequences of

Pin1 inhibition on immune function should be taken into account when considering Pin1 inhibitors in the clinic. Cross-presentation of tumor-associated antigens is important for eliciting tumor-specific CD8+ T cell responses (Melief, 2008), and CD8+ cDC have been demonstrated to be particularly adept at cross-presentation (Belz et al, 2005; den Haan et al, 2000). Additionally, Batf3-null mice, which lack the CD8+ subset of cDC, exhibit an impaired response to tumor challenge (Hildner et al, 2008). Due to the capacity of DC to prime T cells, DC-based immunotherapies have been in development for the treatment of cancer patients for many years (Delamarre & Mellman, 2011; Rescigno et al, 2007). In light of the important contribution of CD8+ cDC to tumor immunity, Pin1 inhibitors may prove to be less effective at eradicating tumors than anticipated due to a dampening of tumor-specific immune responses. Furthermore, Pin1 inhibition may be detrimental, and therefore inappropriate, if combined with DC-based therapies that aim to elicit CD8+ T cell-based responses.

## **5.2 Future Directions**

### **5.2.1 Cellular Defects**

Upon continuous *in vivo* administration of BrdU to mice, the CD8+ cDC subset of cDC exhibits the most rapid labeling kinetics, indicating that these cells are produced and turned over more quickly than other cDC subsets (Kamath et al, 2000).

Additionally, under conditions that stimulate DC expansion *in vivo*, such as challenge with monophosphoryl lipid A, injection of FL, and bone marrow transplantation, the

CD8+ subset of cDC has been demonstrated to exhibit the greatest degree of expansion (Kamath et al, 2000; O'Keeffe et al, 2002). It is conceivable that retarded development in the absence of Pin1 could produce a more pronounced defect in the accumulation of CD8+ cDC, which are more quickly turned over *in vivo*. Similarly, if rapid expansion of this subset is induced in response to various stimuli, delayed development of new CD8+ cDC would manifest itself as a decrease in CD8+ cDC under such conditions. Further supporting a possible timing defect in the production of Pin1-null CD8+ cDC are reports that forced overexpression of PU.1 can inhibit the proliferation of both murine erythroleukemia (MEL) cells and the human K562 myeloid leukemia cell line. Such results are perhaps not surprising in light of the general role for PU.1 in promoting terminal differentiation. Additionally, PU.1 overexpression in MEL leads to an accumulation of cells in G0/G1, as indicated by FACS analysis. Since PU.1 expression is elevated in Pin1-null BMDC, and cells lacking Pin1 have previously been reported to exhibit a delay in G0 exit, it seems likely that a similar mechanism may impair CD8+ cDC production in the absence of Pin1 (Delgado et al, 1998; Yamada et al, 1997). Further investigations should be conducted to determine whether the absence of Pin1 induces a similar lengthening of the cell cycle in pre-cDC. BrdU labeling *in vivo* followed by staining for pre-cDC in blood and fully developed cDC subsets in the spleen under both steady-state and inflammatory conditions should be carried out to address this

possibility. BrdU labeling could also be carried out in *ex vivo* bone marrow cultures in the presence of either FL or GM-CSF.

Lymphoid-resident DC subsets exist not only the spleen, but also populate the lymph nodes. The data we present in this dissertation, however, is derived solely from spleen DC, as the spleen is both easy to acquire and contains much greater numbers of DC than lymph nodes. Despite the potential difficulty in obtaining sufficient numbers of cells to identify the various DC subsets, such experiments should be carried out to confirm a general defect in DC development that permeates multiple lymphoid organs. As such analysis is routinely performed by other groups, it should also be performed in Pin1-null mice.

In addition to lymphoid-resident DC, there exist DC in non-lymphoid tissues, such as the lung, gut, and skin. In particular, the CD103+Mac1- subset of non-lymphoid dermal DC has been shown to exhibit similar patterns of gene expression to CD8+ spleen cDC (Ginhoux et al, 2009; Hildner et al, 2008; Jackson et al, 2011). Indeed, Batf3-null mice were also found to lack this subset of DC as well as CD8+ spleen DC (Hildner et al, 2008). Because of these similarities, the presence of CD103+Mac1- dermal DC should be examined in Pin1-null mice. This can be done by staining for this population in skin-draining lymph nodes, or by enzymatic digestion of skin to produce a single-cell suspension followed by subsequent staining and analysis by flow cytometry.

The majority of work described in this dissertation addresses the role of Pin1 in DC development. With the exception of determining the expression of co-stimulatory molecules in BMDC stimulated with LPS, we did not directly address DC function. The induced expression of CD40 and CD86 observed in BMDC should be confirmed *in vivo* by administering LPS to mice and staining spleen DC with antibodies against these markers. Additionally, further experiments should be conducted to address the ability of Pin1-null DC to secrete cytokines. Specifically, FL cultured BMDC subsets should be stimulated with different TLR ligands, including LPS (TLR4), Poly (I:C) (TLR3), and CpG (TLR9), to induce production of cytokines such as IL-6, TNF $\alpha$ , IL-12, and IL-10, which can be measured by ELISA. Any identified defects in cytokine production should then be confirmed by isolating cDC subsets from spleen (this can be done either by flow cytometry, or by enrichment using magnetic bead-based kits) and stimulating each subset *ex vivo*. To finally validate results *in vivo*, WT and Pin1-null mice should be challenged with the same TLR ligand or with a pathogen that elicits a similar response, followed by serum cytokine measurement and local cytokine production in spleen by RT-qPCR. It would be interesting to determine if defects exist in the absence of Pin1, and to compare these results to those obtained by Tun-Kyi et al. from BMDC generated using GM-CSF (Tun-Kyi, 2011).

Although the results obtained from infection with *Listeria monocytogenes* indicated that Pin1-null mice were defective in eliciting robust CD8+ T cell proliferation,

we did not demonstrate that this defect was the direct consequence of CD8+ cDC deficiency. To confirm that this is the case, defects in T cell proliferation in Pin- null mice should be rescued by injecting mice with WT CD8+ cDC. Furthermore, if the reduced number of CD8+ T cells is truly a reflection of fewer Pin1-null CD8+ cDC and does not result from impaired function, injection of additional Pin1-null CD8+ cDC should also be able to rescue the defect in T cell proliferation.

## **5.2.2 Molecular Mechanism**

### **5.2.2.1 PU.1 binding partners and activity**

PU.1 activity is regulated by multiple mechanisms, including phosphorylation and protein-protein interactions (Lloberas et al, 1999; Marecki & Fenton, 2000; Rosenbauer & Tenen, 2007). Three putative Pin1 binding sites (S/T-P) exist in the PU.1 protein, two of which reside in the PEST domain, which has been shown to participate in the binding of IRF transcription factors, including IRF8 (Gangenahalli et al, 2005; Nakano et al, 2005). Thus far, two phosphorylation events have been described within the PEST domain of PU.1: S142 and S148. S148 is the most extensively studied, and has been shown to be phosphorylated by CKII *in vitro* (Lodie et al, 1997). S142 was shown to be phosphorylated by p38MAPK (Wang et al, 2003). Recently, Akiyama et al. investigated the contribution of three S/T-P sites within the PU.1 protein to Pin1 binding. The numeration of the amino acid substitutions was difficult to follow, however, as the described mutations did not correspond to the amino acids of mouse PU.1. If one shifts

the numbering, however, the S119A mutant described by this group corresponds to S126, which is the first serine present in the PEST domain of PU.1. The S119A mutant of PU.1 was unable to bind Pin1, although T92A and S132A maintained the interaction with Pin1 (Akiyama et al, 2011). Because the exact mutations were not properly documented by this group and remain questionable, these results should be confirmed by generating point mutants of PU.1 at each of the putative Pin1 binding sites, expressing the constructs in hematopoietic cell lines that are amenable to transfection/infection, such as THP-1 macrophages or RAW296.4 cells, and determining the ability of each mutant to bind Pin1.

Pin1 has previously been shown to regulate dephosphorylation of its substrates. It is therefore of interest to determine whether loss of Pin1 impacts PU.1 phosphorylation. Since phospho-specific antibodies to PU.1 are not commercially available, total PU.1 protein should be immunoprecipitated from WT and Pin1-null cells and probed with antibodies specific for phospho-serine-proline or phospho-threonine-proline. Additionally, interactions between Pin1 and PU.1 should also be compared between cells that are resting and cells that are acutely stimulated with LPS or other TLR ligands to determine whether Pin1 binding to PU.1 is constitutive or inducible. These investigations should be carried out in untransformed cells such as FL-generated BMDC and MEF.

Given that a point mutation has been described in IRF8 that inhibits its ability to interact with PU.1 and produces a defect in CD8+ cDC, but not pDC (Tailor et al, 2008), it is not difficult to imagine that a corresponding defect in PU.1 could similarly disrupt the PU.1-IRF8 interaction and deregulate CD8+ cDC production. Further experiments should investigate this possibility by determining whether the interaction between PU.1 and IRF8 is altered in the absence of Pin1. Additionally, a more thorough analysis of PU.1 target genes should be conducted to determine whether loss of Pin1 results in impaired PU.1 activity.

In addition to investigating PU.1 dimerization with IRF8, other proteins should be investigated for their ability to bind PU.1 in the absence of Pin1. Indeed, the previously identified Pin1 substrates c-jun and Bcl6 have also been shown to interact with PU.1 and modulate the transcription of target genes (Behre et al, 1999; Joo et al, 2009; Wei et al, 2009). It is possible that Pin1 modulates specific complexes that include PU.1 and other proteins, thereby directing the transcription of targets that impinge on CD8+ cDC development.

#### **5.2.2.2 Flt3 Receptor signaling**

Despite identifying a role for Pin1 in modulating FL-dependent expansion of DC *in vivo* and FL-dependent generation of DC in bone marrow cultures, we have not investigated signaling downstream of the Flt3 receptor. Pin1-null BMDC were demonstrated to express Flt3 mRNA to the same extent as WT BMDC, but Flt3 protein

was not measured. Cell surface staining for Flt3 expression was attempted unsuccessfully in BMDC (data not shown), perhaps due to receptor down-regulation in response to supraphysiologic quantities of FL. Flt3 protein should therefore be quantified by immunoblot, as commercial antibodies are available. Signaling events downstream of acute FL stimulation should also be investigated to determine if there exist defects in the absence of Pin1. FL-induced events include tyrosine phosphorylation of SHC, SHP-2, and SHIP, as well as activation of the PI3K and MAPK pathways (Gilliland & Griffin, 2002). Additionally, both Stat5 and Stat3 activity are induced by FL stimulation (Hayakawa et al, 2000; Laouar et al, 2003). As Pin1 has previously been described as a modulator of Stat3 transcriptional activity (Lufei et al, 2007), Stat3 seems a likely first candidate to investigate.

### **5.2.3 Tumor Models**

The ability of the immune system to eradicate tumor cells relies primarily on the CD8+ cytotoxic T cell response. In order to avoid immune destruction, tumors often employ strategies that either directly inhibit T cells or prevent effective T cell activation by antigen presenting cells. Indeed, defects in DC activation have been detected in cancer patients and are thought to contribute to the induction of T cell anergy by failing to provide strong activation signals (Almand et al, 2000; Lenahan & Avigan, 2006; Lin et al, 2010; Pockaj et al, 2004). In addition to defective DC function, tumor progression has been found to be associated with decreased circulating DC and increased immature cells

lacking hematopoietic markers (Almand et al, 2000). Furthermore, the infiltration of mature DC in breast tumors has been found to correlate with a favorable outcome (Allan et al, 2004; Kohrt et al, 2005). These studies suggest that both defects in DC function and decreases in DC numbers may impact tumor formation and clinical outcome.

Cross-presentation of tumor antigens has been shown to be important for activating tumor-specific CD8<sup>+</sup> T cell responses in mice (Hildner et al, 2008; Lorenzi S, 2011; Matheoud et al, 2011). Because CD8<sup>+</sup> cDC have been shown to be particularly efficient at cross-priming CD8<sup>+</sup> T cells (Belz et al, 2005; den Haan et al, 2000; Hildner et al, 2008), we predict that the decrease in CD8<sup>+</sup> cDC observed in Pin1-null mice will impact CD8<sup>+</sup> T cell activation in response to tumor formation *in vivo*. Additionally, recent reports suggest that IFN $\alpha/\beta$  production may enhance CD8<sup>+</sup> cDC survival and cross-presenting ability (Lorenzi S, 2011). The ability of Pin1 to modulate IFN $\alpha$  production by pDC further supports the prediction that Pin1-null mice would be more susceptible to developing tumors than WT mice (Tun-Kyi, 2011).

To address whether Pin1-null mice are more susceptible to tumor formation, two syngeneic tumor models could potentially be evaluated. The B16 melanoma cell line is derived from a spontaneous melanoma that arose in a C57BL/6 mouse, and has been demonstrated to produce tumors when injected subcutaneously into mice. Furthermore, the tumors that develop have been demonstrated to modulate immune function (Becker et al, 2010). A second model that can be employed is the injection of EO771 cells into

mammary fat pads of mice to produce mammary tumors. EO771 cells are derived from a spontaneously occurring estrogen receptor positive mammary adenocarcinoma that developed in a C57BL/6 mouse. These cells eventually give rise to highly invasive mammary tumors that eventually metastasize to the lung (Ewens et al, 2006). Tumor progression should be monitored in WT and Pin1-null mice to determine if loss of Pin1 impacts the timing of tumor formation or the total tumor burden. If results indicated a role for Pin1 in modulation of tumor development, these cell lines could also be introduced to irradiated WT mice that have been reconstituted with Pin1-null bone marrow. Altered tumor progression in recipients of Pin1-null bone marrow would confirm that cells of the immune system were modulating tumor outgrowth.

#### **5.2.4 Summary**

The work in this dissertation describes the identification of a cellular defect in Pin1-null mice that is relevant in the context of immune responses to infection and tumor progression. The data presented are largely descriptive, although biochemical evidence is also provided to suggest that the observed defects may be a result of deregulated PU.1 expression. Future efforts should be aimed towards: 1) gaining a more detailed understanding of both the cellular and molecular defects that give rise to the observed deficiency in CD8<sup>+</sup> cDC numbers in Pin1-null mice, and 2) determining how such defects impact tumor formation. Given the previously described roles for Pin1 in modulating the timing of cell cycle progression, the consequence of Pin1 loss on the

proliferation of CD8<sup>+</sup> cDC precursors should be determined. Additionally, the molecular mechanism by which Pin1 modulates cDC development should be more thoroughly investigated. This includes examining signaling events downstream of the Flt3 receptor, as well as determining the consequence of Pin1 binding on PU.1 function. Finally, in light of the advances in understanding how tumor progression is regulated by cells of the immune system, including dendritic cells and T cells, the consequence of Pin1 deletion in immune cells should be evaluated in the context of tumor formation. Collectively, these experiments will determine the ability of Pin1 to modulate infection and tumor progression; additionally, they will provide biochemical information that could be used to design therapies with the potential to more specifically target CD8<sup>+</sup> cDC-dependent responses in the context of disease.

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## Biography

Theresa J. Barberi was born to Phil and Sharon Barberi on December 1, 1981 in San Diego, CA, and moved to Chesapeake, VA in 1990. In 1999 she began attending The College of William & Mary in Williamsburg, VA, where she received a Bachelor of Science in Biology in 2003. She was then accepted into the NIH Postbac IRTA Program and worked for one year in the laboratory of Dan L. Longo, M.D., within the Lymphocyte Cell Biology Unit of the National Institute on Aging in Baltimore, MD. While in the Longo laboratory, she was the recipient of the 2004 Nathan W. Shock Trainee Award, and contributed to two publications:

- (1) Sasaki CY, Barberi TJ, Ghosh P, Longo DL. Phosphorylation of RelA/p65 on serine 536 defines an IkappaBalpha-independent NF-kappaB pathway. *J Biol Chem.* 2005 Oct 14; 280(41):34538-47.
- (2) Sasaki CY, Slemenda CF, Ghosh P, Barberi TJ, Longo DL. Traf1 induction and protection from tumor necrosis factor by nuclear factor-kappaB p65 is independent of serine 536 phosphorylation. *Cancer Res.* 2007 Dec 1; 67(23):11218-25.

In 2004, Theresa gained acceptance into the Department of Pharmacology & Cancer Biology at Duke University in Durham, NC, where she pursued a Doctor of Philosophy degree in Molecular Cancer Biology in the laboratory of Anthony R. Means, Ph.D. In 2007, she was awarded a Department of Defense Breast Cancer Research Predoctoral Fellowship Award (W81XWH-08-1-0216). Additionally, Theresa presented research posters at two national conferences: Regulatory Networks in Immunity & Inflammation Conference in Napa Valley, CA in 2010, and the Department of Defense Era of Hope

Conference in Orlando, FL in 2011. During her time as a graduate student in the Means lab, she submitted one manuscript for publication:

- (1) Barberi TJ, Dunkle A, He YW, Racioppi L, Means AR. The Prolyl Isomerase Pin1 Modulates Development of CD8+ cDC in Mice. *PLoS One*. *Submitted for Review*