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REGULATION OF TYPE I IFN PRODUCTION IN PLASMACYTOID DENDRITIC
CELLS BY SRC-FAMILY KINASES AND CD28

A thesis submitted in partial satisfaction of the requirements

for the degree of Master of Science

in

Biology

by

Charles R. Hesser

Committee in charge:

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      Professor Michael David
      Professor Stephen Hedrick

2012

The thesis of Charles R. Hesser is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2012

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ABSTRACT OF THE THESIS

REGULATION OF TYPE I IFN PRODUCTION IN PLASMACYTOID DENDRITIC
CELLS BY SRC-FAMILY KINASES AND CD28

by

Charles R. Hesser

Master of Science in Biology

University of California, San Diego, 2012

Professor Elina I. Zuñiga, Chair

Plasmacytoid dendritic cells (pDCs) are a dendritic cell subset specialized to rapidly secrete copious amounts of Type I Interferon (IFN-I), a group of innate mediators that play key roles in antiviral immune defense and autoimmune diseases. Loss of pDC-derived IFN-I during chronic viral infection enhances susceptibility to secondary infection while excessive pDC IFN-I production contributes to autoimmune pathology, demonstrating the need for further investigation into the mechanisms of IFN-I regulation in pDCs. Through comparing gene expression profiles of pDCs and conventional (c) DCs, we found that Fyn, a member of the src-family kinases, and CD28, a prototypic T cell co-

stimulatory receptor, were highly and selectively expressed in pDCs. Fyn acted as a positive regulator of pDC IFN-I and inflammatory cytokine production upon toll-like receptor (TLR) stimulation. In contrast, CD28 acted as a negative regulator of pDC IFN-I production but did not affect production of inflammatory cytokines or maturation. Our data suggests that Fyn and CD28 may play important roles in the regulation of pDC cytokine response during pathogenic challenge. These pathways may have evolved to fine-tune the magnitude of innate responses and to coordinate them with the adaptive immune response. Future studies will determine the mechanisms by which CD28 and Fyn regulate pDC cytokine production, including potential cross-talk with the Phosphoinositide 3-Kinase   and   TLR   signaling   pathways.

Introduction:

Overview of Type I Interferons

Type I Interferons (IFN-I), which comprise twelve isoforms of IFN-α and a single form of IFN-β, constitute an integral component of the innate immune response (1, 2). Potent anti-viral mediators, IFN-I are induced upon recognition of microbes through cytosolic or membrane-bound pattern recognition receptors (2). Secreted IFN-I act in an autocrine and paracrine fashion, binding to their cognate heterodimeric receptor, IFN-αβR, which signals through two Janus family kinases, Tyk2 and Jak1 (1). The resulting Jak/Stat phosphorylation generates a positive feedback loop, driving further IFN-I production (1). Not only does IFN-I serve to stimulate transcription of hundreds of Interferon Stimulated Genes (ISGs) that promote synergistic anti-viral effects (1, 2) but IFN-I also help to activate T cells and NK cells, which produce IFN-γ to promote killing of virally infected cells. While the role of IFN-I is best characterized during infection, IFN-I also contributes to immune homeostasis by way of tonic signaling, enabling the survival, differentiation and proliferation of various cell types (3, 4). In contrast, during autoimmune diseases such as systemic lupus erythematosus (SLE) or psoriasis, excess IFN-I production mediates both symptoms and pathology (3, 5). Thus, IFN-I production must be tightly regulated.

pDCs secrete IFN-I and inflammatory cytokines in response to TLR signaling

While almost all cell types are capable of making IFN-I upon microbial infection, pDCs are specialized to make 100-1000 times more IFN-I than other cells types (6). pDCs also produce inflammatory cytokines and chemokines such as IL-12,TNF-α, Il-1α, MCP1, MIP1β and RANTES, upon endosomal Toll-like receptor (TLR) 7 and 9 recognition (7). Murine pDCs are defined by several key surface markers, including low to moderate expression of CD11c, high levels of B220, the absence of CD11b, and unique expression of Siglec H and pDCA-1 (8). While conventional CD11b+ DCs (cDCs) play a vital role in presenting antigen to T cells, pDCs are poor antigen presenters (7, 9). However, pDCs still play an important role in stimulating the innate and adaptive immune response as the most powerful producers of IFN-I and other cytokines following infection (6, 10). Furthermore, upon antigen exposure, pDCs help to control homeostasis of effector and regulatory CD4(+) T cells, and participate in the initiation of CD8(+) T cell responses (11, 12). In sum, pDCs serve as a critical "frontline" responder in several models of viral infection, orchestrating the function of multiple types of immune effector cells.

pDC cytokine production occurs through a TLR7 and TLR9-dependent, pathway. Upon ligand internalization into the early endosome, a MyD88-dependent pathway induces phosphorylation and nuclear translocation of IRF7, a key transcription factor, which initiates IFN-α and IFNβ transcription. While many cell types upregulate IRF7 following activation, pDCs are characterized by high

basal IRF7 expression, which is further amplified following TLR triggering, allowing pDCs to rapidly produce large amounts of IFN-I (6). Indeed, in contrast to other DC subsets, IFN-I induction in pDCs is independent from other TLRs or the cytoplasmic RNA helicases RIG-I and Mda5 (9, 13). Production of inflammatory cytokines and chemokines by pDCs also occurs via TLR7/TLR9 mediated MyD88-dependent signaling, but instead involves activation of mitogen activated protein kinases (MAPK) and nuclear translocation of NFκB (14). Thus, while NFκB activation upon TLR7/9 engagement occurs in multiple cell types, including cDCs, initiation of IFN-I responses by IRF7 is spatiotemporally distinct and unique to pDCs (10, 15). Finally, both NFkB activation and IFN-I signaling drive up-regulation of pDC maturation markers such as CD80, CD86 and MHCII (16).
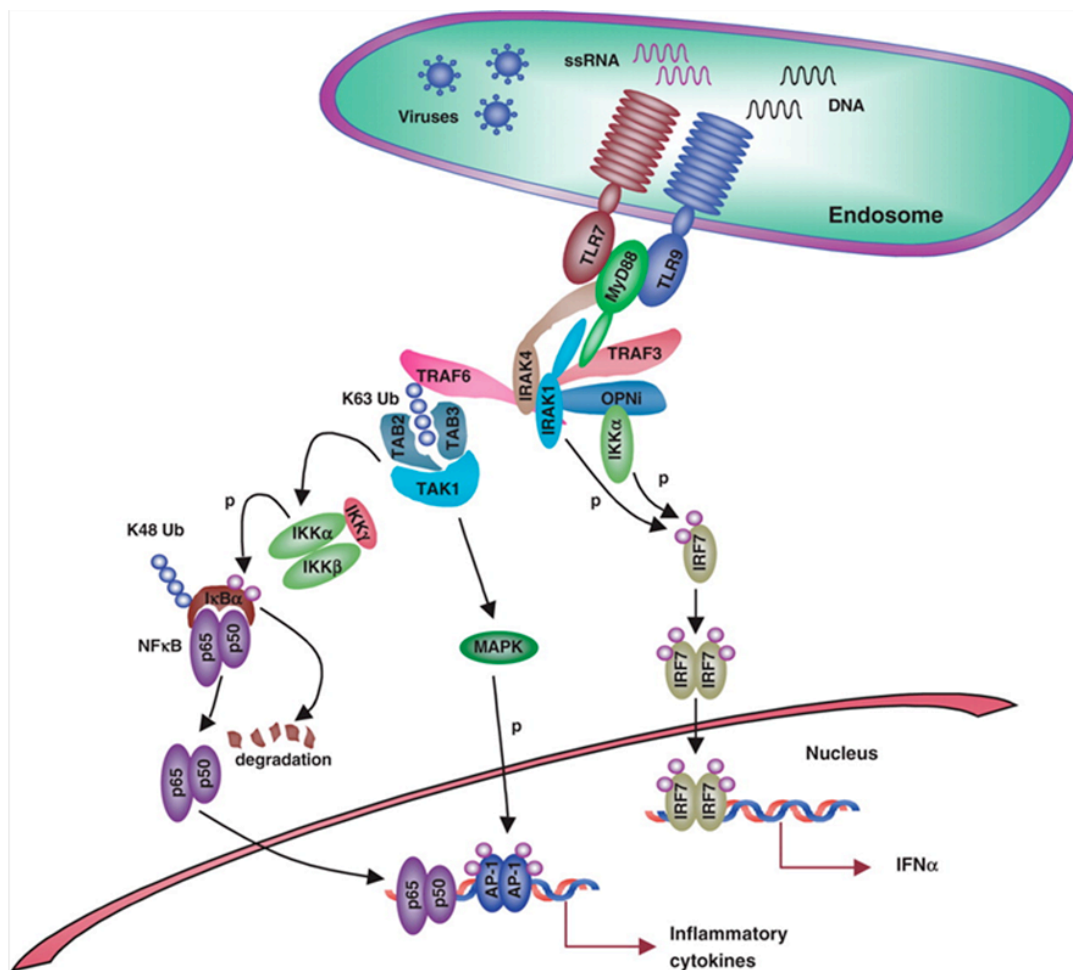
Figure 1. pDCs produce IFNα and inflammatory cytokines in response to TLR7/TLR9 stimulation. Overview of bifurcated MyD88 signaling pathway leading to NFκB and IRF7 activation and nuclear translocation. This panel is reproduced from Kawai, T., and S. Akira. 2007. Antiviral signaling through pattern recognition receptors. *J Biochem* 141:137-145.

pDC IFN-I production is critical to viral control, but is also frequently dysregulated

pDCs rapidly produce IFN-I in response to viral infections recognized by the TLR7 and TLR9 endosomal pathways. TLR7 recognizes ssRNA viruses such as Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV), mouse hepatitis virus (MHV), and the prototypic arenavirus Lymphocytic Choriomeningitis Virus (LCMV), while TLR9 recognizes unmethylated CpG-rich

regions of DNA viruses such as Murine Cytomegalogvirus (MCMV), human CMV, and the related herpes simplex virus (HSV) upon retention in the early endosome (17, 18). Studies demonstrate that pDC-derived IFN-I plays a vital role in viral control during infection with MCMV, where pDCs are responsible for the peak of IFN-I in serum 36 hours post-infection, thus contributing to the activation of T cells and NK cells (19). Furthermore, pDC IFN-I is also necessary for control of MHV, as mice deficient in pDCs fail to generate productive T cell responses and do not clear the otherwise acute infection, once again demonstrating the important role that pDC cytokine production plays in stimulating both the innate and adaptive immune system (11).

Despite its potential in reducing viral burden, pDC IFN-I has been shown to be dysregulated in the context of several chronic viral infections. Early after LCMV Cl 13 infection, for example, murine pDCs are a major early target of infection (8), yet produce copious amounts of IFN-I within the first 24 hours of infection. Despite this rapid and robust response, pDC IFN-I production is rapidly silenced within 3 days of infection, while viral replication persists (20). Similarly, persistent human viruses such as HIV-1 induce substantial IFN-I production upon incubation with pDCs (21). However, silencing of pDC IFN-I is associated with viral spread and immune deficiency (21). It has recently been shown that pDC levels and IFN-α production by peripheral blood mononuclear cells (PBMCs) are higher in "elite controllers," individuals who have maintained low to undetectable levels of HIV in their blood for over a decade without ever having taken

antiretroviral drugs (22). These individuals are better able to reduce viral replication and T cell apoptosis *in vitro.* However, in a race between the host and pathogen, viruses such as HIV-1 have evolved ways to silence IFN-I production to further their own propagation. Indeed, the rapid silencing of IFN-I is a hallmark of pathogenesis, leading to the establishment of a chronic immunosuppressive environment, and increasing the susceptibility of chronically HIV-infected patients to potentially lethal secondary infections (23).

While pDC IFN-I is produced at high levels during the acute phase response to multiple pathogens, it can also be produced aberrantly in the context of autoimmune pathology (24). For example, in patients with SLE, immune complexes consisting of auto-antibodies bound to self-DNA and RNA can stimulate production of pDC IFN-I through the TLR7 and TLR9 pathways (24-29). Furthermore, peripheral blood pDCs from multiple sclerosis patients exhibit functional abnormalities and maturation defects, including lower levels of CD86, CD83 and CD40 (30). Thus, an understanding of the mechanisms of positive and negative regulation of pDC IFN-I production is critical to maintain immunoprotective functions while avoiding autoimmune pathology.

Regulation of pDC IFN-I production is not completely understood

To date, only a handful of receptors that may contribute to positive or negative regulation of IFN-I have been identified on pDCs. For example, PDC-TREM is preferentially expressed on the surface of pDCs following TLR stimulation, and is known to augment IFN-I production (31). In contrast, Siglec-H mediates signals through the associated adaptor DAP12 to reduce TLR-induced IFN-I production (32). In addition, BDCA-2 and ILT7 function as negative regulators of IFN-I production in human pDCs (33, 34). However, the signaling mechanisms of these mediators of pDC IFN-I remain to be fully elucidated, emphasizing the need to both define new targets as well as better characterize the role of known receptors involved in the regulation of pDC IFN-I.

Aims and Objectives

To search for potential molecules involved in pDC IFN-I regulation, we performed a genome-wide DNA microarray on naïve sorted DC subsets, and looked for pDC specific gene expression. Among other pDC-specific genes, we noticed increased expression of the src family kinase (SFK) fyn and the prototypic T cell co-stimulatory molecule CD28 in pDCs. As human pDCs exhibit a BCR-like signalosome which involves SFK (35), **we sought to determine whether SFK play a role in the regulation of pDC cytokine production**. In addition, given that CD28 signaling contributes to activation and proliferation of T cells, **we also decided to investigate a putative role for CD28 in pDC functions.**

To this end, we used murine bone marrow derived DCs (BM DCs) to determine whether SFKs are activated upon TLR stimulation, and whether this activation correlates with increased IFN-I and inflammatory cytokine production (Chapter 1). We also used mice lacking CD28 and agonistic CD28 antibodies to determine whether CD28 played a role in pDC IFN-I function, both *in vitro* and *in vivo* (Chapter 2). Lastly, as CD28 signaling in T cells is known to be dependent on SFK and the Phosphoinositide 3-Kinase (PI3K) pathways, we sought to determine if these pathways contributed to CD28 signaling in pDCs (Chapter 3).

CHAPTER 1: The role of Src-family kinases in pDC cytokine production.

Introduction:

Src-family kinases (SFK) are a group of non-receptor tyrosine kinases that play vital and diverse roles in cytokine signaling via phosphorylation of downstream targets. SFKs are known to be involved in the control of cell proliferation as well as integrin-mediated signaling and cell adhesion (36). Dysregulation of SFKs is known to occur in oncogenic cell transformation and autoimmune diseases. Indeed, c-Src is aberrantly activated in a variety of epithelial and non-epithelial cancers (37). As such, small molecule inhibitors of SFK are in development as cancer treatments (37). Furthermore, Lyn-deficient mice develop antibody (Ab)-mediated autoimmune disease resembling systemic lupus erythematosus involving pathology mediated by hyperactive B cells (38). Additionally, the related spleen tyrosine kinase Syk has been shown to play a role in the progression of rheumatoid arthritis, and a specific-Syk inhibitor is now in phase-II clinical trials in humans (39).Thus, elucidating the molecular mechanisms of SFK signaling is critical to developing better therapeutic treatments for a wide variety of diseases.

Within the context of the immune response, SFK play critical roles in a variety of cell types, including B cells and T cells. Among the SFK, Lyn is highly expressed In B cells, and contributes to the initiation of B-cell receptor signaling and B cell maturation (40). Furthermore, human pDCs are known to express a set of molecules that resemble the BCR signaling cascade, including high levels of Lyn expression (35). Another member of the SFK, Fyn, has been shown to

play a role in both T cell and mast cell function. Engagement of Fyn is involved in mast cell degranulation and involves OX40 and OX40 ligand (L) ligation (41). It has also been shown that Fyn may help regulate the developmental balance between the Th17 and Treg cell subsets (42). We recently identified high levels of expression of SFK in both pDCs and cDCs. However, no studies thus far have directly examined whether SFK contribute to dendritic cell function.

Given the important role of SFK in both immune cell function and disease pathogenesis as well as the novel finding of SFK Fyn in pDCs, we sought to determine whether SFK contributed to the regulation of TLR signaling in dendritic cells. As Fyn is highly expressed in pDCs but not cDCs, we postulated that Fyn might be playing a specific role in pDC cytokine production. To address whether SFK contribute to the regulation of IFN-I as well as pro-inflammatory cytokine production in pDCs, we analyzed src activation in response to TLR stimulation. We then evaluated the functional role of Fyn and Lyn SFKs in both wild type and fyn or lyn deficient mice.   We found that Lyn and Fyn contribute to both IFN-I and pro-inflammatory cytokine production in BM-derived pDCs in response to TLR9 stimulation. Our data suggests the Src family of proteins may play an important role in pDC cytokine response, implying their therapeutic potential during both chronic viral infections, where pDC IFN-I response is silenced, and/or autoimmune diseases where pDC IFN-I response is exacerbated.

Results

Fyn is selectively expressed in bone marrow-derived pDCs

To identify which src-family kinases are highly expressed in DCs, we performed a genome-wide DNA microarray on naïve sorted DC subsets. We observed expression of *lyn*, *fyn*, *fgr* and *syk* in dendritic cells sorted from naïve murine splenocytes (Figure 2a). Of particular note, we observed that while certain SFK appeared to be highly expressed in both pDCs and cDCs (i.e. Lyn and Fgr), Fyn was much more highly expressed in pDCs. To confirm the expression of SFK transcripts observed by DNA microarray, sorted subsets from splenic leukocytes were analyzed by qPCR (Figure 2b). We confirmed that *fyn* transcript was expressed in pDCs, yet was undetectable in splenic macrophages, CD8α conventional (c)DCs or CD11b cDCs   Among the SFKs, *lyn* transcript levels were highest in both pDCs and CD11b cDCs, with the latter having slightly, but non-significantly higher expression. We also confirmed that *hck* was highly expressed in CD11b DCs but not pDCs. While *fgr* had appeared as a potential gene of interest from our microarray, we saw that it was expressed at a relatively low level in pDCs and cDCs. Overall, this data confirmed that significant levels of SFK transcripts are found in both pDCs and cDCs.

Figure 2. Expression of src family kinases in DC subsets. Splenic pDC (CD11c⁺B220⁺PDCA⁺CD3⁻CD19⁻NK1.1⁻CD11b⁻), CD11b⁺cDC (CD11c⁺CD11b⁺B220⁻PDCA⁻CD3⁻CD19⁻NK1.1⁻) and CD8⁺cDC (CD11c⁺CD8⁺B220⁻PDCA⁻CD3⁻CD19⁻NK1.1⁻) were FACS purified from C57BL/6 WT mice and processed for DNA microarray analysis. Relative expression of selected genes in pDCs with respect to cDCs is shown for comparison **(a)**. Expression of indicated genes in naïve sorted splenic pDCs and cDCs was confirmed by real time quantitative PCR. Gene expression is normalized to GAPDH **(b)**. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, Student t test.
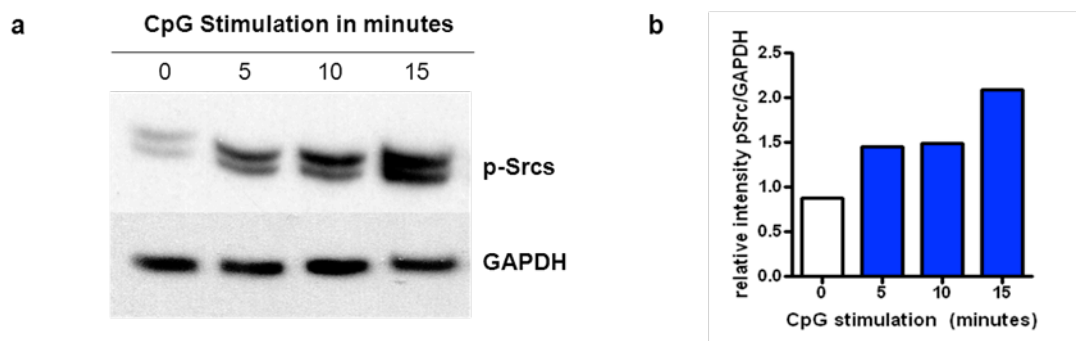
Src phosphorylation increases upon TLR9 Stimulation

Given the high expression of SFKs in DCs at the transcript level, we next investigated whether they become phosphorylated after DC activation. To this

end, we examined whether TLR9 signaling led to increased activation of SFK. We quantified levels of src-phosphorylation between 5 and 15 minutes post-CpG stimulation of Flt3L BM-derived DCs. By Western blot, we observed induction of src-phosphorylation as early as 5 minutes after CpG stimulation (Figure 3a). Densitometry analysis showed that the relative intensity of phospho-src to GAPDH had increased to 1.5 times its original level 5 minutes post-stimulation, and had doubled by 15 minutes post-stimulation (Figure 3b). In summary, src-phosphorylation is triggered rapidly upon TLR9 stimulation in a total bone marrow culture of pDCs and cDCs, supporting the possibility that src-family kinases are playing a role in the DC-TLR stimulation.

Figure 3. Src-phosphorylation increases following CpG stimulation. a. Bone marrow derived DCs were cultured for indicated time points with 0.1 uM CpG ODN 1668. Phosphorylated (p) Srcs were measured in protein cell lysates and GAPDH was used as a loading control. **b.** Relative intensity with respect to GAPDH was quantified by densitometry. A representative of 3 independent experiments is shown.

Src-Family Kinase inhibition decreases DC production of TNFα and IFN-I upon TLR stimulation

We next examined the potential role of SFKs in the pDC functional response to TLR stimulation. To address this question, we pre-treated cells with

a range of doses of PP2, an ATP-competitive inhibitor of SFKs, followed by stimulation with TLR9 agonist CpG. We first considered whether treatment with the chemical inhibitor caused a loss of cell viability due to DMSO toxicity. We observed that the range of doses we used did not have a significant impact on cell viability. Upon staining with propidium iodide (P.I.) an average of approximately 60% of cells were viable after 15 hours in culture with CpG, and viability was only slightly reduced upon treatment with 10 µM PP2, compared to equivalent dose of DMSO control (Figure 4b). Furthermore, we found that treatment with PP2 caused a dose dependent inhibition of both IFN-I and TNF-α, as levels of both cytokines were reduced to background levels when a dose of 5 µM was used (Figure 4c &d). Importantly, even at the highest dose of DMSO control, reduction in IFN-I or TNF-α was negligible, indicating that the observed reductions in cytokine production did not relate to chemical toxicity. Thus, a chemical inhibitor of SFK reduced IFN-I and TNF-α production in a dose dependent fashion, suggesting that SFK act as positive regulators of IFN-I and inflammatory cytokine production.
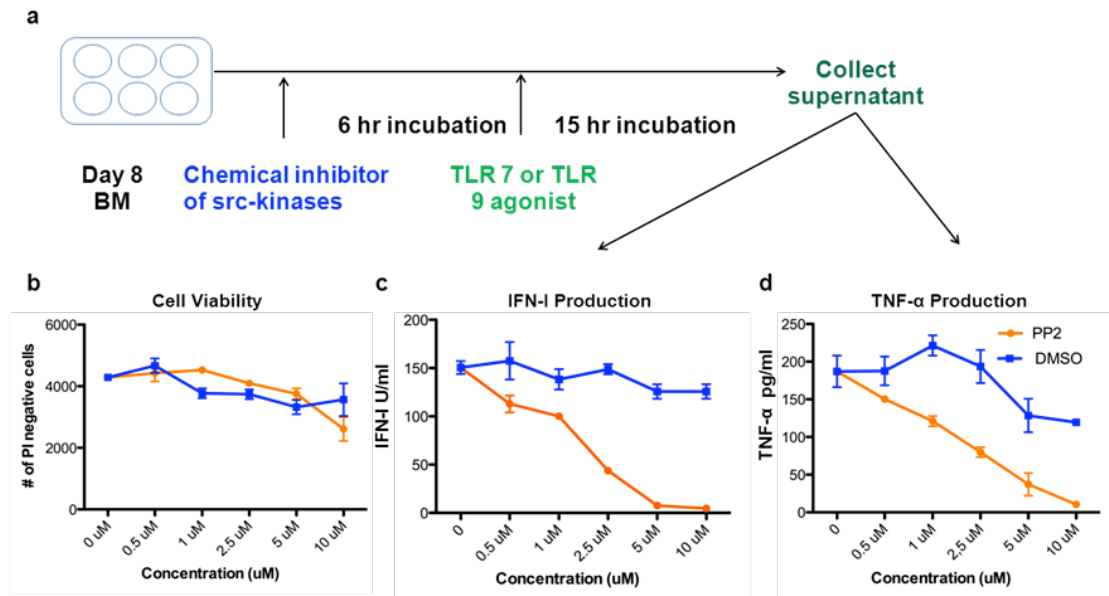
Figure 4. Inhibitor of src-kinases reduces cytokine production in WT mice. a. Bone marrow-derived DCs were pre-treated for 6 hrs with src inhibitor, or the equivalent concentration of DMSO control, followed by 15hrs in media in presence or absence of 0.1 µM CpG-B ODN 1668. **b.** To assay cell viability, cells were stained with PI and analyzed by FACS. **c.** IFN-I production was measured by bioassay. **d.** TNF-α was measured by ELISA. Mean ± SD of ≥3 independent experiments is shown.

Reduced IFN-I and TNF-α production by pDCs from Lyn ko and Fyn ko mice

Owing to the activation of Srcs following TLR stimulation and the high level of expression of Fyn and Lyn in pDCs, we next investigated their potential role in pDC function. To address this question, bone marrow from Fyn ko and Lyn ko mice was differentiated alongside bone marrow from WT mice. To determine if Fyn ko BM-derived DCs had differences in IFN-I or TNF-α in comparison to WT mice, IFN-I was measured 15 hours after stimulation with CpG or the TLR7 agonist loxoribine. BM-derived DCs from Fyn ko mice were found to have significantly lower IFN-I production at Day 7 and Day 9 post-Flt3L culture compared to WT in response to both CpG and loxoribine, with more modest reductions in TNF-α (Figure 5a). Similarly, Lyn ko mice showed a trend toward

reduced IFN-I and TNF-α production (Figure 5b). To investigate whether these differences in cytokine production were due to differences in dendritic cell subset composition or cell viability, FACS staining for DC surface markers was performed, followed by PI staining. The percentage of viable cells both pre and post-TLR agonist stimulation was similar between WT, Lyn ko and Fyn ko DCs. Likewise, pDC and cDC percentages were also similar (Figure 5c, d). Altogether, these results indicate that TLR7 and 9 mediated IFN-I production is significantly decreased in the absence of Fyn and to a lesser extent Lyn, while both Fyn and Lyn deficient mice show a trend toward modest reductions in TNF-α production. Our data suggests that Fyn signaling promotes TLR7 and TLR9 mediated IFN-I and TNF-α production in pDCs.
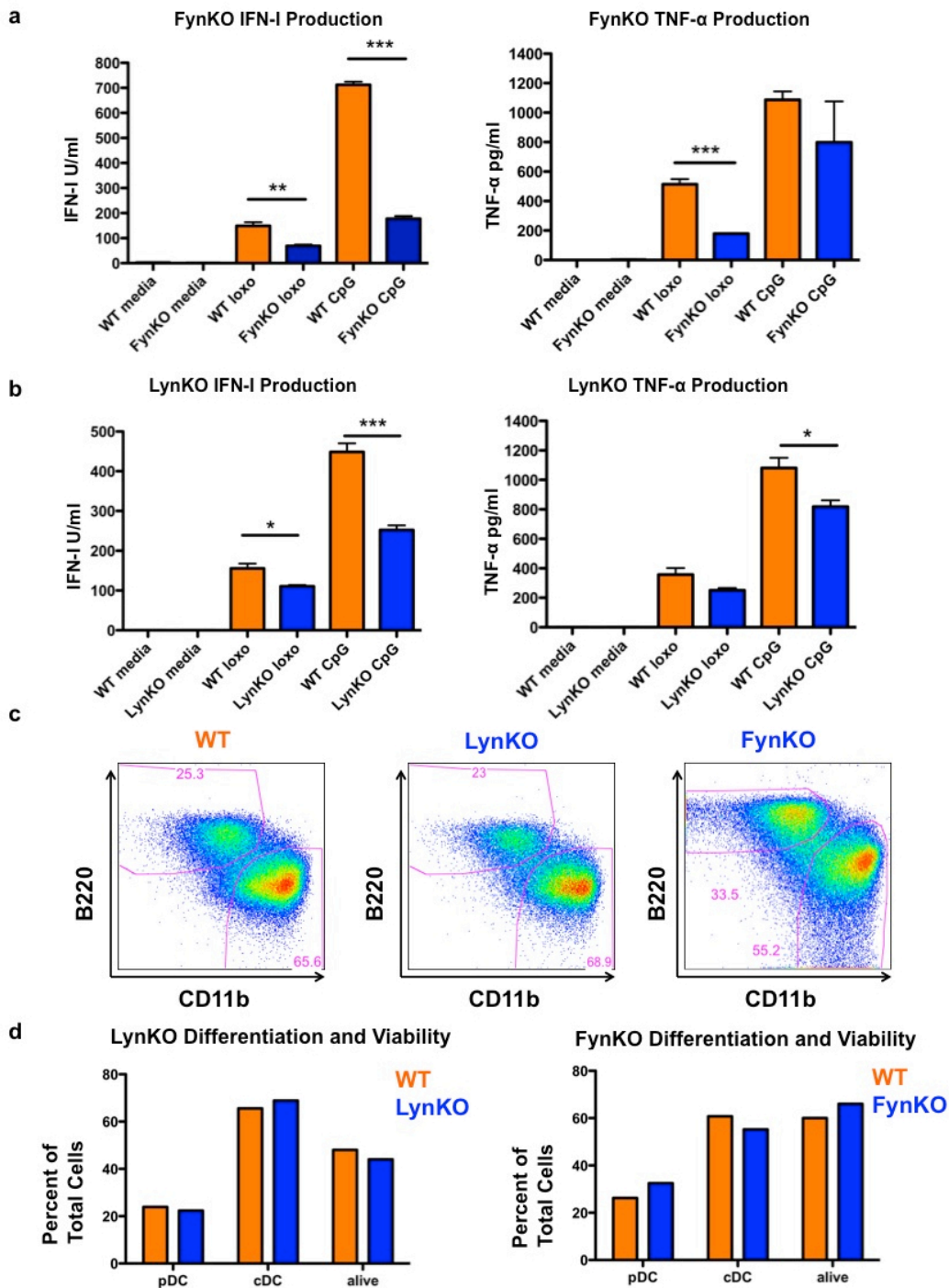
Figure 5. Reduced IFN-I and TNFα in Bone Marrow-derived DCs from LynKO and FynKO mice, despite similar differentiation and viability. Bone marrow from WT and FynKO mice **(a)** or WT and LynKO mice **(b)** was cultured in Flt3L containing media for nine days prior to harvest. Cells were then cultured for 15h in media alone or stimulated with either 100 nM loxoribine (loxo) or 0.1 µM CpG-B ODN 1668. Supernatants were collected and IFN-I levels were measured by bioassay, and TNF-α was measured by ELISA. **c.** Representative FACS plot showing pre-stimulation viability and distribution of DC subsets **d.** Relative pDC and CD11b+ proportions in WT, LynKO and FynKO mice were quantified as a percentage of total viable dendritic cells. After 15h stimulation, cells were stained with PI and analyzed for viability. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Student t test.

Discussion:

In this study, we sought to determine whether SFK play a specific role in pDC cytokine production. We confirmed that SFKs are expressed in both pDCs and cDCs, and are also rapidly phosphorylated upon TLR9 stimulation. Furthermore, we showed that both chemical inhibition of SFK and genetic ablation of SFKs Lyn or Fyn leads to reduced IFN-I and TNF-α production. Our data suggests that Fyn and Lyn act as novel mediators of pDC TLR signaling with the former one exerting a greater effect.

Considering the rapid activation of SFK following CpG stimulation and the reduction of IFN-I upon SFK inhibition, it seems plausible that SFK act to augment pDC TLR signaling. However, as recent research has shown that SFK inhibitor PP2 may also non-specifically inhibit other kinase signaling pathways at high concentrations, other alternative src-family kinase inhibitors should also be used in parallel with PP2, to confirm that observed reductions in cytokine production are not due to off-target effects (3, 43). Furthermore, transfection of a pDC cell line with a constitutively active mutant of src kinase could test whether increased activation of SFK is sufficient to induce increased cytokine production upon TLR stimulation (43). Thus, a combination of treatments with chemical inhibitors and activators of SFK are necessary next steps to confirm that src-activation in DCs leads to increased IFN-I and inflammatory cytokine production.

The trend toward reduced IFN-I and TNF-α in Fyn ko and Lyn ko mice suggests that the SFKs are important not only for IFN-I production but also for

the generalized inflammatory cytokine production upon microbial stimulation. However, as we have only looked at total levels of src-phosphorylation in total BM DCs, the exact contribution of SFK phosphorylation to cytokine production in cDCs versus pDCs remains to be determined. While the IFN-I produced 15 hours post-CpG stimulation is primarily pDC-mediated, both pDCs and cDCs may be contributing to TNF-α production (44). Due to the lack of commercially available highly specific inhibitors of Lyn and Fyn kinases, TLR stimulation of sorted DC subpopulations from Fyn and Lyn ko mice could assess the relative importance of these individual kinases to individual DC subset cytokine production. Such studies would be of particular relevance in the case of Lyn, as it is highly expressed in both cDCs and pDCs. Thus, evaluating Lyn-phosphorylation post-CpG stimulation in sorted pDCs and cDCs would elucidate whether Lyn is equally activated in both cell types. Future experiments should also assess the specific levels of Fyn phosphorylation following TLR stimulation. Additionally, generation of Fyn/Lyn double-deficient mice could test whether the reduction in cytokine production seen in individual Lyn and Fyn deficient mice would be compounded when both genes are removed.

The mechanisms through which TLR stimulation affects SFK activation in pDCs remain unknown, but such TLR signaling has been shown to activate SFK in other cell types. In B cells, TLR9 stimulation has been shown to activate SFK and lead to a MyD88 dependent signaling cascade (45). Furthermore, two SFKs, Hck and Lyn, are phosphorylated upon monocyte stimulation with CpG-ODN,

also leading to a MyD88-dependent endosomal signaling cascade (45). However, use of the endosomal acidification inhibitor chloroquine to inhibit interaction between CpG and TLR9 did not affect levels of Src-phosphorylation. To test whether CpG internalization also activates SFK in pDCs, we could perform similar studies with TLR signaling inhibitors, in conjunction with imaging studies using fluorescently labeled CpG oligonucleotides and endosomal and lysosomal markers. Such studies could elucidate whether SFK play a specific role in the trafficking of CpG into the endosome and subsequent endosomal acidification in pDCs. Retention of CpG in the early endosome promotes downstream IRF7 phosphorylation, nuclear translocation and IFN-I production. In contrast, transient passage through the early endosome results in both IFN-I production and increased NFkB-mediated inflammatory cytokine (13, 46). As Fyn ko mice exhibited a more pronounced defect in IFN-I production compared to TNF-α production, one possible explanation is that Fyn activity contributes to retention in the early endosome. Understanding the relationship between SFK and endosomal trafficking will greatly improve our ability to study the downstream molecular pathways that lead to increased cytokine production.

An important remaining question is which are the targets of SFK in DCs. Research in monocytes has shown that Src-phosphorylation leads to downstream phosphorylation of Syk (46). Syk is known to transmit signals from the B cell receptor, is required for B cell development and also plays a role in direct DC maturation under the specific circumstances of FcR engagement (47).

Indeed, herpes viruses such as Epstein Barr virus are known to encode Immunoreceptor Tyrosine Activation Motifs (ITAM) that lead to constitutive activation of Syk and B cell oncogenic transformation (48). Importantly, our microarray data demonstrates Syk expression is significant in both pDCs and cDCs. While homozygous Syk deficiency is embryonic lethal, specific chemical inhibitors of Syk do exist (50). Thus, treating WT cells with either src-kinase or syk-kinase inhibitors, or a combination of the two, should elucidate whether Syk is also involved in the SFK-TLR signaling pathway in dendritic cells.

While our data is preliminary, it is noteworthy that Fyn is almost exclusively expressed in pDCs among DC subsets, and that Fyn-mediated signaling appears to promote IFN-I production to a greater extent than TNF-α. Future studies could determine whether downstream targets in the IRF7 pathway leading to IFN-I production or the NFkB pathway leading to inflammatory cytokine production are differentially regulated in Fyn ko mice. However, therapeutic applications for Fyn in the context of human disease are likely to be limited by the very low expression levels observed on human pDCs (35). Nonetheless, the possibility remains that a highly similar pathway may exists in human pDCs involving Lyn activation as opposed to Fyn activation. Human pDCs express a BCR-like signalosome, consisting of Lyn, Syk, Btk, among the SFKs (35). Thus, it would be interesting to confirm in human tissues whether Lyn is indeed a positive regulator of IFN-I and TNF-α production. If so, small molecule inhibitors of Lyn

could serve as a therapeutic agent during pDC-mediated autoimmune diseases such as lupus and psoriasis.

Chapter 2: CD28 acts as a negative regulator of pDC IFN-I production

Introduction:

Like any important (and potentially pathogenic) biological process, pDC IFN-I production must be tightly regulated. We recently identified that the src-family kinase Fyn acts as a positive regulator of IFN-I and TNF-α production, and is uniquely expressed on pDCs among BM DC subsets. Thus, we searched splenic pDC and cDC genome-wide expression profiles to identify other unique pDC genes that could be candidate regulatory molecules. Our preliminary findings demonstrated the gene encoding the prototypic T cell co-stimulatory molecule CD28 was highly expressed among DC subsets.

CD28 is known to play an important role in the activation of T cell responses, but its function in pDCs remains unknown. Upon pathogen recognition, APCs upregulate CD80, CD86, and MHC molecules, and subsequently migrate to lymphoid organs to present antigen to T cells (49). As CD28 is known to be constitutively expressed on T cells, interactions between CD80 and CD86 on APCs and CD28 on T cells provide a necessary "second signal" in T cell activation, helping to prevent the initiation of destructive autoreactive T cell responses in the absence of infection (50). Furthermore, CD28 signaling has also been shown to be necessary for long-lasting immunity, as mice deficient in CD28 and CD80/86 exhibit impaired memory T cell responses to LCMV (51).

While CD28 is best known for its role in T cell activation, CD28 is also expressed on murine and human NK cells, in which anti-CD28 stimulation

enhances production of IL-12-induced IFN-γ (52). Notably, CD28 and CD80/86 interactions are vital for both T cell and NK cell responses during MCMV infection (52). However, none of these studies looked at early time points after infection during which pDC function is known to contribute significantly to systemic IFN-I levels. As CD28 is uniquely expressed in pDCs among DC subsets (Fig. 6), we hypothesized that CD28 may contribute to regulation of pDC IFN-I. Thus, we sought to test whether CD28, or its natural ligands, CD80 and CD86, influenced IFN-I and inflammatory cytokine production upon TLR stimulation of BM Flt3L DCs. Furthermore, we sought to determine whether CD28 affected IFN-I and inflammatory cytokine production early after MCMV infection.

Results:

CD28 transcript is expressed in splenic and Flt3L BM-derived pDCs

To assess CD28 expression among the DC subsets, we compared genome-wide expression profiles of sorted splenic pDCs, CD8α DCs and CD11b cDCs. We noticed that expression of the gene encoding the prototypic T cell co-stimulatory molecule CD28 was more than 50 times higher in pDCs compared to CD11b$^+$ and CD8$^+$ cDCs (Fig. 6a). Other CD28 family receptors (53) were either undetectable in pDCs (i.e. *ctla4*, *pdcd1* and *icos*) or equally expressed in all DC subsets (i.e. *btla*). Notably, DC subset or T cell specific gene transcripts were selectively expressed or absent, respectively, validating our microarray data. To confirm CD28 expression in pDCs, we next determined *cd28* levels by quantitative PCR in sorted splenic DC subsets. Again, we observed that *cd28*

transcript was significantly higher in pDCs and almost undetectable in cDCs (Fig. 6b). To verify that the observed CD28 expression was not due to contamination from other splenic cell populations, we also analyzed CD28 expression in sorted BM Flt3L-derived pDCs. Once again, we saw that CD28 was expressed in pDCs but not cDCs (Fig. 6c). In summary, CD28 was uniquely expressed on pDCs among the dendritic cells subsets, suggesting that it may play a unique role in pDC function.
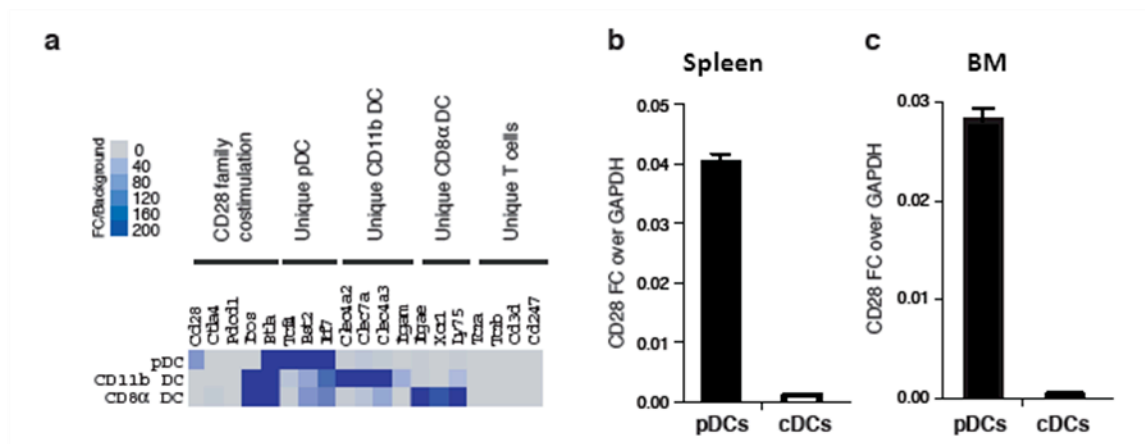
Figure 6. Splenic and bone marrow derived pDCs express CD28. a. Splenic pDC (CD11c+B220+PDCA+CD3-CD19-NK1.1-CD11b-), CD11b+cDC (CD11c+CD11b+B220-PDCA-CD3-CD19-NK1.1-) and CD8+cDC (CD11c+CD8+B220-PDCA-CD3-CD19-NK1.1-) were FACS purified from C57BL/6 WT mice and processed for DNA microarray analysis. Heat map depicts fold of change (FC) of indicated genes over the background. b. cd28 expression relative to gapdh was determined in pDCs and cDCs (CD11chighB220-CD3-CD19-NK1.1-) from FACS purified spleens c. cd28 expression relative to gapdh was determined from BM cultured for 8 days in the presence of Flt3L. Data is representative of 2 independent experiments.

CD28 protein is expressed on splenic pDCs and Flt3L BM pDCs.

Based on the presence of *cd28* transcript in splenic pDCs, we next sought to determine CD28 cell surface expression in various tissues. We detected CD28 expression in naïve splenic pDCs, while expression was low to undetectable in freshly isolated BM and blood pDCs (Fig. 7a). Notably, CD28 was expressed at high levels in CD4 T cells in all three subsets, validating our

CD28 staining. In sharp contrast to naïve bone marrow, CD28 expression was greatly upregulated in BM-derived DC differentiated for 8 days in the presence of Flt3L, with levels approaching those found in T cells (Fig. 7b.). Overall, our data suggests that CD28 is most highly expressed in fully differentiated pDCs.
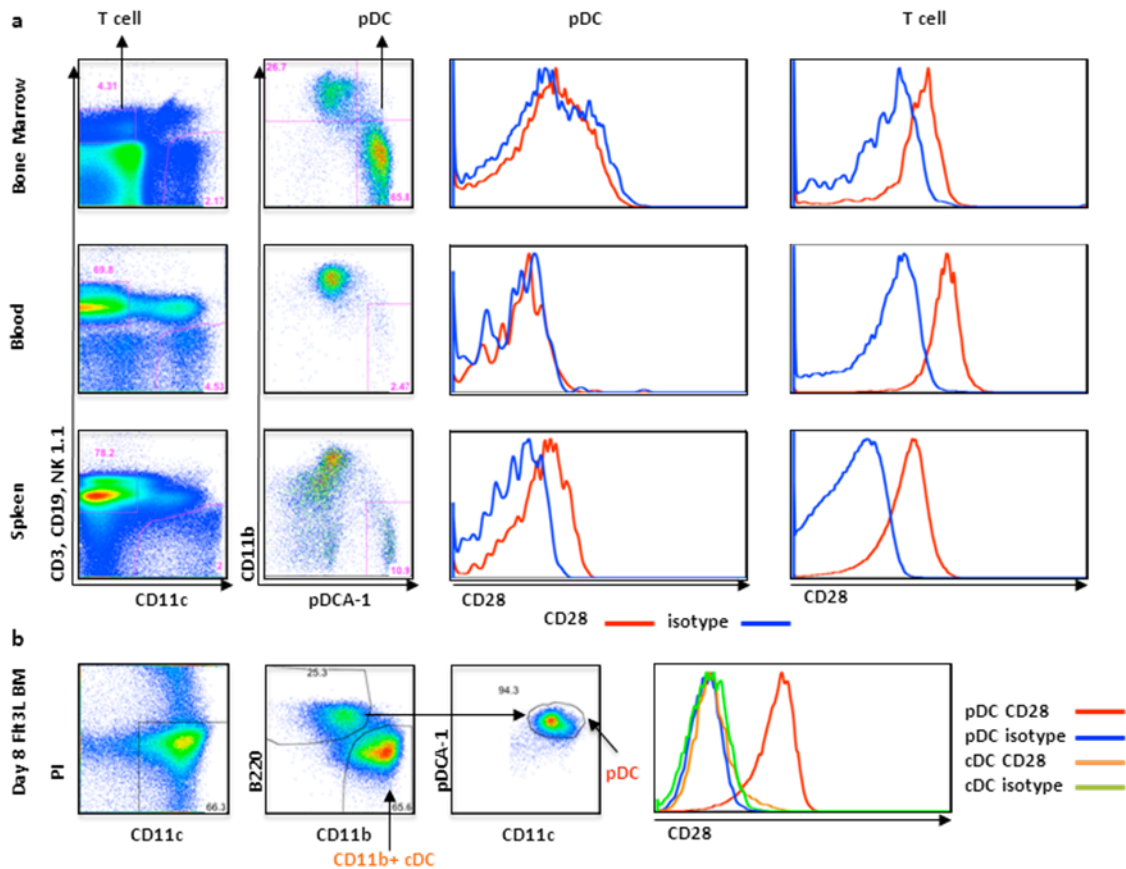
Figure 7. Comparison of CD28 protein expression in pDCs from bone marrow, blood and spleen. a. Bone marrow, blood (pooled from n=20) and spleens were obtained from C57BL/6 WT mice. Surface CD28 expression was determined in CD11c+pDCA+CD11b-CD3-CD19-NK1.1- pDCs by FACS in freshly isolated cells. **b.** BM DCs were differentiated in presence of Flt3L for 8 days. Surface CD28 expression was determined on CD11c+B220+CD11b-pDCA-1+CD3-,CD19-,NK1.1- pDCs in freshly isolated cells. Results are representative of 3 independent experiments with n≥2 mice/experiment.

CD80/86-CD28 interactions downregulate pDC IFN-I production in a cell-autonomous manner after TLR stimulation

We next investigated a putative role for CD28 and CD80/86 in pDC differentiation and/or function. Given that CD80 and CD86 engage CD28 to promote T cell activation, are constitutively expressed in pDCs and cDCs, and are upregulated after TLR stimulation (51), we sought to examine CD28 and CD80/CD86 participation in pDC regulation. To this end, we compared WT, CD28 ko and CD80/CD86 double ko BM-derived DCs after CpG stimulation. The levels of IFN-α protein and IFN-I bioactivity upon CpG stimulation were enhanced to comparable levels in both CD28 ko and CD80/CD86 ko BM cultures with respect to WT controls (Fig. 8a). In contrast, secretion of TNF-α was indistinguishable in WT versus CD28 ko and CD80/CD86 ko BM cultures (Fig 8b). This data suggested that CD28 and CD80/86 interaction negatively regulated IFN-I production, but not inflammatory cytokine production. Of note, in all experiments we observed that WT, CD28 ko and CD80/86 ko bone marrow cultures exhibited similar percentages of pDCs and cDCs, and that the viability of pDCs and cDCs was similar following overnight culture with CpG (Fig. 8c). Overall, these results indicated that CD80/CD86-CD28 interactions exert selective down-regulation of pDC IFN-I production after TLR stimulation.
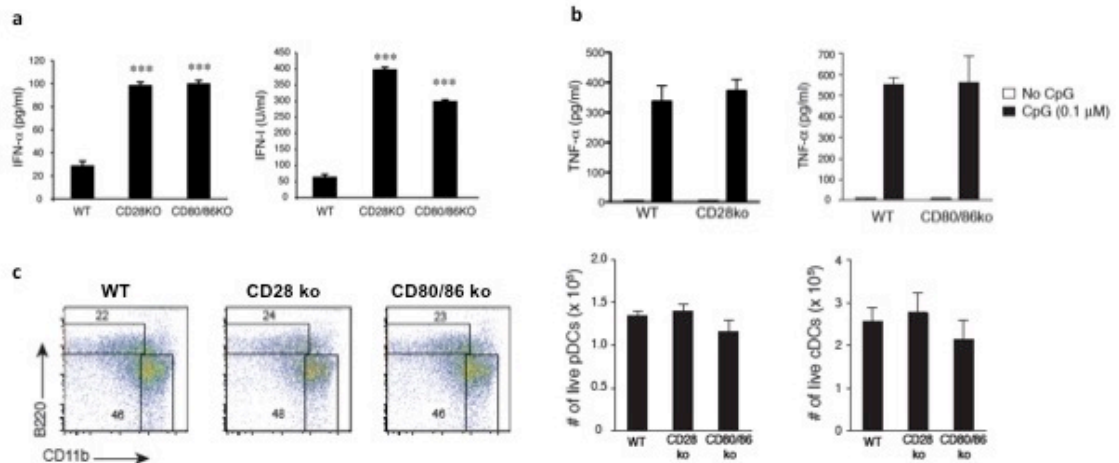
Figure 8. CD80/CD86-CD28 interactions down-regulate IFN-I. BM cells were obtained from WT, CD28, CD80/86 ko mice and differentiated in the presence of Flt3L for 8 days. **a.** Total cells were stimulated with 0.1 µM of CpG. After 15 h, levels of IFN-α protein and IFN-I bioactivity were measured in culture supernatant by ELISA and bioassay, respectively. **b.** Levels of TNF-α were measured in culture supernatants by ELISA **c.** Dendritic cell composition from cultures shown in (a) was determined by FACS, while total live cells were determined via PI staining.

CD86 and MHCII, but not CD28 expression increases following TLR stimulation.

We next examined whether CD28 expression was modulated by TLR signaling. To address this question, we stimulated both WT and CD80/86 ko BM Flt3L DCs with CpG. As CD80/86 are the natural ligands for CD28, we wanted to test whether CD80/86 expression affected CD28 expression on pDCs. We observed that CD28 was expressed at similarly high levels on unstimulated pDCs from WT and CD80/86 ko bone marrow, but was barely detectable on cDCs (Fig 9a). CD28 expression decreased slightly after CpG stimulation in both WT and CD80/86 ko mice, but remained at near background levels in cDCs. Thus, expression of CD28 on pDCs was not affected by the absence of its endogenous ligands, CD80 and CD86. Overall, our data suggests that CD28 is expressed

homeostatically on bone-marrow derived pDCs, and that this expression is minimally downregulated in response to microbial stimuli.

To address whether CD28 expression might affect DC maturation, we measured CD86 and MHC-II levels following CpG stimulation in WT and CD28 ko BM DCs. We observed that CD86 (a hallmark of DC maturation) was expressed at low levels in WT and CD28 ko pDCs prior to stimulation, and was significantly upregulated after CpG stimulation in both groups (Fig. 9b). Likewise, MHC-II levels increased to a similar extent in WT and CD28 ko pDCs. Furthermore, CD86 and MHC-II were comparably expressed and upregulated post-stimulation on CD11b+ cDCs, showing that a lack of CD28 expression did not affect maturation of either pDCs or CD11b+ DCs. Overall, our data suggests that CD28 is dispensable in the TLR-9 induced upregulation of pDC maturation markers.
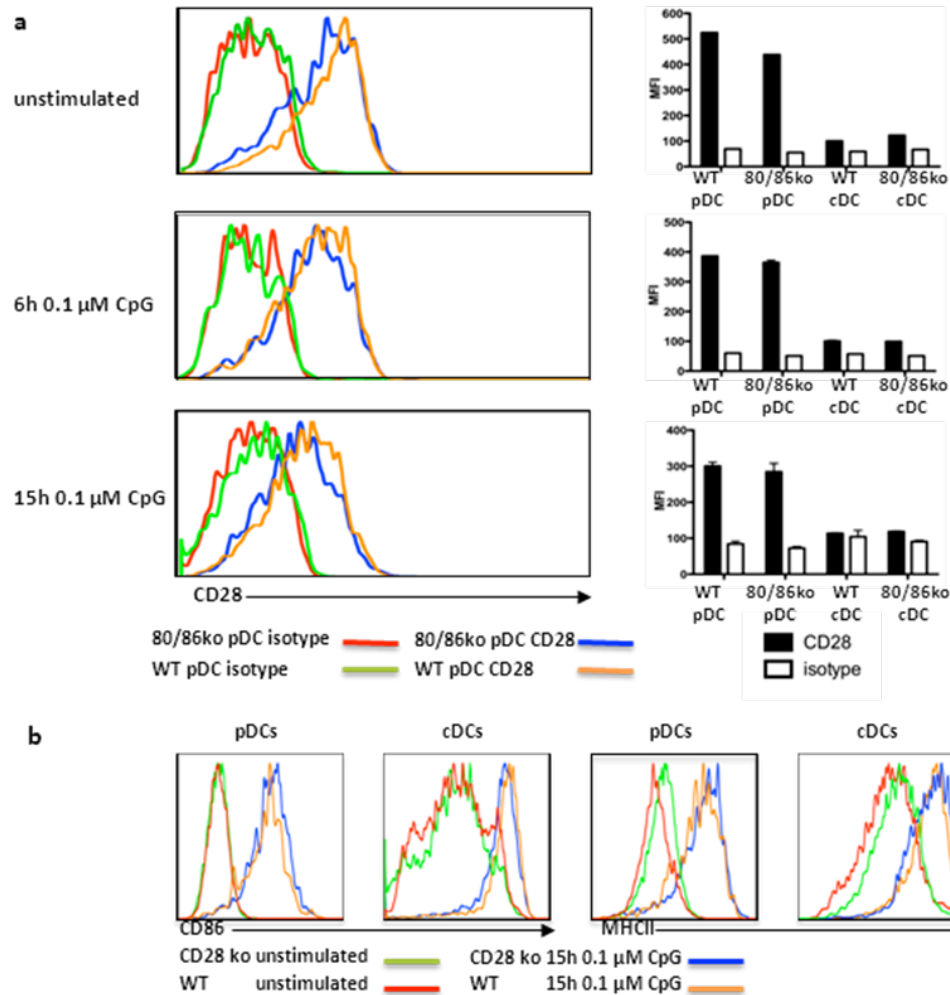
Figure 9. CD28 is expressed homeostatically in BM Flt3L pDCs, while CD86 and MHCII are upregulated upon TLR-9 stimulation. BM cells were obtained from WT, CD28 ko or CD80/86 ko mice and differentiated in the presence of Flt3L for 8 days. a. Cells were stimulated with CpG at 0.1 µM for the indicated times. Histogram overlays depict expression of CD28 and isotype control in WT and CD80/86 ko mice, gated on viable pDCs. Bar graphs on right quantify MFIs for CD28 and isotype control in pDCs and cDCs from the same cultures. b. Expression of CD86 and MHC-II in pDCs was determined by FACS. Representative histograms are shown. Bar graphs show mean ± SEM. Data are representative of 3 independent experiments. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Direct in vitro CD28 stimulation has modest effects on BM pDC IFN-I production

To confirm the results obtained with CD28 or CD80/86 deficient pDCs, we evaluated the effect of an agonistic anti-CD28 Ab in modulating pDC-IFN-I production. To this end, we incubated WT pDCs with a range of doses of either agonistic anti-CD28 Ab or isotype control during CpG stimulation of BM Flt3L

cultures. Anti-CD28 stimulation in WT mice did not yield consistent results. IFN-I secretion was reduced in a dose dependent manner in two experiments (Fig. 10b), while in three other experiments there was only a minimal effect (Fig. 10c). We also tested plate-bound CD28, but did not see significant inhibition of IFN-I in all but the highest dose of CD28 (Fig. 10d). Next, we examined whether the onset of CD28 signaling may need to occur prior to/post TLR stimulation to optimally inhibit IFN-I production (Fig 10e). We saw a small reduction in IFN-I when CD80/86 ko BM cells were pre-treated with anti-CD28 Ab for 1 or 3 hrs prior to addition of CpG, while no differences were observed in WT mice under the same conditions. Thus, we considered whether anti-CD28 treatment might need to occur throughout differentiation to optimally inhibit pDC IFN-I production. To test this hypothesis, CD28 and isotype were added to BM Flt3L cells at days 1,3 and 5 post-culture (Fig. 10f). While a statistically significant reduction in IFN-I occurred in CD28 treated wells, this reduction did not recapitulate the phenotype of CD28 ko or CD80/86 ko mice. In total, as differences in IFN-I production observed upon treatment with anti-CD28 were inconsistent, we were unable to conclude if anti-CD28 stimulation could inhibit pDC IFN-I *in vitro*.

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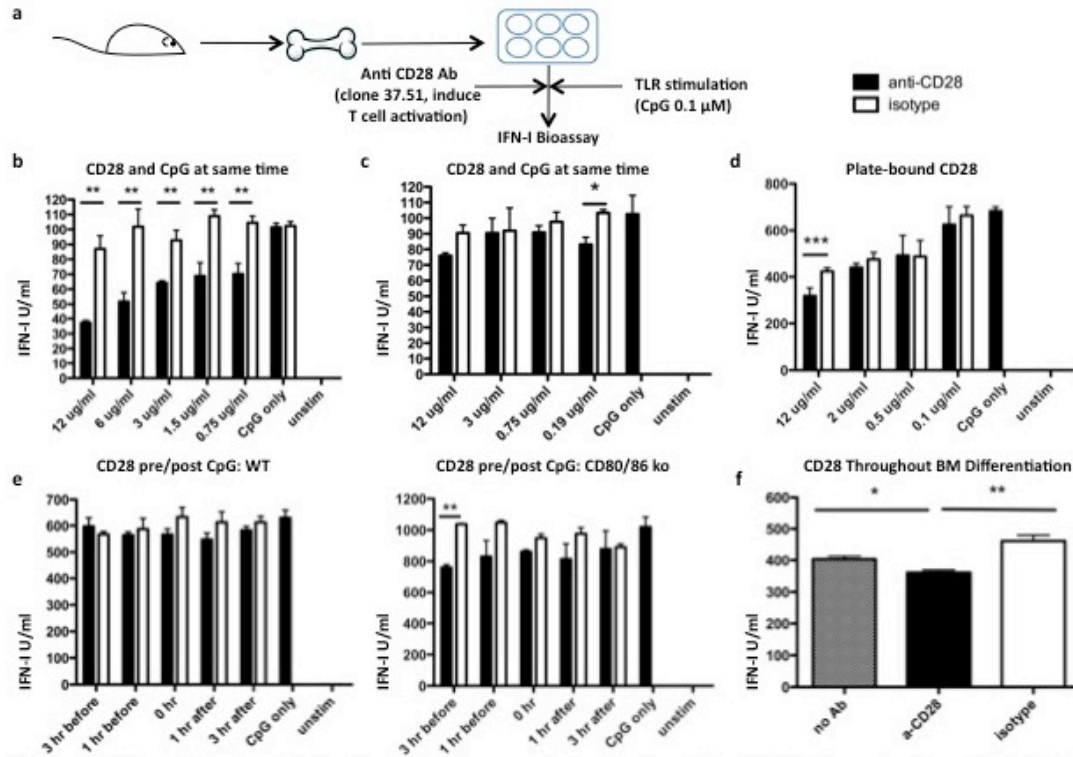
Figure 10. Direct *in vitro* CD28 stimulation has modest effect on BM pDC IFN-I production. Total BM Flt3L cultures were coincubated with anti-CD28 or isotype control and 0.1 µM of CpG as indicated. Supernatant was collected after 15 h and IFN-I production was measured by bioassay **(a)**. Increasing concentration of anti-CD28 or isotype control were added to BM Flt3L cultures at the same time as CpG **(b & c)**. Indicated concentrations of a-CD28/isotype were plate-bound prior to addition of total BM Flt3L cells and CpG **(d)**. 3µg/ml anti-CD28/isotype were added to WT and CD80/86 ko BM cultures at indicated lengths of time prior to/post CpG **(e)**. 3µg/ml anti-CD28/isotype was added at Day 1, 3, 5 post-culture, and IFN-I production was measured **(f)**.

Elevated IFN-I production upon MCMV infection in the absence of CD28

To investigate whether the CD28 inhibitory effect on pDC-IFN-I production also occurred during viral infection, we irradiated WT mice and reconstituted them with 85% *cd11c*-DTR (Diphtheria Toxin Receptor) bone marrow combined with 15% of either WT or CD28 ko bone marrow. The use of *cd11c*DTR-GFP mice allowed us to deplete CD11c+ cells expressing the DTR receptor prior to infection, providing us with a system to test the differential effects of reconstitution with CD28 deficient or WT bone marrow cells (55). Furthermore, the *cd11c*DTR+ cells can be quantified by GFP expression. Chimeric mice

showed similar proportions of total lymphocytes, pDCs and cDCs following reconstitution (Fig. 11b). As a control, we also injected a subset of *cd11c*DTR/CD28 ko with PBS instead of diphtheria toxin (DT) prior to infection with MCMV. Thus, the majority of pDCs at 36 h pi are either of WT or CD28 ko origin in mice treated with DT, while in untreated mice the majority of pDCs should still be of *cd11c*DTR transgenic origin, which should be functionally equivalent to WT pDCs. While cDCs are known to express higher levels of CD11c than pDCs, we found that both DTR expressing pDCs and cDCs were depleted by DT treatment, with GFP fluorescence levels reduced to near background level (Fig. 11c). Notably, 36 h after MCMV infection, DT treated mice reconstituted with BM of CD28 ko origin showed significantly increased serum IFN-I levels compared to DT-treated mice reconstituted with WT BM (Fig. 11d). Furthermore, mice reconstituted with CD28 ko BM, but not treated with DT did not show such an increase. There was also no significant difference between CD86 and MHCII expression in pDCs between the three groups of mice, showing that differences in maturation were not responsible for the differences in IFN-I production (Fig 11e). As IFN-I production 36h post MCMV infection is known to be dependent on pDCs, these results support the conclusion that the reduction in pDC IFN-I is mediated by cell intrinsic effects of *in vivo* CD28 signaling.
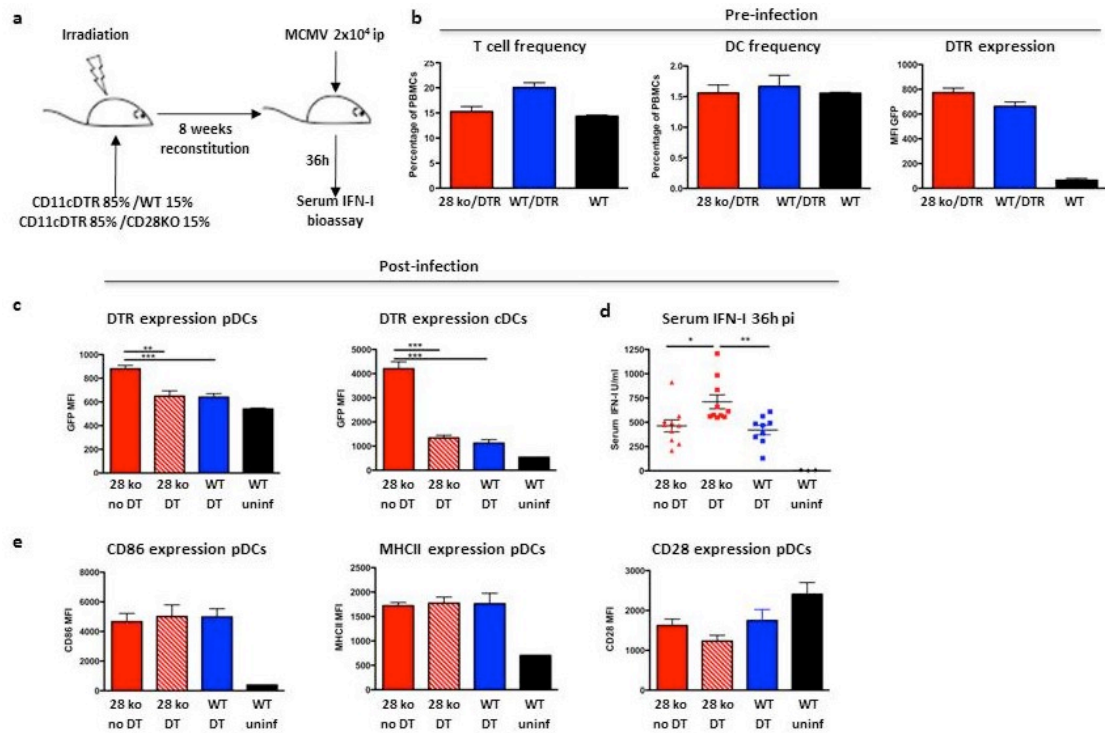
Figure 11. Increased IFN-I in *cd11c*-DTR/CD28 ko Mixed Chimeras. WT mice were lethally irradiated and reconstituted with a mixture of GFP expressing *cd11c*-DTR (85%) and WT (15%) or CD28 ko (15%) bone marrow (**a**). T cell, DC frequency and DTR expression is shown comparing CD28 ko/DTR (red) and WT/DTR (blue) chimeras to WT control (black) mice prior to infection (**b**). Mice were treated with DT (8 ng/g body mass) 56 hours prior to infection and again 8 hours prior to infection with MCMV. GFP DTR expression is shown in pDCs and cDCs with respect to uninfected cells (**c**). Serum IFN-I was measured 36h pi by bioassay (**d**). CD86, MHCII and CD28 expression was measured on splenic pDCs 36 hr pi (**e**).

Discussion:

In this study, we sought to identify unique pDC genes that regulate IFN-I production. Strikingly, CD28 acted as a negative and selective regulator of pDC IFN-I production in response to *in vitro* TLR stimulation and *in vivo* infections with MCMV, restricting early virus control. Both CD28 and its endogenous ligands CD80/CD86 generated similar inhibitory effects on pDC IFN-I, suggesting that an autonomous pDC-IFN-I regulatory loop exists between CD28 and CD80/CD86.

Results showing enhanced IFN-I production in CD80/86 ko and CD28 ko mice strongly support the hypothesis that CD28-CD80/CD86 interactions act to

restrain pDC IFN-I. However, to definitively prove that an autonomous regulatory loop exists between CD28 and CD80, CD86, sorted pDCs and cDCs from either WT or CD80/86 ko mice could be co-cultured in the presence of TLR agonists. As both pDCs and cDCs upregulate CD80 and CD86 upon TLR stimulation, these experiments would show whether CD80 and CD86 on cDCs and/or pDCs constitute the physiological ligands for CD28 on pDCs. To address the role of autocrine CD80/CD86 signalling in pDCs, future studies will examine pDC IFN-I production in WT:CD80/CD86 mixed chimeric mice upon MCMV infection.

Furthermore, the molecular mechanisms of CD28-mediated regulation of IFN-I production by pDCs following TLR stimulation remain to be elucidated. To address this, we will need to develop a more reliable *in vitro* CD28 stimulation assay for pDCs. Our attempts to inhibit pDC IFN-I via direct anti-CD28 stimulation yielded inconsistent results, with some experiments showing a dose dependent inhibition of IFN-I, while others showed no effect. These differences could not be attributed to variation in pDC composition, as even experiments with enriched pDCs did not show consistent results. Several possible explanations exist for the failure of anti-CD28 treatment to recapitulate the phenotype seen with CD28 ko mice. One possibility is that consistent CD28 stimulation throughout bone marrow differentiation is necessary to induce inhibition of IFN-I. However, this hypothesis is not supported by preliminary data showing no significant differences in IFN-I production when anti-CD28 Ab is added to BM

Flt3L throughout differentiation. Additionally, as CD28 is known to exist in several isoforms in humans (56), another possibility is that the anti-CD28 Ab clone, which is shown to activate T cells, does not bind with sufficient affinity to the form of CD28 expressed on pDCs to exert consistent effects. Thus other clones of CD28 antibody should be tested.

Future experiments to modulate pDC CD28 signaling may benefit from use of fusion proteins to either block or stimulate CD80/86 signaling. Fusion proteins have gained popularity in immunotherapies as they generally have longer half-lives compared to traditional monoclonal antibodies and/or higher binding affinities allowing sustained signaling modulation (57). For example, CTLA-4 Ig, which comprises the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4, has been used to help prevent graft rejection in transplant patients (58). With the exception of natural FoxP3+ Tregs, CTLA-4 is not expressed at high levels on the surface of naïve T cells (57). However, CTLA-4 is upregulated upon T cell activation, competing with CD28 for binding to CD80/86 on APCs, and serving to constrain T cell activation after the danger signal has subsided (60, 61). While our microarray data shows that CTLA-4 is not expressed on uninfected pDCs, further experiments are needed to show whether CTLA-4 is upregulated on pDCs following TLR stimulation. Competition between CD28 and CTLA-4 for CD80/86 binding could help explain the variable results seen upon anti-CD28 treatment of pDCs. Based on data that CD80/86 deficiency and CD28 deficiency appear to equally impact pDC IFN-I production, we also

plan to test a murine CD86/human IgG1 fusion antibody to determine whether it would inhibit pDC IFN-I production. Experiments using a combination of CTLA-4 Ig and CD86 Fc chimeric antibody treatments to disrupt CD28-CD80/86 interactions should help us to develop a better system for modulating pDC CD28 signaling *in vitro*. Such a system could allow exploration of how CD28 may contribute to phosphorylation of downstream kinases, affording us a better understanding of CD28 signaling in pDCs.

CD28 modulation of pDC IFN-I production *in vivo* should also be explored in the context of additional viral infections. Studies have shown that enhanced early IFN-I response may be critical for modulating the Effector:Target ratio, in which limiting the number of virally infected cells (target) early after infection can allow immune cells (effector) to effectively prevent viral spread (59). However, due to the fact that CD28 serves to constrain pDC effector function, while simultaneously enhancing NK and T cell effector function, we cannot conclusively evaluate the effects CD28 may be having on pDC IFN-I production at later time points during infection. Indeed, multiple studies have shown that CD28 and CD80/86 deficient mice have impaired viral clearance due to attenuated T cell responses (51, 60). Yet, pDCs are also critical to both viral control and generation of an effective T-cell response upon infection with Murine Hepatitis Virus (MHV) (11). To evaluate the effects of CD28 signaling specifically on pDCs, it will be necessary to create a pDC-specific CD28 conditional knockout mouse, potentially through existing E2-2 Cre technology (11). E2-2 acts as a

specific transcriptional regulator of the pDC lineage in mice and humans (61). These mice could then be infected with a chronic viral infection, such as LCMV Cl 13, to determine if pDC IFN-I production remained enhanced at time points at which it is normally silenced and whether any potential increase in IFN-I production from pDCs at these time points could contribute to enhanced viral clearance (20). Thus, it would be interesting to test whether mice with a conditional deletion of CD28 in pDCs exhibited improved cytokine response and/or faster viral clearance.

In contrast to their beneficial role during the acute phase of viral infections, pDCs become activated in several autoimmune diseases through endogenous TLR ligands, producing excessive amounts of IFN-I that promote a pathogenic cycle of autoreactive B and T cell activation (13, 62, 63). Interestingly, previous studies have shown that inhibition or stimulation of the CD28 pathway may affect the outcome of autoimmune diseases such as Type-1 diabetes. While these effects are known to be mediated by Tregs (64), the possibility exists that CD28 may also function on pDCs to provide a critical suppressive signal that prevents aberrant IFN-I production during autoimmune diseases.

In conclusion, cell-intrinsic CD28 signaling represents a biologically relevant pDC regulatory pathway that selectively limits IFN-I production and restricts anti-viral innate responses and early viral control *in vivo*. Future studies will determine whether CD28 is expressed in human pDCs, or if perhaps a similar

pathway exists in human pDCs that could be targeted for development of therapies to treat autoimmune diseases and/or microbial infections.

CHAPTER 3:

Potential cross-talk between CD28, Src-family kinase and PI3K signaling.

Introduction:

In our search to identify novel regulators of pDC IFN-I production, we have discovered that the SFKs act to enhance IFN-I and TNF-α signaling in DCs while CD28 acts as a negative regulator of pDC IFN-I production. It is well-established that TLR7 and 9 signaling in pDCs signals through a MyD88-dependent pathway involving several downstream signaling molecules including IRAK1/4, TRAF3, IKK- α, and PI3K activation, leading to phosphorylation and nuclear translocation of IRF7 and induction of IFN-I production (15, 65). However, we do not yet understand the molecular mechanisms of cross-talk between TLR7 and 9 signaling and SFKs and/or CD28 for pDC IFN-I production.

To investigate how CD28 might interact with the PI3K and SFK pathways, we first considered T cells, in which two distinct cytoplasmic signaling domains of CD28 have already been well characterized. The PYAP motif in the cytoplasmic tail of CD28 is linked to activation of Lck, a member of the SFKs (66). Signaling downstream of Lck leads to T cell proliferation, survival and activation. In contrast, the YMNM motif links CD28 to PI3K activation, and is dispensable in T cell activation (66). Therefore, we sought to determine whether the SFK or PI3K pathways directly contribute to CD28-mediated regulation of pDC IFN-I production. To test this hypothesis, WT, CD28 ko and/or CD80/86 ko mice were treated with specific chemical inhibitors of the PI3K and SFK pathways. We found that while both PI3K and SFK inhibitors silence IFN-I in a dose dependent fashion, this silencing occurred in all 3 genetic strains of mice. Our data

suggests that CD28-mediated reduction in pDC IFN-I is not dependent on either the SFK or PI3K pathways.

Results:

Inhibition of PI3K signaling reduces IFN-I in WT and CD80/86 ko BM DCs

To investigate whether CD28 signaling influenced PI3K mediated IFN-I production in dendritic cells, BM-DC cultures derived from WT and CD80/86 ko mice were stimulated with CpG in the presence of both a reversible (Ly294002) and an irreversible (Wortmannin) inhibitor of PI3K signaling. While both inhibitors have been published to silence both mouse and human IFN-I (65, 67), we sought to determine whether this IFN-I silencing could be recapitulated in CD80/86 ko mice which lack endogenous CD28 ligands. We found that upon treatment with Ly294002, no significant differences in cell viability were seen between WT and CD80/86 ko mice, although viability was reduced upon treatment with the highest doses of Ly294002 when compared to the equivalent concentrations of DMSO (Fig. 12a). Furthermore, both WT and CD80/86 ko mice exhibited a comparable dose-dependent reduction in IFN-I production with respect to equivalent dilutions of DMSO control (Fig. 12b). Of note, significant inhibition of IFN-I occurred upon treatment with 10 μM Ly29004, while cell viability was not significantly impaired at this dose. Importantly, while the presence of DMSO alone in CpG-stimulated samples appeared to augment IFN-I production, IFN-I production was consistently reduced in samples treated with Ly294002. To test whether sustained inhibition of PI3K signaling might be necessary to induce changes in

IFN-I, we also conducted the same experiments in the presence of an irreversible inhibitor of PI3K signaling, Wortmannin (Fig. 12c&d). Upon treatment with a 1 μM dose of Wortmannin, we observed that IFN-I production was significantly reduced in both WT and CD80/86 ko mice, without significant impairment of cell viability. In summary, our data confirms that PI3K signaling is necessary for pDC IFN-I production in mice. However, a role for CD28-mediated signaling in this process cannot be ascribed, as IFN-I silencing occurred at equivalent doses in both WT mice and mice deficient in both CD80 and CD86. Overall, our data suggests that inhibition of pDC IFN-I via CD28 and CD80/86 interactions may involve mechanisms other than direct modulation of TLR7 and 9 signaling via PI3K.
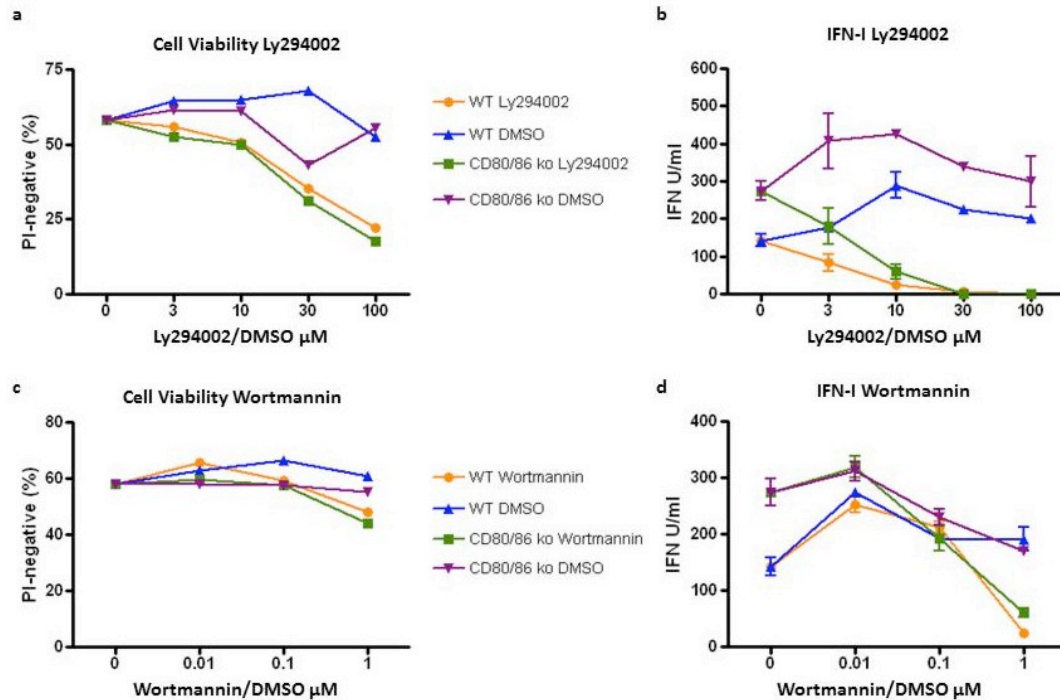
Figure 12. Inhibition of PI3K signaling results in reduced IFN-I production upon CpG stimulation. BM-derived DCs were pre-treated for 1 h with indicated doses of Ly294002 **(a&b)** or Wortmannin **(c&d)**, or the equivalent concentration of DMSO control, followed by 15 h in the presence of 0.1 μM CpG-B ODN 1668. IFN-I production was measured by bioassay following incubation with Ly294002 **(b)** and Wortmannin **(d)**. Cell viability was determined by PI staining following incubation with Ly294002 **(a)** and Wortmannin **(c)**. Data is representative of 2 independent experiments.

Inhibitor of Src-kinases reduces cytokine production in WT and CD28 ko BM DCs

Given that SFKs are positive regulators of IFN-I production (Chapter 1), we next looked to see whether specific interactions between CD28 and SFK could be responsible for the negative regulation of IFN-I observed. WT and CD28 ko mice were treated with the pan SFK inhibitor PP2. Consistent with the results in WT mice (Fig. 4), treatment with higher doses of inhibitor resulted in minimal effects on cell viability compared to DMSO control in both WT and CD28 ko mice (Fig. 13a). Similar to our results with PI3K inhibitors, IFN-I production was reduced to near background levels in WT and CD28 ko mice (Fig. 13b). While IFN-I production in the absence of inhibitor was higher in CD28 ko mice,

we could not find a concentration of inhibitor that consistently generated significant reduction of IFN-I in WT mice but not CD28 ko mice. Furthermore, TNF-α production was also comparably reduced in both groups of mice (Fig. 13c). In summary, despite the prominent role of SFKs in promoting pDC IFN-I production, using this methodology, it is difficult to interpret the direct involvement of SFKs in CD28-mediated inhibition of pDC IFN-I.
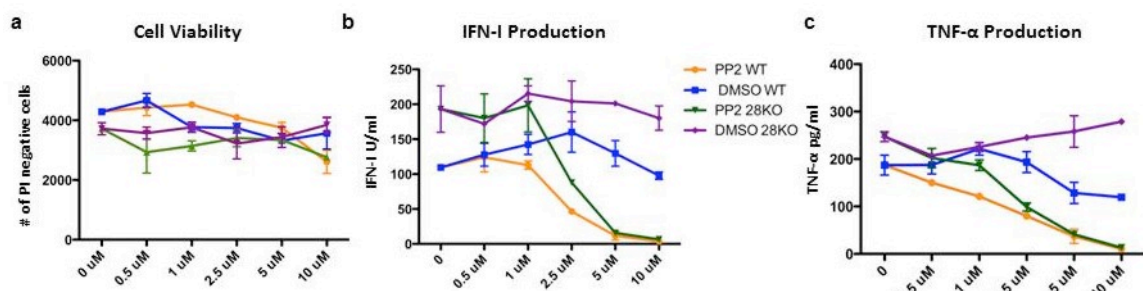
Figure 13. Inhibitor of src-kinases reduces cytokine production in WT and CD28KO mice. BM-derived DCs were pre-treated for 6 hrs with src inhibitor PP2, or the equivalent concentration of DMSO control, followed by 15h in the presence of 0.1 μM CpG-B ODN 1668. Cell viability was determined by PI staining (a). IFN-I production was measured by bioassay (b). TNF-α was measured by ELISA (c). Representative results from 3 independent experiments are shown.

Discussion

Having set out to search for potential novel regulators of IFN-I in pDCs, we have shown that the SFK pathway acts as a positive regulator of BM DC IFN-I production, while CD28 negatively regulates pDC IFN-I both *in vitro* and early after *in vivo* viral infection. Due to the unreliability of α-CD28 treatment of BM Flt3L DCs, we based our study on the treatment of BM DCs from CD28 ko and CD80/86 ko mice with chemical inhibitors of the PI3K and SFK pathways. We observed that disruption of CD28 and CD80/86 interactions did not yield any obvious alteration to IFN-I signaling via either pathway. However, these observations also do not rule out involvement of CD28 in PI3K or SFK signaling.

Thus, alternative approaches to test CD28 involvement in these pathways will be necessary, including consideration of the phosphorylation of downstream signaling targets, and evidence of protein-protein interaction between CD28 and either PI3K or SFK-associated molecules.

Of note, CD28 signaling in pDCs appears to selectively impact IFN-I production, and not inflammatory cytokine production. This observation could support the argument for PI3K involvement as opposed to SFK involvement in CD28-regulated IFN-I signaling. Inhibitors of PI3K have been shown to selectively inhibit TLR7- and 9-mediated IFN-α response in human pDCs, limiting IRF7 nuclear translocation, while leaving inflammatory cytokine production unaltered (65, 67). In contrast, our data shows that inhibition of the SFK pathway results in reduction in both IFN-I and TNF-α production. PI3K activity is known to induce phosphorylation of Akt on two epitopes, Ser473 and Thr308 (68), leading to a downstream signaling cascade that causes IRF7 nuclear translocation and increased IFN-I production. However, we have yet to determine whether direct CD28 stimulation of BM DCs has any effect on Akt phosphorylation. To test for potential cross-talk between CD28 and the TLR pathway in pDCs, we intend to measure Akt phosphorylation induced by CD28 stimulation both in the presence or absence of synthetic TLR ligands such as CpG and loxoribine. As the PI3K pathway positively regulates IFN-I, we would expect to see increased Akt phosphorylation upon TLR stimulation. Thus, it would be interesting to see if a combination of TLR and CD28 stimulation

abrogated any potential increase in Akt phosphorylation. Furthermore, negative regulation of the PI3K pathway in hematopoietic cells is primarily mediated by phosphastases, including PTEN and SHIP-1 and SHIP-2 (69). Thus, it would be informative to test whether anti-CD28 signaling specifically induced any of the aforementioned phosphatases, and whether this corresponded to a downstream reduction in Akt phosphorylation, and reduced IFN-I production. While further evaluation of the PI3K pathway is important, we also cannot form definitive conclusions about CD28 involvement in the SFK pathway. The possibility exists that the SFK inhibitor PP2 may non-specifically affect other pathways known to regulate IFN-I production, such as the MAPK pathway, suggesting specific inhibitors for MAPK should also be tested. Should such experiments confirm that inhibition of IFN-I occurs in both WT and CD28 ko pDCs, further experiments to test for changes in Akt phosphorylation should be performed. As CD28 signaling in T cells via Lck leads to phosphorylation of Akt at Thr308 (69), simultaneous inhibition of the PI3K and SFK pathways may synergize to further restrain Akt phosphorylation.

It is also noteworthy that interactions in T cells between CD28 and the SFK Lck are critical to T cell proliferation and function. While Lck is not shown to be expressed amongst our microarray targets, Fyn is highly expressed in T cells, and has been shown to be an important signal secondary to Lck upon TCR engagement (66). For example, it has been shown in T cells that upon anti-CD3 and anti-CD28 stimulation, Fyn deficient mice show significant defects in

downstream phosphorylation of Zap70 and NFAT nuclear translocation, important downstream signaling molecules for T cell activation (70). Furthermore, another CD28 costimulatory family member, CTLA-4, has been demonstrated to associate with Fyn and Lck in T cells (74).   To test for CD28 and SFK interactions *in vitro*, immunoprecipitation of CD28 prior to and post-TLR stimulation would allow us to determine the level of direct protein-protein interaction between CD28 and Fyn or Lyn. If such interaction exists, we can also evaluate changes in phosphorylation between SFK and downstream targets upon anti-CD28 treatment to determine if CD28 negatively regulates IFN-I via impaired SFK-phosphorylation. Future studies using mass-spectrometry would also provide valuable information on novel protein interactions with CD28 following TLR stimulation of pDCs.

In summary, preliminary experimental data has not revealed cross-talk between CD28 and SFK, nor between CD28 and PI3K-mediated TLR signaling. Thus, use of alternative kinase inhibitors, as well as evaluation of relative levels of kinase activity and phosphorylation of downstream targets will be required to unravel mechanisms of CD28 and SFK regulation of pDC IFN-I.

CONCLUSION:

Potential mechanisms of CD28 and SFK signaling in pDCs

The prolific production of IFN-I and inflammatory cytokines and chemokines by pDCs play crucial roles in containing viral infections at their earliest stages. Yet, unfettered IFN-I production by pDCs also contributes to autoimmune pathology in Systemic Lupus Erythematosus (SLE) and psoriasis, (26), demonstrating the need to characterize genes that regulate pDC cytokine production. Here, we identified two membrane-associated proteins that are unique to pDCs among DC subsets and act to specifically modulate pDC cytokine production. Fyn acts as a positive regulator of pDC IFN-I and inflammatory cytokine production, while CD28 selectively suppresses pDC IFN-I production. Understanding the signaling mechanisms downstream of these genes could highlight useful therapeutic targets in the battle to combat immunosuppressive viruses, as well as in the restraint of autoimmune pathology. Thus far, only three other surface receptors with the ability to modulate murine pDC IFN-I production have been described. pDC-TREM and Ly49Q lead to augmented IFN-I production (31, 71), while Siglec-H inhibits IFN–I production (32). Furthermore, the regulatory mechanisms governing these pathways are poorly understood, opening the possibility that CD28 and/or Fyn could mediate regulation of cytokine production via these surface receptors or through overlapping signaling mechanisms following TLR stimulation.

Future Avenues of Study for Src-Family Kinases

While Siglec-H and pDC-TREM have opposing effects on pDC IFN-I production, there may be substantial similarities in the downstream signaling pathways. pDC-TREM is upregulated after TLR stimulation and triggers a DAP12-dependent phosphorylation cascade involving PI3K , ERK 1 / 2 and IKK-α (31). In contrast, Siglec H functions as an endocytic receptor, mediating antigen uptake, yet signaling via Siglec-H leads to impaired IFN-α production. Interestingly, signaling via both pDC-TREM and Siglec-H has been shown to require signaling through the same adaptor, DAP12 (32). Indeed, DAP12 has been shown to have activating functions upon high affinity ligand interactions, but inhibitory functions upon low affinity ligand or antibody interactions (72). DAP12 and FcRγ contain ITAM motifs that serve as the substrates and docking sites for SFK (72). Importantly, DAP12 activation involves phosphorylation by members of the SFK. As the selective expression of Fyn in pDCs suggests a specific role in cytokine signaling, future investigations could identify whether Fyn specifically phosphorylates and activates DAP12, and whether such interactions constitute a necessary component of the pDC-TREM signaling pathway in pDCs. Furthermore, as binding of autoimmune complexes to FcRγ can activate SFK signaling, investigation of downstream Fyn phosphorylation targets could yield insight into potential mechanisms for regulating autoimmune pathology.
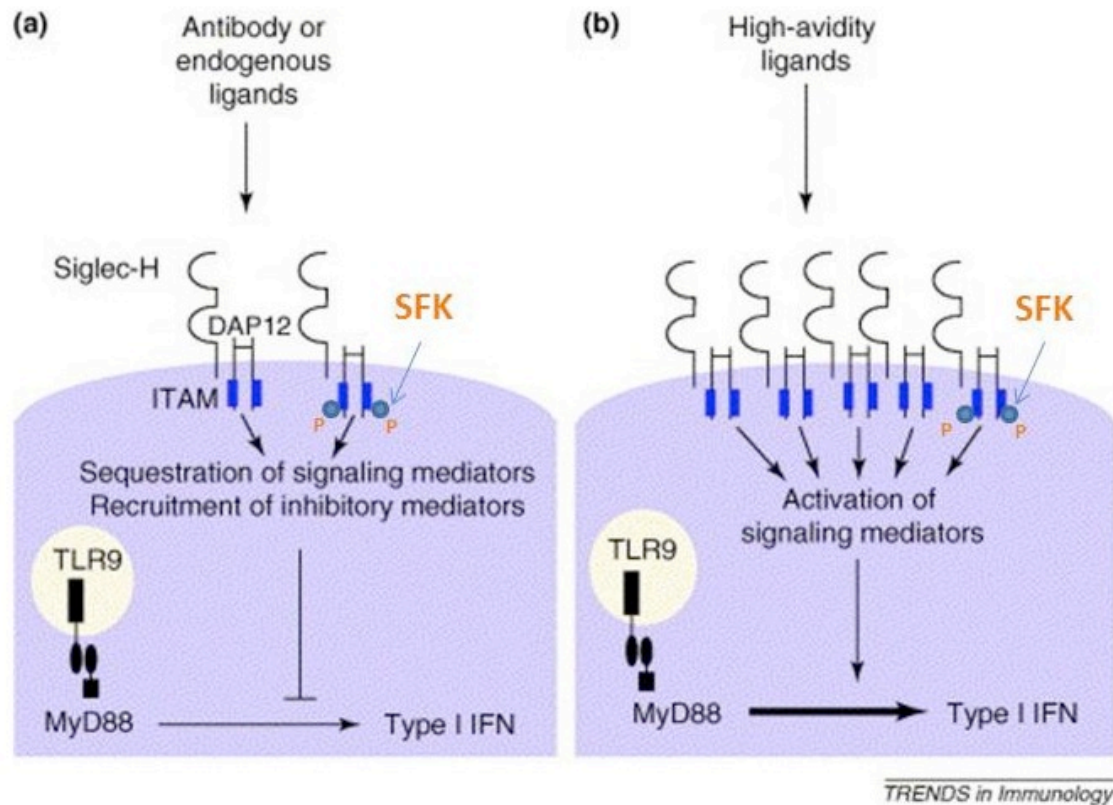
Figure 14. Potential mechanism for involvement of SFK in Siglec-H–DAP12 signaling in pDCs. (a) The TLR9–MyD88 pathway stimulates the secretion of IFN-I by pDCs. Siglec-H can mediate inhibition of this pathway when engaged by antibody or low-avidity endogenous ligands, which induce suboptimal receptor clustering. **(b)** By contrast, high-avidity ligands for Siglec-H optimally engage the receptor, resulting in multivalent clustering, activation of signaling mediators and cytokine secretion. SFK may act as key mediators of this pathway via their phosphorylation of ITAM motifs. *Adapted from Hamerman, J. A., M. Ni, J. R. Killebrew, C. L. Chu, and C. A. Lowell. 2009. The expanding roles of ITAM adapters FcRgamma and DAP12 in myeloid cells. Immunol Rev 232:42-58.*

Lyn has also been shown to act as a positive or negative regulator of signal transduction, depending on a variety of factors including the type of stimulation and developmental state of the cell (73). Positive regulation occurs in the form of ITAM phosphorylation, which can then lead to the recruitment and phosphorylation of signal amplifiers such as Syk kinase (19). In contrast, Lyn-mediated phosphorylation of ITIM motifs generates docking sites for phosphatases such as SHIP-1 and SHIP-2 (77). Owing to the high levels of expression of Lyn in pDCs, it is likely that tyrosine phosphorylation events

induced by Lyn have pleotropic effects on downstream signaling pathways. Interestingly, mutation of mice causing constitutive upregulation of Lyn resulted in considerably enhanced dendritic cell inflammatory cytokine production in response to LPS, triggering enhanced NK cell activation (20). Thus, future studies exploring the phosphorylation targets of Lyn are needed to address whether Lyn kinase activity triggers differential activation of TLR4 versus TLR7/9 signaling in BM DCs.

Future Avenues of Study for CD28 signaling in pDCs

CD28 co-stimulation in T cells is one of the best established events that bridge innate and adaptive immunity, linking up-regulation of CD80/CD86 molecules in antigen presenting cells upon pathogen encounter and presentation to adaptive immune cells (74). The co-option of this very same pathway to hamper IFN-I production by pDCs suggests that CD28-CD80/CD86 interactions act as a rheostat that fine-tunes the magnitude of the innate response according to the needs in place. For instance, many pathogens suppress the up-regulation of CD80/CD86 molecules to dampen CD28 signaling in T cells and avoid their priming (75). If CD28 signaling in pDCs is mitigated through CD80/CD86 interactions, down regulation of these danger signals would trigger higher IFN-I production to more effectively contain the immunosuppressive microbe. This enhanced IFN-I production could provide a stronger "signal 3" that could compensate for the debilitated signal 2 (i.e. CD80/CD86) during T cell priming (74, 76).

To further investigate the molecular mechanisms through which CD28 signaling restrains pDC IFN-I production, it would be useful to explore whether key mediators of pDC-TLR signaling are differentially regulated between WT and CD28 ko pDCs during chronic viral infection. pDCs become functionally exhausted by immunosuppressive viruses such as LCMV Cl 13 in mice and HIV and HCV in humans. In LCMV Cl13 infection, functional exhaustion of pDCs involves rapid silencing of key mediators of pDC IFN-I production in the absence of viral control. In contrast, while pDC IFN-I production capacity is also silenced in acute LCMV Armstrong infection, it is restored by 9 days post-infection, correlating with viral clearance of Armstrong virus. Utilizing both LCMV strains of infection (acute and chronic), genome-wide microarray studies in our laboratory have uncovered a potential pDC transcriptional signature of exhaustion correlating with pDC IFN-I silencing in chronic LCMV Cl13 infection. To determine if CD28 signaling is involved in pDC exhaustion, naïve and Cl 13 infected WT and CD28 ko pDCs expression profiles could be analyzed by microarray and compared to our current microarray results. Genes found to be differentially regulated between infected WT and CD28 ko pDCs could be considered potential mediators of CD28 downstream signaling. Further studies would be required to determine whether CD28 signaling acts through the sequestration or degradation of key TLR signaling genes, or via the direct recruitment of inhibitory mediators. If indeed a candidate "adaptor gene" linking CD28 to pDC-TLR signaling is found, confirmation of its role would involve

showing that mice deficient in this gene recapitulate the phenotype of CD28 ko and CD80/86 ko mice.

In conclusion, our findings reveal novel regulatory roles for two molecules not previously reported to be expressed on pDCs. The prototypic T cell costimulatory receptor CD28 acts to suppress innate immunity through selective and cell-intrinsic inhibition of pDC IFN-I production. In contrast, src family kinase Fyn acts as a positive regulator of pDC IFN-I and inflammatory cytokine production. Future studies revealing the molecular mechanisms, including potential cross-talk, between CD28 and SFK signaling pathways could yield therapeutic mechanisms to maximize early viral control and/or interrupt the pathogenic IFN-I cycle during autoimmune diseases in pDCs.

MATERIALS & METHODS

Mice and Viruses

C57BL/6 (WT), CD28 deficient mice (CD28 ko), CD80/86 double knockout mice (CD80/86 ko), and Fyn deficient (Fyn ko) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *cd11c*DTR-GFP mice were a gift from Lars Eckmann (UCSD Department of Medicine). Lyn deficient mice (Lyn ko) were a gift from Dr. Toshi Kawakami (LIAI). All ko mice used were in C57BL/6 background. Mice were bred and maintained in a closed breeding facility and mouse handling conformed to the requirements of the National Institute of Health and the Institutional Animal Care and Use Guidelines of UCSD. Mice (6–8 weeks old) were infected intraperitoneally (ip) with 2×10^4 PFU of MCMV Smith (Obtained from Bruce Beutler, TSRI). MCMV was propagated and quantified as described (19).

Generation of bone marrow culture

Bone marrow (BM) cells were isolated from femurs and tibias and a single cell suspension was prepared and seeded in 6-well plates at a concentration of 2×10^6 cells/ml, in a total volume of 5 ml of RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 µM β-mercaptoethanol and 100 ng/ml fms-like tyrosine kinase receptor-3 ligand (Flt3L), (Amgen, Thousand Oaks, CA). After 5 days, half the volume of medium was replaced with fresh medium and 3

days later cells were harvested. These conditions consistently render a bone marrow culture containing approximately 20-35% plasmacytoid dendritic cells (pDC) and 55-70% of conventional dendritic cells (cDC).

Stimulation of BM culture

Bone marrow derived pDCs and cDCs were generated as described above. Functional grade purified anti-CD28 (Clone: 37.51; e-Bioscience, San Diego, CA) or isotype control (Golden Syrian Hamster IgG, e-Bioscience) was used for *in vitro* stimulation assays. Total BM culture or enriched BM pDCs were stimulated with CpG ODN 1668 (Integrated DNA Technologies, San Diego, CA) at 0.1 µM, or Loxoribine (Invivogen, San Diego, CA) at 100 µM. Stimulated cells were seeded at a concentration of 1×10^5 in a total volume of 200 µl in a round bottom 96-well plate, unless otherwise stated. Cells and/or supernatant were collected at time points indicated for cytokine analysis.

Cytokine detection

Total IFN-I bioactivity was measured by luciferase bioassay with reference to a recombinant mouse IFNβ standard (Research Diagnostics, Concord, MA) using a L-929 cell line transfected with an interferon-sensitive luciferase as previously described (7). IFN-α protein was measured by ELISA optimized using a commercially available ELISA as reference (PBL, Piscataway, NJ). 96-well plates were coated with anti-IFN-α monoclonal antibody (clone F-18, Hycult, Plymouth Meeting, PA) in PBS. Plates were blocked with 10% FCS in PBS and

samples and standards (mouse Interferon alpha 1, PBL) were incubated at 4°. Plates were developed following anti-rabbit IgG-HRP (Sigma, St Louis, MO) incubation and TMB substrate (BD Bioscience, San Diego, CA). TNF-α content was measured by ELISA (e-Bioscience).

Inhibition of Kinase Activity

To inhibit src-family kinases, 1×10^5 cells/well were incubated in complete RPMI media plus src-family kinase inhibitor PP2 (CalBiochem, San Diego, CA) at indicated doses for 6 hours in a total volume of 190 µl. For inhibition of PI3K, cells were pre-treated for 1 hr with either Wortmannin (CalBiochem), or the reversible inhibitor Ly294002 (Sigma-Aldrich, St. Louis, MO) at indicated doses. CpG was then added in a volume of 10 µl to avoid dilution of the inhibitor. Addition of DMSO was used as a control. Cells were then cultured for an additional 15 hours in the presence of CpG.

Flow Cytometry

The following antibodies purchased from e-Bioscience or BD-bioscience were used to stain bone marrow culture cells or spleen cells: anti- CD3-, CD19-, NK 1.1-, PerCP-Cy5.5, Thy 1.2-, CD3-, CD19-, CD11c-, NK 1.1-, CD86-, MHCII-PE, CD11c-, CD28-APC, B220-Pacific Blue, CD11b-,B220-APC-eFluor 780, pDCA-1-FITC. Syrian Hamster-APC was used as isotype control when staining with anti-CD28-APC was performed. Propidium Iodide (PI) was used to excluded dead cells and to measure cell viability where indicated. Cells were acquired with

an LSR II flow cytometer (BD Bioscience). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Cell Purification

Spleens were incubated with collagenase D (1 mg/mL, Roche, Indianapolis, IN) for 20 min at 37°C and passed through a 100 µm strainer to achieve a single-cell suspension. Splenocytes were enriched with PanDC or PDCA microbeads using an Automacs system (Miltenyi, Auburn, CA). PanDC$^+$ or PDCA+ fractions were FACS purified using a BD ARIA (BD Biosciences, San Jose, CA) for pDCs (CD11c^{intermediate}CD11b$^-$B220$^+$PDCA$^+$),CD8$^+$ and CD11b$^+$ cDCs (CD11c$^+$B220$^-$CD11b$^+$CD8$^-$) after B (CD19), T (Thy1.2), and NK (Nk1.1) cell exclusion. Purity of the cells was always > 92%.

Generation of cd11c/DTR mixed bone marrow chimeras

To obtain mixed bone marrow chimeras, WT C57BL/6 recipient mice were lethally irradiated with 1000 rads and reconstituted 1 day after with a 85:15 mix of bone marrow cells from *cd11c*/DTR-GFP and either CD28 ko or WT mice, respectively. Bone marrow cells were isolated from femurs and tibia of donor mice and 10 million total cells were transferred intraveneously into the irradiated recipient mice. Recipient mice were treated with antibiotics (Trimethoprim 8 mg/ml and Sulfamethoxazole 40 mg/ml supplied in the drinking water) for 3 weeks to prevent infection and allow immune reconstitution. Reconstitution was analyzed 8 weeks after bone marrow transfer and DTR-GFP expression was

determined by flow cytometry. 56 hours prior to infection, mice were weighed and treated with Diphtheria Toxin (Sigma-Aldrich) 8 ng DT/g body mass i.p. in a volume <200 ul, or the equivalent volume of PBS (control). Mice were treated with a second dose of DT or PBS 8 hours prior to infection. Mice were then infected with MCMV as described above.

Real-time RT-PCR

Total RNA was extracted using RNeasy kits (Qiagen, Valencia, CA), digested with DNase I (RNase-free DNase set; Qiagen) and reverse transcribed into cDNA using Superscript III RT (Invitrogen). cDNA quantification was performed using SYBR Green PCR kits and a Real-Time PCR Detection System (Applied Biosystems, Carlsbad, CA). The RNA levels of *lyn, fyn, hck, fgr, cd28*, *ifna (ifna4 and ifna6),* and *ifnb* were normalized to cellular *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*). All primers were designed using online tools (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Table 1 shows the primers used.

SDS-PAGE and Immunoblotting

Cells were lysed in RIPA buffer (Thermo Scientific, Waltham, Mass) containing protease and phosphatase inhibitors (Calbiochem). Protein homogenates were run on 10% SDS-PAGE gels (BioRad, Hercules, CA). Blots were blocked in 10% milk and incubated with 5% milk containing anti-phospho-Src (Tyr 416) (1:2000; Cell Signaling, Danvers, MA), 4°C overnight with gentle

rotation. Rabbit anti-mouse GAPDH (1:5000; Cell Signaling) was used as loading control. HRP-conjugated anti-rabbit IgG (Cell Signaling; 1:5000 in 5% milk) was then added for 1h at room temperature and protein levels were visualized by chemiluminescence using ECL Plus (Pierce Biotechnology, Rockford, IL).

Microarray

RNA extracted from FACS-purified splenic pDCs (Thy1.2$^-$CD19$^-$NK1.1$^-$ CD11c$^{intermdiate/dim}$CD11b$^-$B220$^+$PDCA$^+$), CD8$^+$DCs (Thy1.2$^-$CD19$^-$NK1.1$^-$ CD11c$^+$CD11b$^-$CD8$^+$) and CD11b$^+$ DCs (Thy1.2$^-$CD19$^-$NK1.1$^-$ CD11c$^+$CD11b$^+$CD8$^-$) from uninfected WT mice were utilized for DNA microarray using mouse Affymetrix chips (Santa Clara, CA).

Statistical Analysis

Unpaired student's t-tests or ANOVA tests were performed using the GraphPad Prism software (Graphpad, La Jolla, CA).

Table 1. Quantitative PCR primers

Gene	Forward sequence	Reverse sequence
ifnb	CTGG CTTCCATCATGAACAA	AGAGGGCTGTGGTGGAGAA
ifna4/6	TATGTCCTCACAGCCAGCAG	TTCTGCAATGACCTCCATCA
cd28	ACAGTTGGGCCACTTGTTGTCCTTT	GCTCCCAATGGTGCCTTCTGGA
gapdh	TCCCACTCTTCCACCTTCGA	AGTTGGGATAGGGCCTCTCTT
lyn	CTTTTAACCGAAGTCACCGT	GAAATTCAGGAACTGGCCTT
fyn	TGGCAAAAGAGCTTGGATAATGGGC	GCTCTGGTTCAGGCTGCCGT
fgr	CACTGAACTGATCACCAAGG	GATTGGCAAGAGCAAGTACA
hck	CGTTGTCTGTTCGAGACTTT	TGCTCCTTGGAGAGATGTAG

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