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Stat3 Inhibits Type I Interferon Signaling In Type I Conventional Dendritic Cells

Taylor Chrisikos

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by

Taylor Thomas Chrisikos, B.S.

APPROVED:

Stephanie S. Watowich, Ph.D.
Advisory Professor

Shao-Cong Sun, Ph.D.

James Allison, Ph.D.

Gregory Lizee, Ph.D.

Danielle Garsin, Ph.D.

Gheath Alatrash, D.O./Ph.D.

APPROVED:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

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Taylor Thomas Chrisikos, B.S.
Houston, Texas

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Dedication

To my parents who always supported me, wherever my interests took me.

And to Nicole, for being with me on this journey, and the next.

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Taylor Thomas Chrisikos, B.S.

Advisory Professor: Stephanie S. Watowich, Ph.D.

Conventional dendritic cells (cDCs) are an essential immune population, responsible for controlling adaptive immunity and tolerance. Recently, type I cDCs (cDC1s) have been delineated as a distinct cDC subset, uniquely responsible for coordinating T cell-mediated immunity against pathogens and tumors. Although the importance of cDC1s is now well established, the mechanisms that regulate cDC1 function remain largely unknown. Signal Transducer and Activator of Transcription 3 (STAT3) mediates the intracellular signaling of interleukin 10 (IL-10), an immunosuppressive cytokine. Therefore, we hypothesized that STAT3 and IL-10 inhibit cDC1 function and induction of T cell-mediated immunity. Herein, we show that IL-10 inhibits polyinosinic:polycytidylic acid (poly I:C)-induced cDC1 maturation in a STAT3-dependent manner. Transcriptome analyses further revealed that although poly I:C induces numerous inflammatory pathways in cDC1s, interferon (IFN) signaling was selectively inhibited by IL-10 and STAT3. Furthermore, assessment of the relative contribution of each IFN type indicated that type I IFN is the primary target of STAT3-mediated inhibition. To determine the impact of these signaling events on cDC1 induction of T cell-mediated immunity, we utilized a cell-based cDC1 anti-tumor vaccine strategy. STAT3 and IL-10 were found to impede the ability of cDC1 vaccination to restrain tumor growth. In addition, both $CD8^+$ T cell and $CD4^+$ T helper cell responses induced by cDC1 vaccination were inhibited by STAT3. Taken

together, we conclude that STAT3 inhibits cDC1-induced anti-tumor immunity and cDC1 type I IFN signaling. As cDC1s are essential for the induction of T cell-mediated immunity, these findings could provide rationale for development of novel immunotherapies for cancer and other immune diseases.

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1.1 – Dendritic cell roles in host defense, tolerance, and anti-tumor immunity

1.1.1 Dendritic cell subsets

Dendritic cells (DCs) are a crucial immune population, responsible for coordinating both host defense against pathogens and immune tolerance [1]. DCs serve this function by arching the innate and adaptive immune systems through their unmatched ability to activate naïve T cells and guide their polarization [1-5]. The DC lineage comprises multiple, developmentally distinct subsets, that differ based on their function and anatomical location. At steady-state, DCs arise from the common DC progenitor (CDP) in the bone marrow and can be divided into two main populations: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [1]. cDCs are categorized into type 1 cDCs (cDC1s) and type 2 cDCs (cDC2s) [1]. cDC1s and cDC2s can be further divided based on their anatomical location. Migratory, or non-lymphoid organ cDCs reside in peripheral non-immune tissues and migrate to lymph nodes (LNs) upon maturation, while lymphoid organ resident cDCs populate immune tissue such as the spleen and LNs. pDCs circulate in the blood and reside in LNs and spleen [1]. Moreover, during active inflammatory settings, DC-like cells can arise from the committed monocyte progenitor (cMoP). This population is termed monocyte-derived DCs (moDCs) and is capable of acquiring cDC-like phenotype and function [1].

cDC1s, cDC2s, and pDCs can be delineated by expression of specific transcriptional regulators that drive their development [6]. cDC1 development is driven by interferon regulatory factor (IRF) 8, basic leucine zipper ATF-like factor (BATF) 3, nuclear factor IL3 regulated (NFIL3), inhibitor of DNA binding (ID) 2, and B cell lymphoma 6 protein (BCL6) [7 8 9 10 11]. In addition, non-lymphoid organ cDC1s require the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF; encoded by *Csf2*) and its signal transducer, Signal Transducer and Activator of Transcription (STAT) 5 [12-14]. cDC2s require IRF4, IRF2, and RELB proto-oncogene, NF-κB subunit (RELB) [15, 16 17 18]. Interestingly, cDC2s appear to be more heterogenous than cDC1s or pDCs, as only certain populations of cDC2s rely on Notch receptor 2 (NOTCH2) and recombination signal binding protein for immunoglobulin kappa J region (RBPJ) signaling, or Kruppel like factor 4 (KLF4) [19, 20 21]. Finally, pDCs require E2-2 (encoded by *Tcf4*), IRF8 and zinc finger E-box binding homeobox 2 (ZEB2) [22 23 24].

In addition to transcription factor-based delineation, DC subsets can also be distinguished from each other based on surface marker expression profiles [1]. All DCs express cluster of differentiation (CD) 11c, and major histocompatibility complex (MHC) class II (MHC II) [1]. pDCs are distinguished from cDCs by expression of CD45R, and sialic acid binding Ig-like lectin (SIGLEC)-H [25]. cDC1s are selectively identified by expression of X-C motif chemokine receptor (XCR) 1, and cDC2s by expression of CD172α [25]. In addition, migratory cDC1s and resident cDC1s can be distinguished by expression of CD103 and CD8α, respectively [25]. Migratory cDC2s are distinguished from resident cDC2s through higher expression of MHC II [26]. Although MoDCs share many markers with cDCs, they can be specifically identified

by expression of CD64, which is a shared marker of all monocyte-derived cells [25]. In addition, while unsupervised high-dimensional clustering has shown these main DC populations are conserved in humans and non-human primates, surface marker expression is different between species [25].

1.1.2 – DC functions in T cell activation and tolerance

DCs bridge the innate and adaptive immune systems by being the primary activators of naïve T cells. T cell activation is a multistep process that requires three main signals provided by DCs: signal 1 – antigen presentation; signal 2 – costimulation; and signal 3 – cytokines [1]. Initially, DCs are in an "immature" state, and although they are constantly phagocytosing and presenting antigen, they express low levels of signals 2 and 3, and thus lack the ability to activate naïve T cells [1]. Nonetheless, immature DCs express an array of receptors that bind evolutionarily conserved pathogen- and danger-associated molecular patterns (PAMPs, DAMPs) termed pattern recognition receptors (PRRs), such as Toll like receptors (TLRs), that allow for detection of potential infection and tissue damage [1]. Upon binding their respective PAMPs or DAMPs, PRRs rapidly induce signals 1, 2, and 3, in a process termed DC maturation, transforming an immature DC into a "mature" DC, proficient in T cell activation [1].

The first signal required for T cell activation is antigen presentation [1]. DCs are the most proficient antigen-presenting cells (APCs) of the immune the system [1]. Antigen-presentation consists of displaying peptides on MHC molecules on the cell surface for recognition by antigen-specific T cells via their T cell receptor (TCR) [1].

DCs constantly degrade proteins in their cytosol with the proteasome and peptides of 8 to 18 amino acids are transferred into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) complex [27]. Once in the ER peptides may undergo further processing and are eventually loaded on to MHC class I (MHC I) molecules and transported to the cell surface [27]. At steady-state, only host-derived peptides are presented, promoting immune tolerance. Upon intracellular infection, however, pathogen-derived peptides are presented. These can be recognized by $CD8^+$ T cells, followed by killing of the cell and resolution of the infection [1].

Although all nucleated cells in the body present their own peptides via MHC I to aid in immune surveillance and pathogen clearance, DCs also have specialized machinery, giving them the ability to present antigen derived from DC-extrinsic sources by phagocytosis [1]. DCs present antigen via two distinct processes: direct presentation, a process by which extracellular antigen is presented on MHC II molecules for recognition by $CD4^+$ T cells; and cross-presentation, in which extracellular antigen is instead presented on MHC I molecules for recognition by $CD8^+$ cytotoxic T cells [1].

Antigen cross-presentation grants DCs the ability to activate naïve $CD8^+$ T cells against intracellular pathogens that have not infected the DC itself, as well as soluble or cell-associated factors [1]. Being phagocytic cells, DCs uptake soluble proteins from the extracellular space, as well as cell-associated antigen from dead cells [1]. Compared to other phagocytic populations like macrophages, DCs have specialized machinery that allows them to efficiently cross-present antigen. For

example, DCs have delayed phagosomal acidification and lysosomal fusion, and decreased expression of lysosomal proteases, in order preserve antigen integrity [28, 29]. DCs maintain altered phagosomal acidification through expression of nicotinamide adenine dinucleotide phosphate (NADP)H oxidase 2 (NOX2)[30-32]. NOX2 generates reactive oxygen species (ROS) inside the phagosome, elevating the pH [30, 32]. Increased phagosomal pH inhibits the activity of acid-sensitive proteases, thus reducing the degradation of peptides that could otherwise be loaded on to MHC I molecules [30, 32]. In DCs, NOX2 is recruited to phagosomes by the member RAS-oncogene family 27A (RAB27A), as well as soluble N-ethylmaleimide-sensitive fusion attachment protein receptor proteins (SNAREs) such as Sec22 homolog B, vesicle trafficking protein (SEC22B), and vesicle associated protein 8 (VAMP8) [31 33, 34]. Moreover, DC maturation signals, such as TLR4 activation, can inhibit phagosomal acidification by decreasing lysosomal fusion, thereby promoting cross-presentation [35, 36]. After internalization into specially regulated DC phagosomes, peptides are transferred to the cytosol where they are integrated into the MHC I antigen presentation pathway described above. This mechanism is termed the “cytosolic pathway” of cross presentation [28]. In addition, there is evidence that peptides generated in phagosomes can be loaded directly onto MHC I molecules also present in the phagosome, in a process termed the “vacuolar pathway” of cross-presentation [37]. However, it is currently believed that the cytosolic pathway is the major contributor to cross-presentation in vivo [28].

Direct presentation enables DCs to present exogenous antigen on MHC II for activation of $CD4^+$ T cells, which further regulate the adaptive immune response [1].

Unlike MHC I, which is ubiquitously expressed, MHC II expression is primarily restricted to DCs, macrophages, monocyte-derived cells, and B cells [27]. Direct presentation begins with the transfer of MHC II molecules from the ER to an acidified phagosome or late endosomal compartment termed the MHC II compartment (MIIC) [27]. Similar to the vacuolar pathway of cross-presentation, peptides derived from phagocytosed proteins are processed by proteases [27]. The peptides are then directly loaded onto MHC II molecules within the MIIC, without requiring cytosolic export and processing [27]. From the MIIC, MHC II – peptide complexes are then transferred to the cell surface for activation of $CD4^+$ T cells [27]. Immature DCs constantly synthesize large quantities of MHC II and, upon maturation, MHC II – peptide complexes are increasingly transported to the cell surface [27]. Interestingly, as opposed to its role in cross-presentation, phagosomal acidification enhances MHC II antigen presentation [38]. The expression of transcription factor EB (TFEB), a transcription factor that promotes lysosomal biogenesis and phagosomal acidification, was found to increase MHC II antigen presentation and inhibit cross-presentation [38].

In summary, direct antigen presentation and cross-presentation are highly specialized functions performed most efficiently by DCs and are absolutely essential for the activation of naïve T cells and the initiation of the adaptive immune response [1]. Although peptide-MHC complex binding by the TCR generates the initial signal driving proliferation and activation, naïve T cells also require costimulation [1].

Signal 2 or costimulation, refers to the activation of CD28 on the surface of a naïve T cell with CD80 or CD86, also known as B7-1 or B7-2, respectively, on the surface of the DC, concurrent with TCR binding of the peptide-MHC complex [1].

Immature DCs express low levels of CD80 and CD86 and rapidly upregulate both proteins upon exposure to maturation stimuli such as TLR agonists [1]. Without signal 2, antigen-presentation fails to completely activate naïve T cells, rendering them anergic [39]. When coupled with TCR activation, however, CD28 ligation supports naïve T cell activation by upregulating crucial survival signals, cytokines, and chemokines necessary for the initiation of a T cell response [40]. For example, CD28 is required for induction of the long isoform of BCL2 Like 1 (BCL2L1) BCL-XL, as well as interleukin (IL)-2, both of which are T cell survival factors [40]. In addition to serving as a ligand for CD28, there is evidence that CD80 and CD86 ligation with CD28 induces signaling in DCs themselves, resulting in increased expression of cytokines that promote immunity against fungal infection and cancer [41].

Finally, signal 3 consists of the cytokines required to promote T cell survival and guide the polarization of specific T helper (Th) subsets and T regulatory (Treg) cells [1]. Without these cytokines, activated $CD8^+$ T cells have poor survival and effector function [42]. Moreover, $CD4^+$ Th cells fail to polarize towards specific subsets without exposure to fate-specifying cytokines [43]. During homeostasis, DCs express low levels of inflammatory cytokines [1]. Upon exposure to TLR agonists or other maturation stimuli, DCs rapidly upregulate many inflammatory and regulatory cytokines [1]. The specific cytokines induced vary, based on DC subset- and stimuli-dependent effects, to initiate the correct type of immune response necessitated by the stimuli [1]. For example, double-stranded RNA viruses activate TLR3 signaling in cDC1s, resulting in upregulation of IL-12 which promotes Th1 cell polarization and $CD8^+$ T cell-mediated cytotoxic immunity [1, 44]. Furthermore, commensal microbiota in

the colon induce IL-6 in cDC2s resulting in the polarization of Th17 cells promoting mucosal immunity [45]. In addition, DCs aid in the polarization of Treg cells through expression of transforming growth factor β (TGF-β), promoting immune tolerance [45].

Although DCs responding to PAMPs or DAMPs produce cytokines that promote T cell activation and function, DCs also require cytokines themselves to fully mature [1]. Some of the most important cytokines promoting DC maturation are the type I interferons (IFNs), which are induced by DCs, and many other cell types, in response to DAMPs and PAMPs [1, 46, 47]. Type I IFNs in mice consist of 14 interferon (IFN)-α subtypes, IFN-β, IFN-ε, IFN-κ, and IFN-ζ, while humans express 13 subsets of IFN-α, IFN-ε, IFN-κ, and IFN-ω [48]. All type I IFNs signal through the heterodimeric interferon-α/β receptor (IFNAR), encoded by interferon alpha and beta receptor subunit 1 (*Ifnar1*) and interferon alpha and beta receptor subunit 2 (*Ifnar2*) [48]. DCs responding to type I IFNs induce all three signals required for T cell activation [1, 46]. After exposure to TLR agonists, IFNAR-deficient DCs have decreased antigen uptake, and reduced expression of complexes involved in antigen presentation such as MHC-II and TAP [47, 49, 50]. IFNAR-deficient DCs also have reduced costimulatory molecule expression in response to TLR agonists [47]. Meanwhile, treatment with type I IFNs induces CD80 and CD86 [51-53]. In addition, TLR agonists fail to fully induce inflammatory cytokines such as IL-6, and IL-15 in IFNAR-deficient DCs [49, 54]. Furthermore, treatment with type I IFNs induces chemokines and cytokines like C-X-C chemokine motif ligand (CXCL)10 and IL-12 [55, 56]. Because of these type I IFN-mediated effects on DC function, type I IFN signaling is necessary for DCs to induce full activation and proliferation of T cells [47, 49, 50, 55]. As

a result, IFN-Is promote the T cell-mediated clearance of infection from virus and other intracellular pathogens [48, 50].

Finally, in addition to being important inducers of T cell mediated immunity against pathogens, DCs also play important roles in immune tolerance [1, 57]. Immune tolerance is divided into two main processes; central tolerance, which consists of shaping developing T and B cell populations in the thymus and bone marrow to prevent the emergence of self-reactive clones that promote autoimmunity; and peripheral tolerance, which encompasses many mechanisms mediated in secondary immune structures and peripheral organs that inhibit immune responses towards self and innocuous antigens [57]. DCs in the thymus contribute to central tolerance by presenting self-antigen to promote the negative selection of self-reactive $CD4^+$ T cells or their development into Treg cells, which further promote peripheral tolerance [58-61]. DCs also contribute directly to peripheral tolerance [57]. Steady-state DCs presenting self-antigen and expressing low amounts of costimulatory molecules or inflammatory cytokines induce deletion, or an anergic state in potentially autoreactive, naïve $CD8^+$ T cells [39, 57, 62, 63]. The ability of steady-state DCs to tolerize $CD8^+$ T cells depends upon MHC-II – dependent, suppressive interactions with Tregs [64, 65]. In addition, overall DC numbers at steady-state are tightly linked with total Treg amounts [66]. Mice which lack the DC growth factor FMS-like tyrosine kinase 3 (FLT3) and are deficient in all DC subsets were found to have greatly reduced splenic Treg amounts [66]. Furthermore, expansion of prostate-specific Tregs was found to rely on migration of DCs to prostate-draining LNs and MHC II expression, demonstrating that DCs actively promote Treg abundance during

homeostasis [67]. The process of DCs undergoing maturation at steady-state, in the absence of normal maturation factors, to induce Treg activation and promote tolerance has been termed “homeostatic maturation” [57, 63, 68]. Taken together, DCs not only perform crucial tasks in inducing the adaptive immune response towards pathogens, but also actively promote immune tolerance during steady-state, preventing autoimmunity.

1.1.3 – Subset-specific functions of cDC1s in host defense and tolerance

Each DC subset is functionally specialized for the induction for certain immune responses. cDC1s are the main inducers of $CD8^+$ T cell-mediated immune responses [8, 69-74]. BATF3-deficient mice, which selectively lack the cDC1 subset, have greatly reduced $CD8^+$ T cell-mediated responses to many viruses and intracellular pathogens such as *Toxoplasma gondii* or *Leishmania major* [8, 75-83]. Similarly, cDC1 depletion using XCR1-diptheria toxin receptor- (DTR) expressing mice reduces $CD8^+$ T cell responses against intracellular infection by *Listeria monocytogenes* [84]. In addition, mice that selectively lack cDC1s due to G protein coupled receptor 141b (GPR141b)-deficiency have reduced $CD8^+$ T cell-mediated immunity during primary and secondary challenges to *Listeria monocytogenes* and multiple viruses [85]. Taken together, cDC1s are necessary to generate protective and long lived $CD8^+$ T cell-mediated immunity towards viruses and other intracellular pathogens.

cDC1s function as the main initiator of $CD8^+$ T cell mediated immunity through their unmatched ability to cross-present antigen [8, 69, 70, 72-74, 86-88]. In direct

comparisons between DC subsets, cDC1s induce greater $CD8^+$ T cell proliferation than cDC2s, after in vivo exposure to ovalbumin (OVA)-loaded splenocytes, OVA-conjugated antibodies, or *Plasmodium berghei* infection [69, 70, 72]. In vitro studies have shown that cDC1s are more proficient at promoting $CD8^+$ T cell proliferation after treatment with cell-associated antigen, than cDC2s or moDCs [73, 74, 86]. In addition, cDC1s are more capable than cDC2s at cross-presenting antigen after in vitro treatment with antigen-conjugated beads, or cell-free, soluble antigen [73, 74, 87]. Taken together, cDC1s are uniquely able to cross-present antigen and activate $CD8^+$ T cells.

cDC1s retain increased cross-presentation capacity using specialized machinery [70, 74, 86-88]. For example, cDC1s use Rac family small GTPase 2 (RAC2) to assemble NOX2 at the membrane of the phagosomes [87]. This results in increased phagosomal pH and inhibits antigen degradation [87]. However, cDC2s use Rac family small GTPase 1 (RAC1) to assemble NOX2 at the plasma membrane [87]. Differential NOX2 subcellular localization results in a NOX2-dependent increase in cross-presentation in cDC1s versus cDC2s [87]. Similarly, human cDC1s were found to have lower expression of lysosomal proteases compared to cDC2s, which correlates with increased cross-presentation by cDC1s [88]. In addition, member Ras oncogene family 34(RAB34)-deficient cDC1s are unable to cross-present cell-associated or soluble antigen, while RAB34-deficient moDCs retain their modest capacity for $CD8^+$ T cell activation [86]. Therefore, there exists subset-specific mechanisms of cross-presentation and not just differences of efficiency [86]. Likewise, WDFY family member 4 (WDFY4)-deficiency rendered cDC1s, but not moDCs, unable to cross-present

cell-associated antigen [74]. In summary, cDC1s employ unique mechanisms to maintain antigen integrity and promote cross-presentation, resulting in their unmatched ability to activate $CD8^+$ T cells.

cDC1s also perform roles independent of cross-presentation that are important for the induction of $CD8^+$ T cell mediated responses [73]. This was made abundantly clear when *Batf3*$^{-/-}$ mice were rescued from cDC1-deficiency by forced overexpression of IRF8 [73]. Although transgenic IRF8 overexpression restored cDC1 development and cross-presentation abilities, these mice were not able to reject tumors that are normally cleared in a cDC1- and $CD8^+$ T cell -dependent manner [73]. This finding indicates there are important cross-presentation – independent functions mediated by cDC1s [73]. Undoubtedly, one important cross-presentation – independent function of cDC1s is to act as a platform for Th1 cell-mediated help of the $CD8^+$ T cell response [89, 90]. In a mouse model of vaccinia virus infection it was found that depletion of cDC1s with XCR1-DTR did not alter the early activation of $CD8^+$ or $CD4^+$ T cells [90]. This was likely due to direct presentation by other actively infected APC subsets, as well as dissemination of viral particles throughout the LNs. Thus, this system allowed for the investigation of antigen presentation – independent roles of cDC1s [90]. Vaccinia virus infection requires Th1 cells for full activation of the $CD8^+$ T cell-mediated antiviral immune response. Using this model, cDC1s were found to be essential for transmission of Th1-mediated help [90]. At later time points after vaccinia virus infection, cDC1s were required for the formation of cDC1-Th1-$CD8^+$ T cell tri-cell clusters, which are essential for the Th1-mediated help of $CD8^+$ T cells [90]. Furthermore, cDC1-depleted mice had reduced $CD8^+$ T cells at 8 days post

infection, or after secondary antigen challenge, mimicking results found in $CD4^+$ T cell-depleted animals [90]. Taken together, these results suggest that cDC1s are a critical mediator of Th1-dependent immunity, independent of their function as a cross-presenting APC.

Although cDC1s are important for mediating Th1 to $CD8^+$ T cell cross talk, cDC1s also play important roles in promoting the development of Th1 cells [82, 89]. Early during murine Herpes Simplex Virus 1 (HSV-1) infection, migratory cDC1s activate and polarize Th1 cells [89]. Furthermore, cDC1s are the primary source of IL-12, a Th1 polarizing cytokine, during infection with *Listeria monocytogenes*, vaccinia virus, *Toxoplasma gondii*, and *Leishmania major* [85 82 91]. In addition, cDC1s directly activate and induce anti-tumor Th1 cells [92]. Taken together, cDC1s play an important role in activating $CD4^+$ T cells, polarizing them towards Th1 differentiation through IL-12 production, and promoting their crosstalk with $CD8^+$ T cells.

In addition to promoting the activation of $CD8^+$ T cells and the polarization of Th1 cells in inflammatory settings, cDC1s play important roles in promoting immune tolerance at steady-state [57]. cDC1s that have undergone homeostatic maturation and cross-present self-antigen prevent autoimmunity by inducing anergy or deletion of self-reactive $CD8^+$ T cells, in a process termed “cross-tolerance” [93-95]. The ability of cDC1s to cross-tolerize $CD8^+$ T cells requires MHC-II – dependent interactions with Tregs, as MHC-II – deficient cDC1s are incapable of cross-tolerizing self-reactive $CD8^+$ T cells and instead promote their activation and subsequent autoimmunity [64, 65, 94]. Furthermore, studies targeting antigen to various subsets of DCs have revealed that cDC1s efficiently induce Tregs that inhibit experimentally

induced autoimmunity [96, 97]. In summary, cDC1s act as major regulators of $CD8^+$ T cell tolerance through their ability to cross-tolerize and both mediate and induce Treg-dependent immunosuppression. These tolerogenic functions, in addition to roles for cDC1s in directly activating $CD8^+$ T cells, polarizing Th1 cells, and acting as the platform through which Th1s deliver help, place cDC1s as crucial regulators of $CD8^+$ T cell mediated immune responses.

1.1.4 – The role of cDC1s in anti-tumor immunity

cDC1s are essential for generating $CD8^+$ T cell – mediated anti-tumor immunity, as their absence prevents the rejection of immunogenic tumors in mice [8]. Likewise, the intratumoral abundance of cDC1s correlates with improved survival in many human cancers [98-101]. In line with their role in generating $CD8^+$ T cell – mediated immunity, cDC1 accrual in human tumors correlates with $CD8^+$ T cell abundance and expression of $CD8^+$ T cell-associated cytolytic factors such as granzyme B [102, 103]. cDC1s induce anti-tumor $CD8^+$ T cells through their unmatched ability to cross-present antigen, as evidenced by a lack of naïve $CD8^+$ T cell activation by DCs from BATF3-deficient mice [8]. In addition, comparison of many APC-subsets purified from tumor-draining lymph nodes (TdLNs) demonstrated directly that cDC1s are the most capable at inducing the proliferation of naïve $CD8^+$ T cells [98]. Similar to infectious-disease contexts, tumor microenvironment (TME) and TdLN cDC1s express specific factors, such as WDFY4 [74], and phenotypes, such as increased endocytic pH [98], that are associated with improved cross-presentation ability. Migratory cDC1s, specifically, are required for initiation of anti-tumor immunity

due to their ability to be recruited to tumor tissue via C-C motif chemokine ligand (CCL)3, CCL4, and X-C motif ligand (XCL)1 [100, 104, 105]. After seeding the tumor, migratory cDC1s internalize tumor antigen and traffic to the TdLNs in a C-C motif chemokine receptor (CCR)7 – dependent manner, followed by activation of naïve $CD8^+$ T cells [26, 106].

In the context of the TME, cDC1s also perform several cross-presentation – independent roles that are important for induction of anti-tumor immune responses [1, 73]. In addition to activating anti-tumor $CD8^+$ T cells, cDC1s are a major source of chemokines that recruit $CD8^+$ T cells to the TME, such as C-X-C motif chemokine ligand (CXCL)9 and CXCL10 [107, 108]. Furthermore, cDC1s express the highest amount of the Th1-polarizing cytokine IL-12 in the TME, compared to other APC populations [98]. Moreover, cDC1s directly activate Th1 cells in the TME [92]. Taken together, cDC1s promote anti-tumor Th1 responses and T cell recruitment to the TME, in addition to their role in activating $CD8^+$ T cells.

To perform these essential functions and promote anti-tumor immunity, cDC1s require IFN-I signaling [109, 110]. This is likely due in part to the ability of type I IFNs to promote the ability of cDC1s to cross-present antigen to $CD8^+$ T cells and induce their proliferation [109]. In addition, type I IFN treatment induces CXCL9 and CXCL10 expression in cDC1s [110]. Furthermore, type I IFN expression is induced in migratory cDC1s in the TME by exposure to tumor-associated DNA [111]. Type III IFNs, which activate signaling pathways and upregulate genes very similar to type I IFNs [48], are also induced in migratory cDC1s in the TME [101]. Moreover, type III IFN abundance in the human TME is correlated with T cell chemoattractants such as

CXCL9 and CXCL10, the Th1-polarizing cytokine IL-12, and improved patient survival [101]. In summary, cDC1s require type I and perhaps type III IFN signaling to promote their ability to induce the anti-tumor $CD8^+$ T cell mediated immune response.

Given these crucial functions cDC1s perform in inducing anti-tumor immunity, cDC1s are also essential for mediating positive responses to many immunotherapy treatments for cancer [1]. Intratumoral abundance of cDC1s prior to treatment with anti-programmed cell death protein 1 (PD-1) therapy predicts patient responsiveness [99]. Furthermore, anti-PD-1 treatment efficacy requires cDC1 expression of CXCL9 [112] and IL-12 [113]. In addition, mice that lack cDC1s altogether do not respond to multiple tumor immunotherapies [114, 115] [107]. For example, *Batf3*$^{-/-}$ mice do not respond to anti-PD-1 or adoptive T cell therapy in mouse models of colon cancer or melanoma, respectively [107, 114]. Collectively, cDC1s are essential for the development of spontaneous anti-tumor immunity, as well as the efficacy of many tumor immunotherapies.

Due to their essential role in promoting spontaneous and pharmacologically induced anti-tumor T cell-mediated immunity, cDC1s themselves have recently been utilized as a cell-based vaccine strategy for the treatment of cancer [116, 117]. Interestingly, vaccination with the cDC1-based vaccine led to increased efficacy in murine melanoma when compared to a moDC-based vaccine that closely resembles those used to treat human patients with modest efficacy [116]. Thus, the use of cDC1s in human DC-based vaccine strategies could provide increased efficacy compared to the moDC vaccines used previously. In addition, further characterization of the

mechanisms regulating cDC1-vaccine efficacy may reveal novel aspects of cDC1 biology that could be targeted pharmacologically for improved therapeutic benefit.

1.2 – The role of STAT3 in dendritic cell development and function

1.2.1 – Cytokines and the JAK-STAT pathway

STAT proteins are an essential transcription factor family that translates extracellular signals into regulation of gene expression[118]. In collaboration with Janus kinases (JAKs), STATs enact a crucial signal transduction pathway downstream of many cytokine receptors, termed the JAK-STAT pathway [118]. Specifically, in their ligand unbound state, cytokine receptors exist as dimers or multimers in a confirmation that does not support receptor-associated JAK activity or downstream signal transduction [118]. However, upon binding of the ligand, the receptor undergoes a confirmational change that brings receptor-associated JAKs in close proximity and promotes their activation through transphosphorylation [118]. Activated JAKs then phosphorylate specific tyrosine residues on the intracellular domains of the receptor [118]. Utilizing their Src homology 2 (SH2) domains which bind phophsotyrosines (pTyr), STAT proteins bind to these newly formed, intracellular pTyr sites [118]. Receptor-bound STATs are then phosphorylated by JAKs at specific tyrosine residues in their C-terminal domain [118]. Following phosphorylation, phospho-STATs dissociate from the receptor and form homo- or hetero-dimers through reciprocal pTyr-SH2-dependent interactions. Dimerized phospho-STATs

then translocate to nucleus where they bind specific DNA sequences to directly regulate gene expression [118]. In addition to playing a crucial role in cytokine receptor signaling, STATs are also critical for signal transduction downstream of many other receptor tyrosine kinases such as FLT3 [118]. Furthermore, STATs have been shown to mediate indirect modes of gene regulation through interactions with other transcriptional regulators such as chromatin remodeling proteins [118].

The STAT protein family has 7 members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. All STATs share a similar structure. However, each STAT is associated with the transcriptional response of a distinct suite of cytokines and receptors [118]. For example, STAT1 is necessary for the induction of most IFN stimulated genes (ISG) in response to all 3 types of IFNs, while STAT6 is required for induction of genes downstream of IL-4 and IL-13 [119, 120]. The selectivity of individual STATs in mediating the intracellular signaling of certain cytokines is due to the amino-acid sequence of the STAT-binding site on the receptor. For example, pYxxP sequences are associated with STAT1 recruitment while pYxxQ sequences preferentially recruit STAT3 [119, 121]. In addition, cytokine receptors selectively associate with specific JAKs. The JAK family is composed of 4 proteins: JAK1, JAK2, JAK3, and TYK2. Therefore, each JAK is responsible for transducing the cytokine signal from a unique set of receptors to a specific group of STATs. For example, JAK1 activates STAT3 downstream of IL-6 or IL-10 family cytokines, among others [122, 123].

There are a diverse array of cytokines, and they perform a variety of functions within the immune system. Cytokines can be loosely classified as being pro- or anti-

inflammatory. An individual cytokine may display both pro- and anti-inflammatory effects depending on the specific context. For example, IL-6 is required to clear infection with *Listeria monocytogenes*, an intracellular bacterial pathogen [124]. However, IL-6 also inhibits anti-tumor immunity and the levels of inflammatory cytokines in circulation after endotoxin exposure [125, 126]. Likewise, individual STATs can mediate both pro- and anti-inflammatory functions. As is the case with STAT3, where it is required for neutrophil production and mobilization during acute inflammatory settings but also dampens the immune response to tumors [127, 128]. These somewhat contradictory findings demonstrate the complexity with which cytokines and STATs regulate the immune response.

1.2.2 – STAT3 signal transduction

STAT3 was originally described as the acute phase response factor (APRF), which induces genes downstream of IL-6 signaling in rat livers and human liver cells [129]. Shortly thereafter, the APRF was determined to be closely related to STAT1 in both structure and function and was designated as STAT3 [122, 130, 131]. Alternative splicing of the mRNA gives rise to two STAT3 isoforms: STAT3α, the full-length protein; and STAT3β, a truncated version lacking the full transactivation domain (Figure 1.2A). As with the other STATs, STAT3 is activated by many cytokines and growth factors (Figure 1.2B). Particularly, STAT3 is important for signaling downstream of IL-6 and IL-10 family cytokines [132]. In addition, STAT3 is required for responding to growth factors such as granulocyte colony-stimulating factor (G-CSF; encoded by *Csf3*) and FLT3L [132].

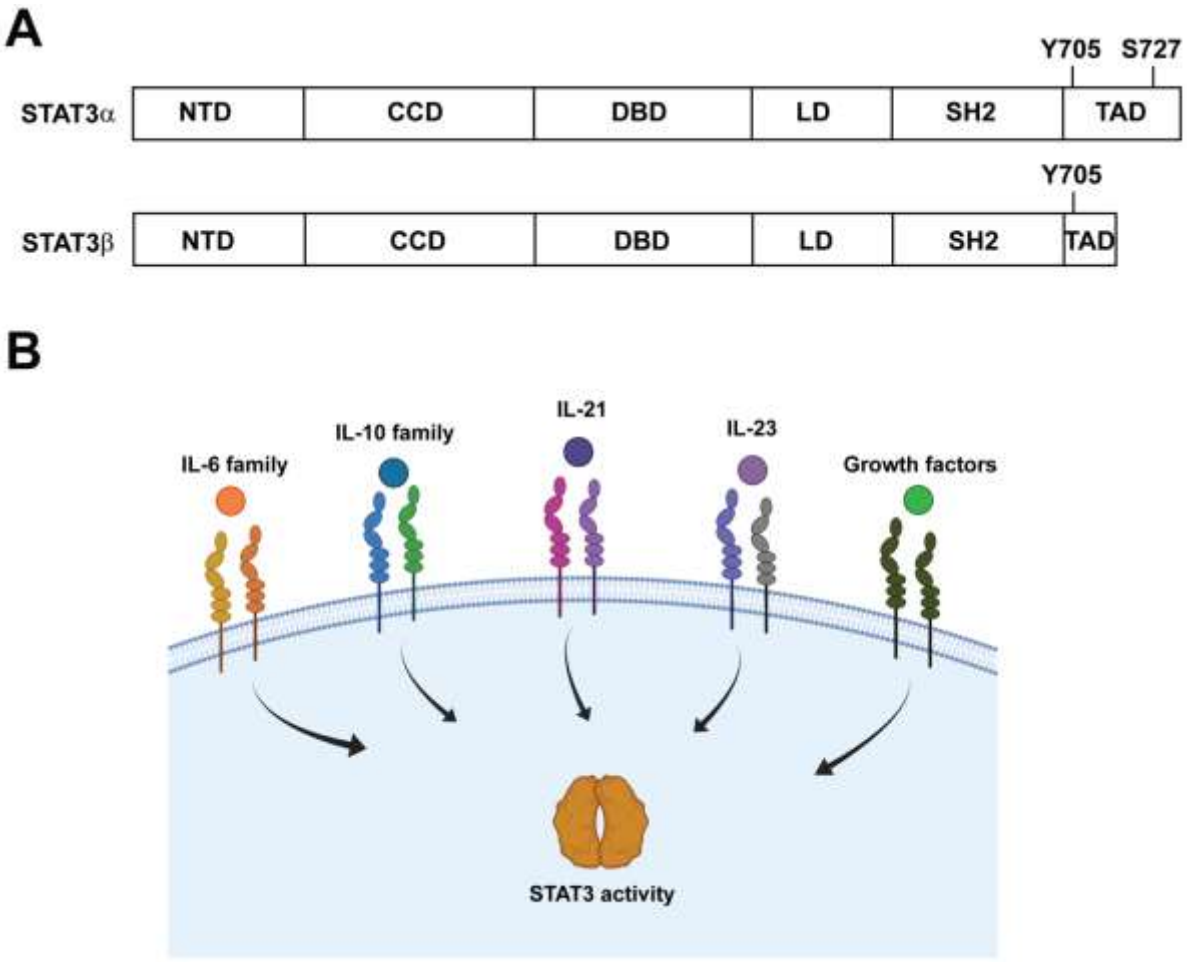

Figure 1.1. Schematic of the functional domains of STAT3 and depiction of the cytokine families that signal through STAT3. (A) The six functional domains and important phosphorylation sites in STAT3α (upper) and STAT3β (lower). N-terminal domain (NTD), coiled coil domain (CCD), DNA-binding domain (DBD), linker domain (LD), SH2 domain (SH2), transactivation domain (TAD). (B) The major cytokine families that require STAT3 for downstream transcriptional control.

STAT3 is first recruited to ligand-bound receptors though SH2-dependent interactions that rely on its coiled coil domain [133]. Activation of STAT3 is induced by phosphorylation of an evolutionarily conserved tyrosine residue near the C-terminal region (Y705). Following activation, STAT3 primarily forms homodimers through reciprocal SH2-dependent interactions [130, 131]. In addition, STAT3 forms heterodimers with STAT1 in response to IL-6 and IFN-α, among others [130, 131, 134]. Activated STAT3 dimers are then translocated to the nucleus by importin proteins [135, 136]. Once in the nucleus, the DNA-binding domain of STAT3 binds directly to specific DNA sequences (TTCnnnGAA) resulting in control of transcription [137-139]. In addition to binding DNA, STAT3 can also bind other transcriptional coactivators, such as p300 which remodels chromatin to promote transcription, through interactions with the transactivation domain in its C-terminus [140].

Although Y705 phosphorylation is the essential signal regulating STAT3 activation, other post-translational modifications are capable of regulating STAT3 transcriptional control. Serine-phosphorylation at position 727 (S727) promotes maximum induction of STAT3 target genes in response to cytokines and growth factors [141]. In addition, STAT3 acetylation and methylation have been reported to regulate induction of STAT3 target genes; however, much remains to be determined in regards to the roles these acetylation and methylation play in controlling STAT3 function [142, 143].

Interestingly, STAT3 maintains functions independent of its role as a cytokine-activated transcription factor. For example, S727 phosphorylation of STAT3 in response to TLR agonists results in mitochondrial translocation and regulation of

cellular respiration [144, 145]. STAT3 may also directly regulate cytoskeletal dynamics during cellular migration [146]. Moreover, unphosphorylated STAT3 can regulate transcription of genes normally regulated by phospho-STAT3, as well as noncanonical STAT3 target genes in collaboration with other transcription factors [147, 148].

1.2.3 – STAT3 mutations and human disease

The importance of STAT3 in the human immune system is demonstrated by the various immune diseases associated with STAT3 mutations [149]. Loss-of-function (LOF) mutations in *STAT3* can cause autosomal dominant hyper immunoglobulin E syndrome (AD-HIES) [150]. AD-HIES is characterized by elevated circulating immunoglobulin E (IgE), recurrent skin and lung infections, bone and connective tissue abnormalities, and increased cytokine production in innate immune cells after TLR agonist exposure [150, 151]. The recurrent skin and lung infections in *STAT3* LOF AD-HIES result from an inability to mount STAT3-dependent neutrophil– and Th17 cell–mediated antimicrobial responses [152]. Furthermore, an inability to generate functional memory $CD8^+$ T cells in *STAT3* LOF AD-HIES likely contributes to their inability to control viral infection [153]. In addition, another type of human *STAT3* LOF mutation has been described that was associated with a fatal fungal infection [154]. Taken together, *STAT3* LOF mutations reveal protective and anti-inflammatory roles for STAT3.

Gain-of-function (GOF) mutations have also been reported in *STAT3* in humans and are associated with altered immune system functioning [155]. Specifically,

STAT3 GOF mutations are associated with early-onset multi-organ autoimmunity, lymphoproliferation, lymphadenopathy, cytopenia, disseminated bacterial infection, and stunted growth [156-158]. These GOF mutations result in increased transcriptional activity of STAT3 in response to cytokines [156-158]. Likewise, the use of JAK or IL-6 inhibitors was found to alleviate some of the autoimmune symptoms in *STAT3* GOF patients [157, 159]. In addition, the autoimmune symptoms induced by *STAT3* GOF mutations are thought to occur, in part, due to inhibition of Treg cell development and function [160-164].

In summary, the study of patients with STAT3 mutations has suggested that altered STAT3 activity has a large effect on human immunity. This points to diverse roles for STAT3 in regulating the immune response. However, it should be noted that the functions of STAT3 outside of the immune system also contribute to human disease. For example, in a mouse model of *STAT3* LOF AD-HIES, bone marrow transplantation with control *STAT3* bone morrow only partially rescued the mice, indicating involvement of STAT3 outside of the immune system [165]. In addition, somatic mutation of *STAT3* is important for cellular transformation and tumorigenesis in non-immune cell populations [166]. Furthermore, germline *Stat3* deletion in mice results in embryonic lethality, indicated STAT3 has essential roles in areas other than immune regulation [167-169].

1.2.4 – STAT3 roles in host defense and autoimmunity

Neutrophils are an essential innate immune population mediating the clearance of extracellular bacteria and fungi [170]. G-CSF is the primary growth factor

supporting neutrophil production and STAT3 is the main signal transducer activated by the G-CSF receptor (G-CSFR; encoded by *Csf3r*) [171, 172]. Mice that have been genetically engineered to lack the ability to activate STAT3 upon G-CSF – G-CSFR signaling have chronic neutropenia that can be rescued upon expression of a constitutively active mutant of STAT3 [171]. This indicates that STAT3 promotes G-CSF – dependent neutrophil development at steady-state [171]. Furthermore, during acute infection, G-CSF promotes increased production of neutrophils from immature neutrophil progenitors, a process termed "emergency granulopoiesis" [172, 173]. Without STAT3, G-CSF cannot induce the expansion of neutrophil progenitors in the bone marrow, resulting in failed emergency granulopoiesis [127, 174]. STAT3 promotes emergency granulopoiesis by mediating G-CSF – dependent induction of CCAAT-enhancer-binding protein β (C/EBPβ), another important transcription factor downstream of G-CSF signaling [175]. In addition, mature neutrophils that lack STAT3 are unable to mobilize from the bone marrow following acute G-CSF stimulation [174]. Similarly, STAT3-deficient neutrophils are unable to egress from the bone marrow upon administration of CXCL2, a neutrophil chemoattractant [176]. This is due to the role of STAT3 in mediating G-CSF – dependent expression of C-X-C motif chemokine receptor (CXCR) 2, the receptor for CXCL2 [176]. Taken together, STAT3 supports neutrophil development, mobilization, and recruitment, all of which are crucial for host defense against extracellular bacteria and fungi.

In addition to neutrophils, Th17 cells are also dependent on STAT3 for their development [132]. Th17 cells are important for maintaining barrier tissue integrity and defending against pathogens at mucosal sites, but have also been attributed to

promotion of chronic inflammation and autoimmunity [177]. Th17 cell specification upon activation of a naïve CD4 T cell relies on STAT3-dependent IL-6 signaling [178, 179]. STAT3 drives the expression of retinoic acid receptor-related orphan receptor (ROR) α and RORγ, essential lineage-specifying transcription factors for Th17 cell differentiation [179, 180]. In addition, STAT3 induces the expression of multiple cytokines and cytokine receptors important for Th17 cell development and function, such as *Il17, Il21,* and *Il23r* [178, 181]. Furthermore, both IL-21 and IL-23 signal in a STAT3-dependent manner to further support Th17 cell differentiation and function [178, 182]. IL-17 produced by Th17 cells drives protective immune responses by inducing antimicrobial peptides and neutrophil chemoattractants that aid in the clearance of pathogens, particularly bacteria and fungi at mucosal sites [152, 177].

Significantly, Th17 cell-induced immune responses are associated with many autoimmune diseases that are also correlated with alterations in *STAT3* [177, 183-186]. Conversely, Treg cells that are capable of inhibiting autoimmunity are negatively regulated by STAT3 during their differentiation [160-164, 170]. Specifically, STAT3 activity downstream of IL-27 or IL-6 inhibits forkhead box P3 (FOXP3) expression, the key transcription factor driving Treg cell differentiation and function [160, 161]. STAT3-mediated inhibition of Treg cell differentiation results in decreased survival during graft-versus-host disease [162]. Furthermore, STAT3-activity is associated with decreased Treg cell abundance during autoimmune arthritis and decreased proliferative capacity in Treg cells from psoriasis patients [163, 164]. In summary, STAT3 promotes autoimmunity through its dual role in promoting Th17 cell differentiation and inhibiting Treg development.

Along with promoting neutrophil- and Th17 cell-dependent immunity, STAT3 promotes B cell mediated immune responses. Naïve B cells are capable of differentiating into antibody secreting cells, termed plasma cells, as well as memory B cells, to provide long-lived protection against reinfection. Early in B cell development, STAT3 is required for the transition of pre–pro-B cells into successive B cell precursors in a mechanism that may involve FLT3L and IL-7 signaling [132, 187, 188]. In addition, differentiation of activated B cells into plasma cells depends upon STAT3 signaling induced by IL-10 and IL-21 [189-191]. STAT3 induces the expression of genes encoding plasma cell promoting transcription factors such as *PRDM1* and *XBP1* [191, 192].

B cell mediated immune responses are also indirectly regulated by STAT3 due to the role STAT3 plays in the development of T follicular helper (Tfh) cells [132]. Tfh cells are essential for the differentiation of long-lived plasma cells and memory B cells [193]. The differentiation of naïve $CD4^+$ T cells into Tfh cells relies on STAT3 activity downstream of IL-6 and IL-21 [194-196]. STAT3 induces the expression of BCL6, an essential transcription factor for Tfh differentiation [195]. Furthermore, Tfh cells are an important source of IL-21 for B cells, as discussed above [197]. Moreover, Tfh cell IL-21 expression is induced in a STAT3-dependent manner by IL-27 [197]. Taken together, STAT3 is crucial for the proper development and function of B cell-mediated humoral immunity, by promoting B and Tfh cell differentiation and function.

STAT3 also plays an important role in the development of memory $CD8^+$ T cells. Without STAT3, activated $CD8^+$ T cells cannot respond to IL-21 or IL-10, and as a result lack the capacity to differentiate into functional memory T cells [198].

Although STAT3-deficient $CD8^+$ T cells mediate the clearance of lymphocytic choriomeningitis virus (LCMV) during primary challenge, the memory $CD8^+$ T cells that develop post-infection are incapable of proliferating or limiting viral replication upon reinfection [198]. Furthermore, STAT3-deficient $CD8^+$ T cells have reduced BCL6, Eomesodermin (EOMES), and suppressor of cytokine signaling (SOCS) 3 expression, which support memory $CD8^+$ T cell formation and function [198-200]. Moreover, these findings recapitulate the $CD8^+$ T cell phenotype present in human AD-HIES patients with *STAT3* LOF mutations [153]. In summary, STAT3 is essential for $CD8^+$ T cell mediated immune memory. Collectively, STAT3 controls cytokine induced responses in many immune cell types to orchestrate protective immunity against pathogens; meanwhile, STAT3 can also promote autoimmunity in conditions of immune dysregulation.

1.2.5 – Anti-inflammatory functions of STAT3

Removal of STAT3 from the hematopoietic compartment in mice, using the MX Cre or TIE2 Cre systems, revealed STAT3 functions as a negative regulator of inflammation [201, 202]. Specifically, these mice develop spontaneous colitis and have elevated levels of inflammatory cytokines in the circulation [201, 202]. Myeloid cell restricted depletion of STAT3 recapitulates the phenotype displayed by total hematopoietic deficiency [203, 204]. Moreover, the colitis in myeloid-specific STAT3-deficient mice required TLR4 and IL-12, suggesting that TLR agonist-induced cytokine expression is repressed by STAT3 [203]. Similar to the findings in STAT3-deficient models, mice that lack IL-10, an anti-inflammatory cytokine, also develop

colitis [205]. Furthermore, colitis in IL-10-deficient mice is associated with increased signaling downstream of PAMP receptors upon sensing commensal microbiota [206, 207]. Therefore, STAT3 and IL-10 form a potent anti-inflammatory signaling axis that prevents excessive cytokine production induced by the intestinal microbiome.

Indeed, IL-10 treatment inhibits cytokine expression induced by TLR agonists in many myeloid cell populations, including macrophages and DCs (discussed further below) [208, 209]. STAT3 induces the expression of anti-inflammatory mediators upon IL-10 treatment, such as SH2 domain containing inositol polyphosphate 5-phophatase (SHIP1; encoded by *Inpp5d*) [210-213]. In macrophages specifically, STAT3 represses expression of the ubiquitin-conjugating enzyme 13 (UBC13; encoded by *Ube2n*), which mediates signaling downstream of TLR4, resulting in decreased cytokine expression after TLR4 activation [214]. STAT3 and UBC13 also play important roles in regulating lineage-balanced hematopoiesis [215]. The inflammation resulting from hematopoietic STAT3-deficiency leads to an accumulation of DNA damage in HSCs and eventual bone marrow failure [215]. However, upon concomitant deletion of *Ube2n*, HSC and overall bone marrow function was rescued from STAT3-deficiency [215]. Therefore, during inflammation, a STAT3-UBC13 axis maintains proper HSC and bone marrow function.

In addition to inhibiting inflammatory responses in the intestine, STAT3 also restrains anti-tumor immunity [128]. More specifically, in irradiated mice reconstituted with STAT3-deficient bone marrow, transplanted tumors are ultimately rejected as compared to mice harboring STAT3-sufficient bone marrow, where transplanted tumors grow progressively [128]. Notably, hematopoietic STAT3-deficient tumor-

bearing mice exhibit decreased abundance of Treg cells in the TME [128]. Upon isolation of STAT3-deficient neutrophils or natural killer (NK) cells from tumors, both populations demonstrate increased cytolytic capacity compared to STAT3-sufficient cells [128]. Furthermore, hematopoietic STAT3-deficiency results in increased $CD8^+$ T cell amounts due to upregulation of CXCR3 allowing for improved CXCL10-dependent chemotaxis [128, 216]. Taken together, STAT3 acts in many tumor-associated immune populations to inhibit anti-tumor immunity.

STAT3 is constitutively active in most cells in the TME because of an abundance of STAT3-activating cytokines, such as IL-6 and IL-10, that inhibit anti-tumor immunity and promote tumor growth [128, 217, 218]. In addition, STAT3 activating cytokines act in a feed-forward mechanism to further induce other immune-inhibitory cytokines in the TME [219]. For example, STAT3 activity in tumor-associated macrophages induces the expression of IL-23 [219]. Treg cells in the TME express the IL-23 receptor and induce IL-10 in a STAT3-dependent manner, which can inhibit macrophage production of IL-12 and further promote STAT3-mediated inhibition of anti-tumor immunity [219, 220].

1.2.6 – STAT3 in DC development and function

STAT3 has multiple roles in DCs that begin with its function as a key transcription factor regulating DC development. FLT3 and FLT3L, are major promoters of the development of all DC subsets [188]. STAT3 is phosphorylated by FLT3 and is required for FLT3L-dependent differentiation and proliferation of most DC subsets [14, 221, 222]. STAT3 is particularly important in the development of pDCs

where it controls the expression of E2-2, a crucial transcription factor that drives pDC differentiation and function [14]. Interestingly, although STAT3 appears to be dispensable for terminal differentiation of migratory cDC1s in response to hydrodynamic gene transfer of FLT3L, STAT3 is required for lymphoid tissue resident cDC1s [14, 223]. This suggests there are different developmental requirements for the resident and migratory subsets of cDC1s in the context of elevated FLT3L. Depending on the inflammatory context, STAT3 can actually inhibit the development of cDC1s from DC progenitors [224]. During tumor progression, elevated G-CSF levels result in increased phospho-STAT3 in DC progenitors in the bone marrow [224]. This increased G-CSF signaling downregulates IRF8, a key transcription factor for cDC1 development, resulting in decreased cDC1 abundance [224].

In addition to regulating DC development, STAT3 also modulates DC function. Mice that lack STAT3 in CD11c expressing cells, which includes all DCs, as well as some macrophage and monocyte populations, develop spontaneous colon inflammation [223]. This finding is similar to the colitis in total hematopoietic STAT3-deficient mice [223]. Thus, STAT3 restrains DC-mediated inflammation in response to commensal microbiota in the colon [223]. DC-specific STAT3-deficient mice have increased serum abundance of tumor necrosis factor-α (TNF-α) and IFN-γ, both of which are potent inflammatory cytokines [223]. Moreover, a heterogenous mixture of cDC and moDC populations can be generated in vitro using bone marrow cultured in GM-CSF (referred to as GM-DCs). When STAT3-deficient GM-DCs are matured in vitro with TLR agonists, many inflammatory cytokines are secreted at increased amounts compared to that of STAT3-sufficient GM-DCs, demonstrating a

role for STAT3 in inhibiting DC maturation [223]. Furthermore, when GM-DCs are pretreated with IL-10 prior to maturation with TLR agonists, maturation is inhibited in a STAT3-dependent manner [223]. Specifically, MHC II, CD86, IL-6, TNF-α, and IL-12 were all sensitive to IL-10- and STAT3-mediated inhibition [223]. Moreover, STAT3-deficient $CD11b^+$ $CD11c^+$ cells in the TME, which likely includes cDC2s as well as monocyte and macrophage derived-cells, express higher amounts of MHC II, CD80, CD86 and IL-12 [128, 219].

STAT3-mediated inhibition of DC maturation ultimately results in altered T cell responses [223, 225]. STAT3-deficient GM-DCs induce greater proliferation and IFN-γ secretion of naïve $CD4^+$ T cells compared to STAT3-sufficient GM-DCs [223]. In addition, mice that lack STAT3 in DCs exhibit improved anti-tumor immunity, characterized by increases in Th1 and $CD8^+$ T cells, and decreases in Treg cells, compared to control mice [217]. This may be due to release of STAT3-mediated inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent IL-12 expression [219]. Similarly, DCs that overexpress a constitutively active mutant of STAT3 are less efficient in activating $CD4^+$ T cells, as measured by IL-2 secretion, compared to control DCs [225]. Taken together, these findings support that STAT3 inhibits DC function by restraining maturation, leading to decreased induction of T cell mediated immunity [223, 225].

It is important to note that the above studies do not differentiate the roles of STAT3 in specific DC subsets. Although these findings corroborate other reports that STAT3 has an overall role in restraining immune responses, the exact function of STAT3 in separate cell types is distinct [128, 201, 204, 208, 219]. For example, a recent study

using IL-10 exposure to study STAT3-dependent transcriptional responses in multiple cell types demonstrated that IL-10 inhibits primarily NF-κB signaling macrophages, but IRF signaling in splenic cDCs (including both cDC1s and cDC2s) [208]. Therefore, the separate functions carried out by distinct DC subsets may be differentially affected by STAT3-mediated inhibition. Given that cDC1s now appear to be the main DC subset regulating $CD8^+$ T cell mediated immunity, targeted studies will be required to determine whether and how STAT3 governs cDC1 function specifically.

Chapter 2 – Materials and methods

Mouse strains

B6.Cg-Tg(Itgax-cre)1-1Reiz/J mice (CD11c Cre^{+}) from the Jackson Laboratory were bred with *Stat3*$^{fl/lf}$ mice to generate CD11c Cre^{+} *Stat3*$^{fl/fl}$ mice and CD11c Cre^{-} *Stat3*$^{fl/fl}$ littermate controls. C57BL/6J, B6.SJL-*Ptprc*^{a} *Pep*^{b}/BoyJ (CD45.1^{+}), B6.129S2-*Il10rb*^{tm1Agt}/J (*Il10rb*$^{-/-}$), B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax (*Ifnar1*$^{-/-}$), and B6.129S7-*Ifngr1*^{tm1Agt}/J mice were acquired from the Jackson Laboratory. *Xcr1*$^{cre/+}$ mice were kindly provided by Dr. Kenneth M. Murphy [92]. All mice were maintained in a specific pathogen-free animal facility at UT MD Anderson Cancer Center and used in accordance with IACUC-approved protocols.

In vitro generation of cDC1s

Adapted from a protocol developed by Mayer et al [226], murine bone marrow cells (BM) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA, USA), 1 mM sodium pyruvate (Thermo Fisher Scientific), 50 μM β-mercaptoethanol (Thermo Fisher Scientific), and 1% penicillin-streptomycin (Thermo Fisher Scientific), supplemented with 50 ng/mL human FLT3L (PeproTech, Rocky Hill, NJ, USA) and 2 ng/mL murine GM-CSF (PeproTech). BM cultures were initiated at a density of 1.5 x 10^{6} cells/mL and supplemented on day 5 with 5 mL of RPMI 1640 medium per 10 mL of culture. On day 9, non-adherent cells were collected and transferred to fresh medium containing 50 ng/mL human FLT3L and 2

ng/mL murine GM-CSF at a density of 3 x 10^5 cells/mL. On days 15-17, non-adherent cells were collected and cDC1s ($CD11c^+$ $CD45R^-$ $CD24^+$ $CD172\alpha^-$ $CD103^+$) were purified by fluorescence activated cell sorting (FACS) on a FACSAria III or FACSAria Fusion (BD Biosciences, Palo Alto, CA, USA).

Administration of cDC1 vaccines in vivo

FACS-purified cDC1s were cultured at 4.5 x 10^6 cells/mL in RPMI 1640 medium supplemented with 20 μg/mL poly I:C (Millipore Sigma, Darmstadt, Germany), 100 mg/mL ovalbumin (OVA; a surrogate tumor antigen) (Millipore Sigma), and 20 ng/mL murine GM-CSF for 4 hours at 37°C. Cells were washed three times with phosphate buffered saline (PBS) and resuspended in endotoxin-free PBS for injection into tumors. For studies evaluating tumor growth, mouse survival, or immune subsets post vaccination, 2 x 10^6 purified cDC1s were administered via intratumoral (i.t.) injection on d 7 following tumor establishment. For studies examining cDC1 vaccine migration and surface marker expression post vaccination, 5 – 7.5 x 10^6 cDC1s were injected i.t. on day 7 or day 14 following tumor establishment.

Cancer cells and establishment of murine tumors

Murine breast cancer cells stably expressing the model antigen ovalbumin (Polyoma virus middle T-Ovalbumin, PyMT-OVA) were kindly provided by Dr. David G. DeNardo and Dr. Melissa A. Meyer and derived as previously described [227]. Briefly, the cell line was isolated from an end stage tumor in a PyMT transgenic

C57BL/6 mouse and transduced to express OVA [227]. PyMT-OVA cells were cultured in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were washed three times with PBS and resuspended in endotoxin-free PBS prior to injection into mice. Ten to twelve-week old female C57BL/6 mice received bilateral injections of 5 x 10^5 PyMT-OVA cells in the mammary fat pad between the 4^{th} and 5^{th} mammary glands. Tumor length and width were measured every 2-3 d with electronic calipers. Mice were euthanized when tumors reached 15 mm in any direction or when ulceration > 2mm occurred.

For melanoma experiments, B16-F10 cells stably expressing OVA (B16-OVA) were cultured in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were washed three times with PBS and resuspended in endotoxin-free PBS prior to injection into mice. Ten to twelve-week old mice received subcutaneous injections of 3 x 10^5 B16-OVA cells. Tumor length and width were measured every 2-3 d with electronic calipers. Mice were euthanized when tumors reached 15 mm in any direction or when ulceration > 2mm occurred.

Immune cell isolation from mouse tumors and organs

PyMT-OVA tumors were removed and cut into small pieces (~2 mm) with scissors. Tumor pieces were incubated in a digestion buffer containing 1 mg/mL collagenase type IV (Millipore Sigma, Darmstadt, Germany), 0.1 mg/mL hyaluronidase (Millipore Sigma), and 30 units/mL deoxyribonuclease (Millipore Sigma) in RPMI 1640 for 45 minutes (min) in a shaking incubator (Eppendorf New Brunswick Excella E25) at 37°C and 100 RPM. Digested cell suspensions were

passed through 100 μm mesh filters; cells were subsequently washed with PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% FBS (FACS buffer) in preparation for antibody staining. Inguinal lymph TdLNs were dissected; TdLN cells were passed through 100 μm mesh filters and washed with FACS buffer prior to antibody staining. Lungs and livers were removed and cut into small pieces (~2 mm) and incubated in RPMI 1640 medium containing 1 mg/mL collagenase type IV for 45 minutes (min) in a shaking incubator (Eppendorf New Brunswick Excella E25) at 37°C and 100 RPM. Digested lung and liver suspensions were passed through 100 μm mesh filters. Lung suspensions were subsequently washed with FACS buffer in preparation for antibody staining. Liver suspensions were enriched for immune cells using Percoll (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) density gradient centrifugation. Percoll was made isotonic with concentrated PBS and diluted with RPMI 1640 medium. Liver suspensions were resuspended in 37% Percoll, layered on top of 70% Percoll and centrifuged for 25 min at 1200 x g at room temperature, without brake. Cells at the interface of the two Percoll layers were harvested and subsequently washed with FACS buffer in preparation for antibody staining. Spleens were removed and passed through 100 μm mesh filters and washed with RPMI 1640 medium. Spleen suspensions were then exposed to RBC lysis buffer (Tonbo Biosciences, San Diego, California, USA) for 5 min at room temperature and subsequently washed with FACS buffer in preparation for antibody staining.

Immune profiling by antibody staining and flow cytometry

Single-cell suspensions were incubated in FACS buffer containing rat anti-mouse CD16/32 antibody (Fc block, Tonbo Biosciences, San Diego, California, USA) for 15–30 min at 4°C. Subsequently, samples were stained with fluorescently conjugated antibodies against murine cell surface markers for 20 min at 4°C. For samples requiring analysis of intracellular proteins (T cell subsets), single-cell suspensions were incubated initially in RPMI 1640 medium containing 0.5 μg/mL ionomycin (Millipore Sigma), 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Millipore Sigma), and GolgiStop™ (BD Biosciences) for 4 h at 37°C. Cell surface marker staining was followed by fixation and permeabilization according to manufacturer's instruction (eBioscience Intracellular Fixation & Permeabilization Buffer Set, Thermo Fisher Scientific). After fixation and permeabilization, the cells were stained with fluorescently conjugated antibodies for intracellular proteins for 20 min at 4°C. The following reagents were used: BV-421–conjugated CD11c (N418) and XCR1 (ZET) antibodies; APC-conjugated Ly6C (HK1.4), CD86 (GL-1), CD45R (RA3-6B2), CD172α (P84), CD40 (3/23), or CD45.1 (A20) antibodies; redFluor™ 710-conjugated CD45.2 (104) antibody; APC-Cy7-conjugated CD4 (GK1.5), CD11c (N418) or CD11b (M1/70) antibodies; FITC-conjugated IFN-γ (XMG 1.2), Ly6G (1A8), CD172α (P84), MHC I (AF6-88.5) or CD80 (16-10A1) antibodies; Percp-Cy5.5-conjugated CD3e (17A2), CD24 (M1/69), F4/80 (BM8), or CD19 (1D3) antibodies; PE-conjugated FoxP3 (FJK-16S) or CD103 (2E7) antibodies; PE-Cy7-conjugated CD8α (53-6.7) or MHC II (M5/114.15.2) antibodies. All antibodies were

purchased from Tonbo Biosciences, BD Biosciences, Thermo Fisher Scientific, or BioLegend (San Diego, CA, USA). OVA-specific $CD8^+$ T cells were identified by staining with the SIINFEKL/H-2Kb-Pentamer (F093-4A, ProImmune, Sarasota, FL, USA). Dead cells were discriminated in all experiments using Ghost Dye™ violet 510 (Tonbo Biosciences). Stained single-cell suspensions were analyzed on a BD LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo v10 software (FlowJo, Ashland, OR, USA)

Cytokine detection

cDC1 and PyMT-OVA supernatant cytokines were measured using the mouse ProcartaPlex panel 1A in accordance with manufacturer's instruction (Invitrogen, Carlsbad, CA, USA) on a Luminex 200 machine (Luminex, Austin, TX, USA). Cytokines and chemokines analyzed: CXCL5, G-CSF, GM–CSF, CXCL1, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-10, IL-12p70, IL- 13, IL-15/IL-15R, IL-17A, IL-18, IL-2, IL-22, IL-23, IL-27, IL-28, IL-3, IL-31, IL-4, IL-5, IL-6, IL-9, CXCL10, LIF, M-CSF, CCL2, CCL7, CCL3, CCL4, CCL5, and TNF-α.

Cytokine, poly I:C, and other in vitro treatments

cDC1s were treated with murine, recombinant cytokines: 10 ng/mL IL-10 (PeproTech), 10 ng/mL IFN-β (R&D Systems, Minneapolis, MN USA), 10 ng/mL IFN-γ (Peprotech), 10 ng/mL IFN-λ2 (R&D Systems), or 3 μg/mL anti–IFN-λ (R&D Systems) or 20 μg/mL poly I:C (Millipore Sigma), as indicated.

Immunoblotting

cDC1 cell lysates were subject to SDS-PAGE and immunoblotting antibodies to detect Tyr705-phosphorylated STAT3 (Cell Signaling Technology, Danvers, Massachusetts, USA), total STAT3 (Santa Cruz Biotechnology, Dallas, Texas, USA; or Cell Signaling Technology), Tyr701-phosphorylated STAT1 (Cell Signaling Technology), total STAT1 (Cell Signaling Technology), GAPDH (Cell Signaling Technology), or tubulin (clone 12G10).

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA extraction was performed using TRIzol™ (Invitrogen) and RNA reverse-transcription (RT) into cDNA was done using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), each in accordance with the manufacturer's instructions. To evaluate relative gene expression, RT-qPCR was performed using SYBR Green (Millipore Sigma) on a CFX384 Touch™ Real Time PCR Detection System (Bio-Rad) with the following protocol: denaturation at 95°C for 10 seconds (s); annealing and extension at 60°C for 20 s. mRNA expression of target genes was normalized to ribosomal protein L13 (*Rpl13)* mRNA as an endogenous control. The following primers were used: *Rpl13* forward (F) 5'-GGCTGAAGCCTACCAGAAAG-3', *Rpl13* Reverse (R) 5'-TTCTCCTCCAGAGTGGCTGT-3'; *Batf3* F 5'-CAGACCAGAAGGCTGACAAG-3', *Batf3* R 5'-CTGCGCAGCACAGAGTTCTC-3'; *Id2* F 5'-AAACAGCCTGTCGGACCAC-3', *Id2* R 5'-CTGGGCACCAGTTCCTTGAG-3'; *Irf8* F 5'-GAGCCAGATCCTCCCTGACT-3', *Irf8* R 5'-

GGCATATCCGGTCACCAGT-3'; *Irf4* F 5'-ATGGGAAACTCCGACAGTGG-3', *Irf4* R 5'-GGCTCCCTCTGGAACAATCC-3'; *Tcf-4* F 5'-AGACCAAGCTCCTGATTCTC-3', *Tcf-4* R 5'-AGGCTCTGAGGACACCTTCT-3'; *Zeb2* F 5'-GGCAAGGCCTTCAAGTACAA-3', *Zeb2* R 5'-AAGCGTTTCTTGCAGTTTGG-3'; *Cxcl10* F 5'- CCCACGTGTTGAGATCATTG-3', *Cxcl10* R 5'-GAGGCTCTCTGCTGTCCATC-3'; *Ifnb1* F 5'-CTGAGGCATCAACTGACAGG-3', *Ifnb1* R 5'-GGAAAGATTGACGTGGGAGA-3'.

RNA sequencing

RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA sequencing was performed by Novogene using Illumina sequencers. Data were processed and initially analyzed by Li Shen and Dr. Jing Wang of the Department of Bioinformatics and Computational Biology at the UT MD Anderson Cancer Center. Briefly, RNA-seq by Expectation Maximization (RSEM) was used for transcript quantification and the data were normalized by limma-voom. Differentially expressed genes between indicated groups were determined by Welch's T test. Normalized expression values or differential expression gene lists were then used for analysis by Gene Set Enrichment Analysis (GSEA) or Ingenuity Pathway Analysis (IPA), respectively. For IPA analyses, genes were considered differentially expressed using the following cutoffs: fold change ≥ |2| and $p < 0.05$.

Statistics

All statistical analyses were performed using Prism 9 software (GraphPad Software, San Diego, CA, USA). Welch's T test, one-way, or two-way ANOVA with Bonferroni's multiple comparison tests were performed, as indicated. Results were considered significant when $p < 0.05$.

The following chapter is based upon “Chrisikos, Taylor T., et al. "STAT3 inhibits CD103+ cDC1 vaccine efficacy in murine breast cancer." *Cancers* 12.1 (2020): 128. ”

Chapter 3 – STAT3 inhibits cDC1 maturation and cDC1-mediated anti-tumor immunity

3.1 – Background and rationale

cDC1s are an essential APC population, important for coordination of adaptive immunity and tolerance [1]. cDC1s seed LNs and peripheral organs in an immature state and act as sentinels of the immune system, sampling their environment, poised to respond to potential pathogens [1]. Upon sensing PAMPs or DAMPS, cDC1s undergo maturation by upregulating expression of MHC molecules, costimulatory markers, and inflammatory cytokines and chemokines, all of which support optimal activation of naïve T cells and the induction of the adaptive immune response [1].

In the TME, cDC1s are required for the induction of anti-tumor $CD8^+$ T cell-mediated cytotoxic immunity [1]. cDC1s perform this role through their ability to transport tumor antigen from the TME to TdLN, efficiently present antigen to naïve $CD8^+$ and $CD4^+$ T cells, and recruit effector $CD8^+$ T cells to the TME through expression of T cell chemoattractants such as CXCL10 [1, 92]. Importantly, the abundance of the human cDC1 equivalent, $CD141^+$ DCs, is positively correlated with overall patient survival and predicts responsiveness to immunotherapy in human cancer patients [99]. However, despite the important role of cDC1s in mediating immunity against foreign pathogens and tumors, little is known regarding the factors that regulate cDC1 functions in these settings.

STAT3 mediates intracellular signaling elicited by anti-inflammatory cytokines, such as IL-10. Removal of STAT3 from all DCs in mice leads to altered immune homeostasis that results in chronic colon inflammation and increased inflammatory cytokines in circulation [223]. In addition, when GM-DCs are matured in vitro with TLR agonists, STAT3-depletion results in increased inflammatory cytokine secretion [223, 228]. Furthermore, STAT3-deficient GM-DCs are resistant to IL-10 – mediated inhibition of TLR agonist induced costimulatory marker expression and cytokine secretion [223]. However, whether STAT3 has a role in regulating cDC1 function specifically, remains unaddressed.

3.2 – Results

3.2.1 – STAT3 inhibits poly I:C-induced cDC1 maturation

cDC1s are an exceedingly rare cell type making them difficult to study. In addition, culture systems based on incubating bone marrow in the presence of DC growth factors GM-CSF or FLT3L generate a heterogenous mixture of cDCs, pDCs, and moDCs [228]. However, when using FLT3L in combination with a relatively low concentration of GM-CSF, bone marrow cells in culture develop primarily into cDC1s [226]. To validate this finding, bone marrow was harvested from C57BL/6J mice (WT) and incubated with FLT3L and GM-CSF. Analysis of surface marker expression revealed that most cells expressed cDC1-associated lineage markers ($CD11c^+$ $CD45R^-$ $CD24^+$ $CD172\alpha^-$ $CD103^+$) after over 2 weeks of culture (Figures 3.1A and 3.1B). cDC1s purified from this culture system by FACS, based on the surface

marker expression profile described above, showed increased expression of cDC1-associated transcription factors *Batf3*, *Id2*, and *Irf8*, compared to those normally expressed by pDCs or cDC2s, such as Irf4, Zeb2, and *Tcf4*. (Figure 3.1C). Furthermore, in response to poly I:C treatment, a TLR3 agonist that efficiently promotes cDC1 maturation, the FACS-purified cDC1s upregulated surface expression of CD80 and CD86 (Figure 2.1D). Taken together, based on surface marker expression, transcription factor expression, and poly I:C-responsiveness, the data indicate that culture-derived cDC1s resemble their in vivo counterparts. Unless otherwise stated, the cDC1s described in the following experiments are in vitro-derived cDC1s, FACS-purified using the gating scheme shown in Figure 3.1A.

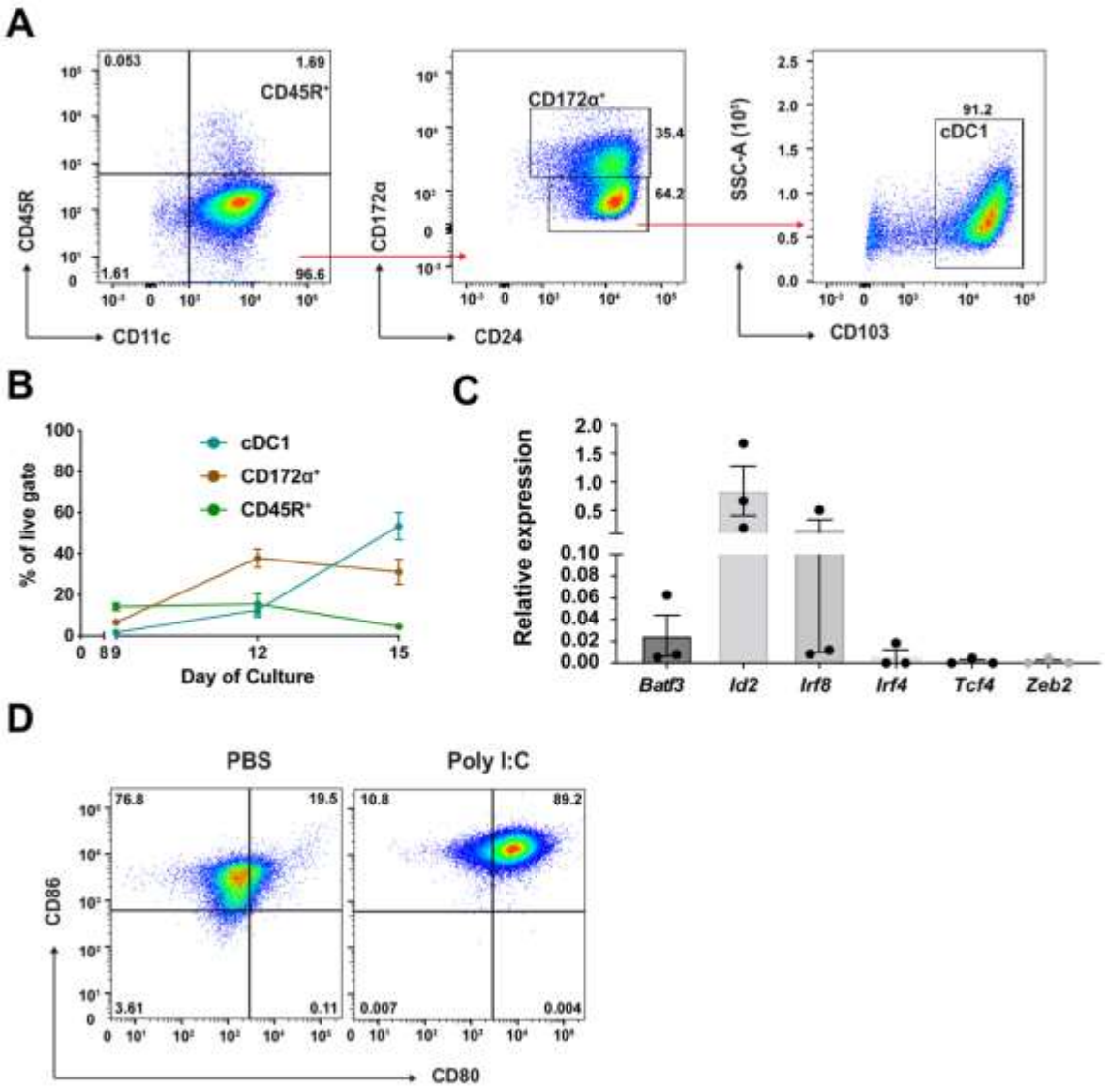

Figure 3.1. In vitro cDC1 generation. Bone marrow from WT mice was cultured for 15 days in the presence of FLT3L and GM-CSF. (A) Flow cytometry plots of DC-associated surface marker expression on day 15 of culture, following gating on live, single cells. Frequency of each parent population is shown, and the major populations are defined (CD45R$^+$, CD172α^+, and cDC1). Data are representative of 3 independent experiments. (B) Relative abundance of the indicated populations in culture over time. Data are combined from 3 independent experiments, *n* = 3. (C) Expression of DC-associated transcription factor mRNA in FACS-purified cDC1s, assessed by RT-qPCR. Data are combined from 3 independent experiments, *n* = 3. (D) Flow cytometry plots showing surface costimulatory marker expression on FACS-purified cDC1s after exposure to PBS or poly I:C for 16 hours. Data are representative of 3 independent experiments, *n* = 3. (B and C) Data are shown as mean ± SEM. (C) Each symbol represents an individual biological replicate.

To investigate the role of STAT3 in cDC1s, culture-derived cDC1s were generated utilizing bone marrow from CD11c Cre *Stat3*$^{fl/fl}$ mice (*Stat3*$^{\Delta/\Delta}$), which deplete STAT3 in all CD11c^{+} cells, or *Stat3*$^{fl/fl}$ control mice [223]. Although a low level of phospho-STAT3 could still be detected by immunoblot in *Stat3*$^{\Delta/\Delta}$ cDC1s exposed to IL-10, total STAT3 abundance was greatly reduced (Figure 3.2A). STAT3 is not required for the homeostatic production of CD103^{+} cDC1s in vivo, however, it was conceivable that depletion of STAT3 could alter development in culture [14]. STAT3 depletion slightly increased the frequency of cDC1s as a percent of all live cells but did not alter the viability of the cultured cells or the overall abundance of cDC1s produced (Figures 3.2B, 3.2C, 3.2D). Furthermore, the expression of cDC1- and pDC-associated transcription factors was comparable between *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s, as judged by mRNA expression (Figure 2.2E). Therefore, these results indicate that CD11c Cre-driven deletion of *Stat3* does not alter cDC1 development in culture with FLT3L and GM-CSF.

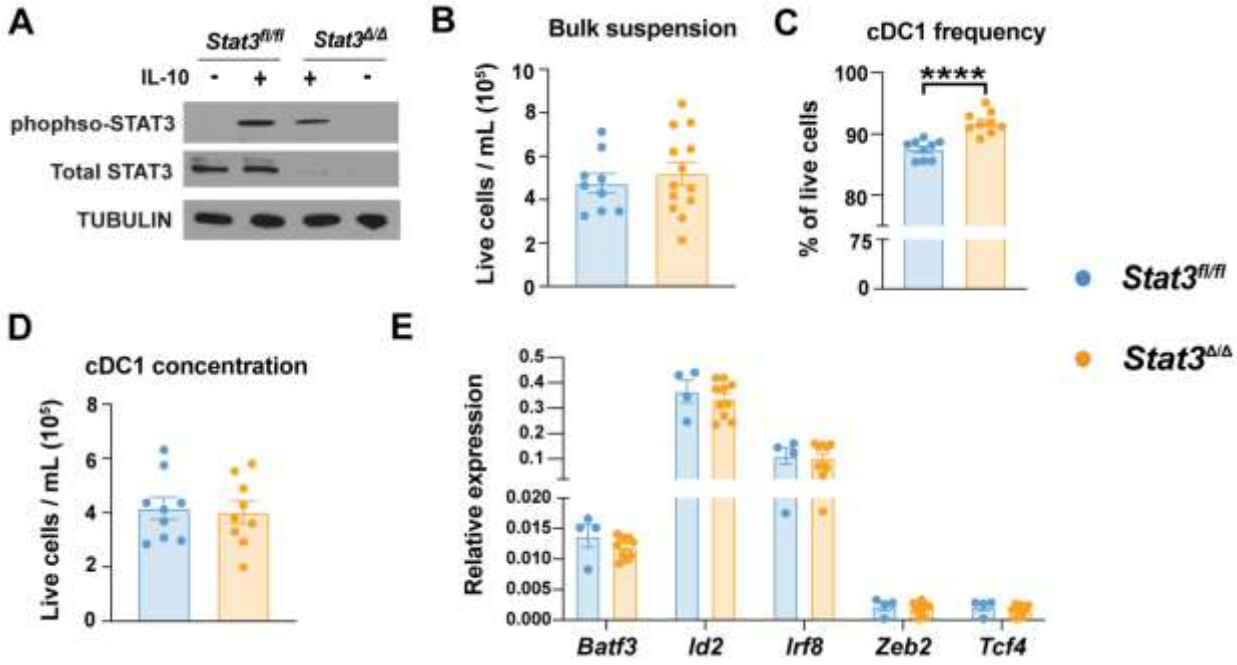

Figure 3.2. STAT3 depletion in cDC1 in vitro cultures. cDC1 cultures were derived from the bone marrow of *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ mice. (A) Immunoblotting for the indicated proteins following exposure to PBS or IL-10 for 0.25 hours. Data are representative of 3 independent experiments. (B) Total live cell concentration in suspension on day 17 of culture, assessed by hemacytometer counts. Data are combined from 4 independent experiments, n = 9 (*Stat3*$^{fl/fl}$), n = 13 (*Stat3*$^{\Delta/\Delta}$). (C) cDC1 frequency of live cells on day 17 of culture, as assessed by flow cytometry. (D) cDC1 concentration in suspension on day 17 of culture, as assessed by multiplying total live cell concentration determined in (B), by cDC1 frequency of live cells, determined in (C). (C and D) Data are combined from 3 independent experiments, n = 9. (E) Relative expression of the indicated transcripts in cDC1s, determined by RT-qPCR. Data are combined from 3 independent experiments, n = 4 (*Stat3*$^{fl/fl}$), n = 10 (*Stat3*$^{\Delta/\Delta}$). (B – E) Data are shown as mean ± SEM. Each symbol represents an individual biological replicate. Results were analyzed by Welch's T test (B – E). Results were considered significant when $p < 0.05$. **** $p < 0.0001$.

To determine whether STAT3 regulates cDC1 maturation, *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were exposed to PBS, IL-10, an immunomodulatory cytokine that induces STAT3 signaling, poly I:C, or a combination of IL-10 and poly I:C. The costimulatory markers CD80 and CD86 were induced by poly I:C to a similar degree in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 3.3A). However, co-treatment with IL-10 inhibited poly I:C-induced upregulation of CD80 and CD86 in *Stat3*$^{fl/fl}$ cDC1s, while *Stat3*$^{\Delta/\Delta}$ cDC1s maintained high expression of CD80 and CD86 in these conditions (Figure 3.3A). MHC I, MHC II, and CD40 were expressed at comparable levels in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s within each treatment group (Figure 3.3A). In addition, the expression of many poly I:C-induced chemokines and cytokines was inhibited by co-treatment with IL-10 in *Stat3*$^{fl/fl}$ cDC1s, but not in *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 3.3B). Notably, IL-1β was the sole cytokine or chemokine induced by IL-10 in *Stat3*$^{fl/fl}$ cDC1s, while none were detected in *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 3.3B). Taken together, surface marker expression and cytokine secretion analyses indicate that IL-10 inhibits poly I:C-induced maturation of cDC1s in a STAT3-dependent manner.

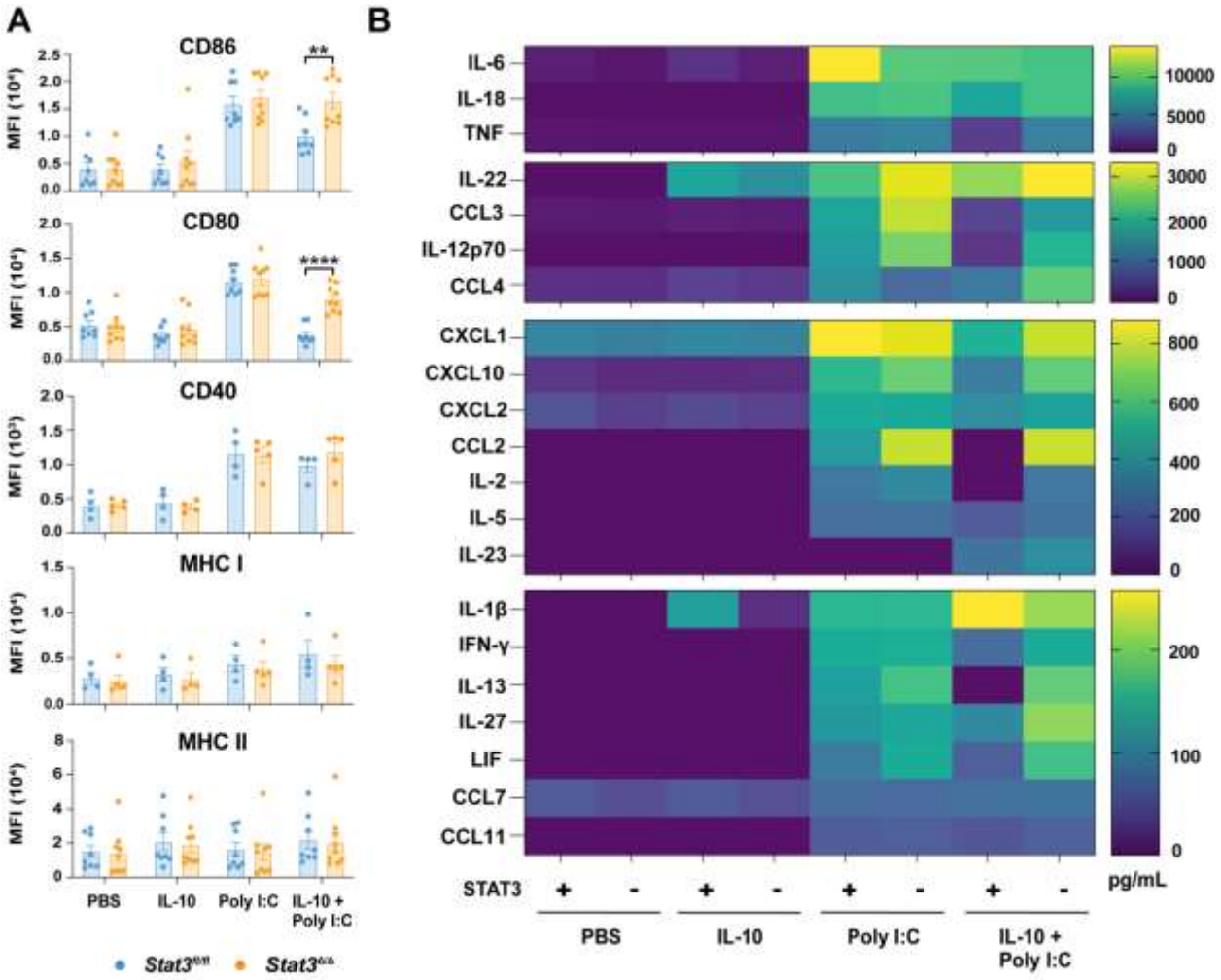

Figure 3.3. IL-10 inhibits poly I:C-induced maturation of cDC1s in a STAT3-dependent manner. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C for 16 hours. (A) Cell surface expression of the indicated proteins as assessed by flow cytometry. For CD86, CD80, and MHC II, data are combined from 4 independent experiments, n = 8 (*Stat3*$^{fl/fl}$), n = 9 (*Stat3*$^{\Delta/\Delta}$). For CD40 and MHC I data are combined from 3 independent experiments, n = 4 (*Stat3*$^{fl/fl}$), n = 5 (*Stat3*$^{\Delta/\Delta}$). (B) Mean cytokine and chemokine concentration in cell culture supernatants determined by Luminex multiplex assay. Data are combined from 4 independent experiments, n = 4 (PBS), n = 5 (IL-10), n = 6 (poly I:C, IL-10 + poly I:C). Graphs of individual analytes can be found in the appendix (Figure A1). (A) Data shown are the mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ** $p < 0.01$, **** $p < 0.0001$.

3.2.2 – STAT3 inhibits cDC1 vaccine efficacy in murine breast cancer

Given that STAT3 inhibits cDC1 maturation, cDC1-mediated anti-tumor immunity could potentially be regulated by STAT3. To test this hypothesis, *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were used in a previously established tumor vaccination strategy wherein cDC1s are treated with poly I:C and GM-CSF, to induce maturation, as well as OVA serving as tumor antigen, followed by i.t. injection [116]. A murine mammary carcinoma was used for these experiments as it models a prevalent cancer in humans that is refractory to many immunotherapeutic approaches [229]. Furthermore, the PyMT-OVA breast tumor cells used in this setting express multiple STAT3-activating cytokines, such as IL-6 and IL-10 (Figure 3.4A). In addition, bilateral injection of PyMT-OVA cells into the mammary fat pad of female mice, followed by injection of the cDC1 vaccine into just one of the tumors, allows for the assessment of local and systemic anti-tumor immune responses induced by the vaccine in an orthotopic setting.

Vaccination with *Stat3*$^{fl/fl}$ cDC1s restrained bilateral tumor growth and improved mouse survival as compared to i.t. PBS (Figures 3.4B and 3.4C). However, *Stat3*$^{\Delta/\Delta}$ cDC1 vaccination inhibited bilateral tumor growth and increased mouse survival to an even greater extent than vaccination with *Stat3*$^{fl/fl}$ cDC1s (Figures 2.4B and 2.4C). These data indicate that STAT3 inhibits the efficacy of cDC1 vaccination in murine breast cancer.

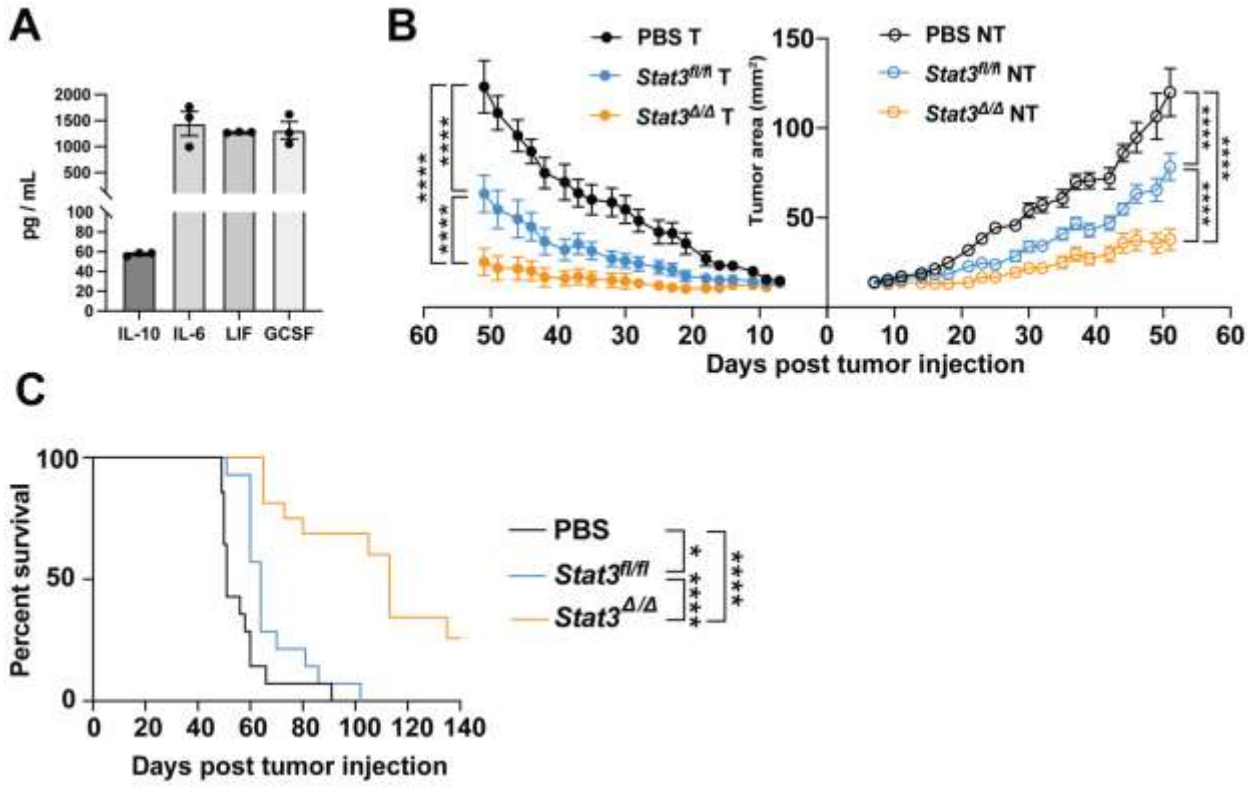

Figure 3.4. STAT3 inhibits cDC1 vaccine efficacy in murine breast cancer. (A) Cytokine concentration in cell culture supernatants of PyMT-OVA cells, determined by Luminex multiplex assay. Data are combined from 3 independent experiments. *n* = 3. (B) Mean tumor area of treated (T) and non-treated (NT) tumors in mice bearing bilateral PyMT-OVA tumors, vaccinated i.t. 7 days after tumor injection, with PBS, *Stat3*$^{fl/fl}$ cDC1s, or *Stat3*$^{\Delta/\Delta}$ cDC1s. Data are representative of 2 independent experiments, *n* = 7 (PBS, *Stat3*$^{fl/fl}$), *n* = 8 (*Stat3*$^{\Delta/\Delta}$). (C) Cumulative mouse survival from 2 independent experiments, described in (B), *n* = 14 (PBS, *Stat3*$^{fl/fl}$), *n* = 16 (*Stat3*$^{\Delta/\Delta}$). (A and B) Data are shown as mean ± SEM. (A) Each symbol represents an individual biological replicate. Results were analyzed by two-way ANOVA and Bonferroni's multiple comparison test (B) or log-rank (Mantel-Cox) test (C). Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗∗∗ $p < 0.0001$.

3.2.3 – Maturation, but not migration, is inhibited by STAT3 in vaccine-derived cDC1s

Since STAT3 inhibits cDC1 maturation in vitro, it could potentially inhibit the maturation of the vaccine-derived cDC1s in the TME or TdLNs. To test this hypothesis, CD45.2^{+} *Stat3*$^{fl/fl}$ or *Stat3*$^{\Delta/\Delta}$ cDC1s were injected i.t. into CD45.1^{+} mice bearing bilateral PyMT-OVA tumors. 40 hours after i.t. injection, vaccine-derived cDC1 abundance and maturation state in the TME and TdLN was assessed by flow cytometry. Vaccine-derived cDC1s could only be detected in the TME and TdLNs of the vaccine treated-side tumors and there were comparable numbers of *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ vaccine-derived cDC1s at both sites (Figure 3.5A). CD86 was expressed to a greater degree on the surface of vaccine-derived *Stat3*$^{\Delta/\Delta}$ cDC1s compared to *Stat3*$^{fl/fl}$ cDC1s in the TME, but the same trend was not evident in the TdLN (Figure 3.5B). In addition, CD80, and MHC II were expressed to a similar degree in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ vaccine-derived cDC1s in both the TdLNs and TME (Figure 3.5B). Taken together these results indicate while STAT3 inhibits surface CD86 expression on vaccine-derived cDC1s in the PyMT-OVA TME, STAT3 does not alter their abundance in the TME or migration to the TdLNs.

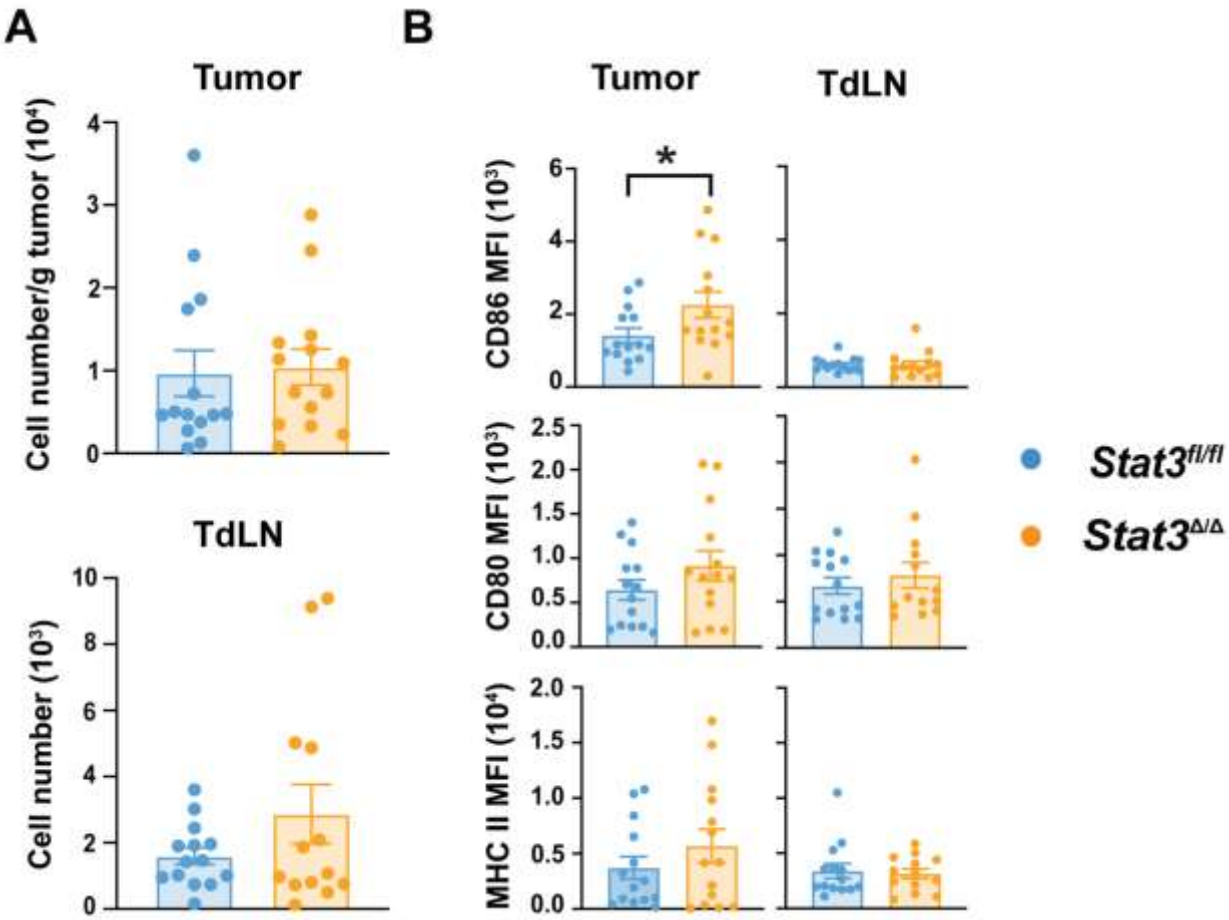

Figure 3.5. cDC1 maturation is inhibited by STAT3 in the TME. Bilateral PyMT-OVA tumor-bearing CD45.1$^+$ mice received i.t. vaccination with CD45.2$^+$ *Stat3*$^{fl/fl}$ cDC1s, or CD45.2$^+$ *Stat3*$^{\Delta/\Delta}$ cDC1s, after 7 days of tumor growth. Vaccine-derived cDC1s were identified in tumors and TdLNs by flow cytometry analysis of CD45.2$^+$ cells, 40 hours after i.t. injection. (A) Vaccine-derived cDC1 abundance in the treated-side tumors and TdLNs. No CD45.2$^+$ cells were detected in non-treated side tumors or TdLNs. (B) Surface marker expression on vaccine-derived cDC1s in treated-side tumors and TdLNs. (A and B) Data are combined from 2 independent experiments, n = 14 (*Stat3*$^{fl/fl}$ tumor, *Stat3*$^{fl/fl}$ TdLN, *Stat3*$^{\Delta/\Delta}$ tumor), n = 13 (*Stat3*$^{\Delta/\Delta}$ TdLN). Date are shown as mean ± SEM and each symbol represents an individual biological replicate. Data were analyzed by Welch's T Test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$.

3.2.4 – STAT3 inhibits cDC1 vaccine-induced anti-tumor T cell responses

Previously, in murine melanoma and osteosarcoma, cDC1 vaccination was shown to induce anti-tumor T cell responses [116]. Since STAT3 inhibits cDC1 vaccine efficacy in the PyMT-OVA tumor model it is plausible that STAT3 inhibits vaccine efficacy by suppressing cDC1-mediated induction of anti-tumor T cell responses (Figures 3.4B and 3.4C). To test this hypothesis, immune cell populations in the TME and TdLNs of mice bearing bilateral PyMT-OVA tumors, vaccinated i.t. with *Stat3*$^{fl/fl}$ or *Stat3*$^{\Delta/\Delta}$ cDC1s, were evaluated by flow cytometry. Immune populations in the TME and TdLNs were assessed at day 4 and day 10 post-vaccination. Tumor size and progression may influence immune populations in the TME and TdLN. Therefore, analysis at day 4 allows for the assessment of early events post vaccination, when tumor sizes are comparable across all groups, whereas analyses at day 10 represent when alterations in tumor growth first become apparent in vaccinated tumors (Figure 3.6A).

At day 4 post-vaccination with *Stat3*$^{fl/fl}$ cDC1s, a trend toward increased tumor antigen (OVA)-specific CD8$^+$ T cells, identified by SIINFEKL/H2-Kb pentamer staining, and Th1 cells, identified as IFN-γ^+ CD4$^+$ T cells, was observed in the TME and TdLNs of treated-side tumors relative to PBS-treated controls, although these data did not reach statistical significance (Figures 3.6B and 3.6C). By day 10 post-vaccination these trends were no longer evident and OVA-specific CD8$^+$ T cell abundance was decreased compared to PBS-treated controls, in the treated-side TdLNs (Figures 3.6B and 3.6C). In contrast, vaccination with *Stat3*$^{\Delta/\Delta}$ cDC1s resulted in a significant increase of OVA-specific CD8$^+$ T cells and Th1 cells in the vaccine-

treated tumors and corresponding TdLNs, 4 days following vaccination compared to PBS-treated controls (Figures 3.6B and 3.6C). Furthermore, at day 10 following vaccination with *Stat3*$^{\Delta/\Delta}$ cDC1s, Th1 cell amounts were increased in the vaccine-treated tumor and the distal, non-treated tumor, relative to PBS-treated controls (Figure 3.6C). In addition, treatment with the *Stat3*$^{\Delta/\Delta}$ cDC1 vaccine resulted in a trend towards increased IFN-γ^+ CD8$^+$ T cells numbers, and an increased CD8$^+$:FoxP3$^+$ CD4$^+$ T cell ratio in the vaccine-treated and distal tumors, relative to PBS-treated controls, although these data did not reach statistical significance (Figure 3.7A). Myeloid population abundance in the TME was largely unaltered by vaccination with either *Stat3*$^{fl/fl}$ or *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 3.7B). Taken together, these results indicate that STAT3 inhibits cDC1 vaccine-induced tumor-antigen specific CD8$^+$ T cell-mediated immunity. Furthermore, STAT3 inhibits the induction of Th1 cells in the tumors and TdLNs of cDC1 vaccinated mice. Therefore the overall induction of T cell-mediated anti-tumor immunity by the cDC1 vaccine is inhibited by STAT3.

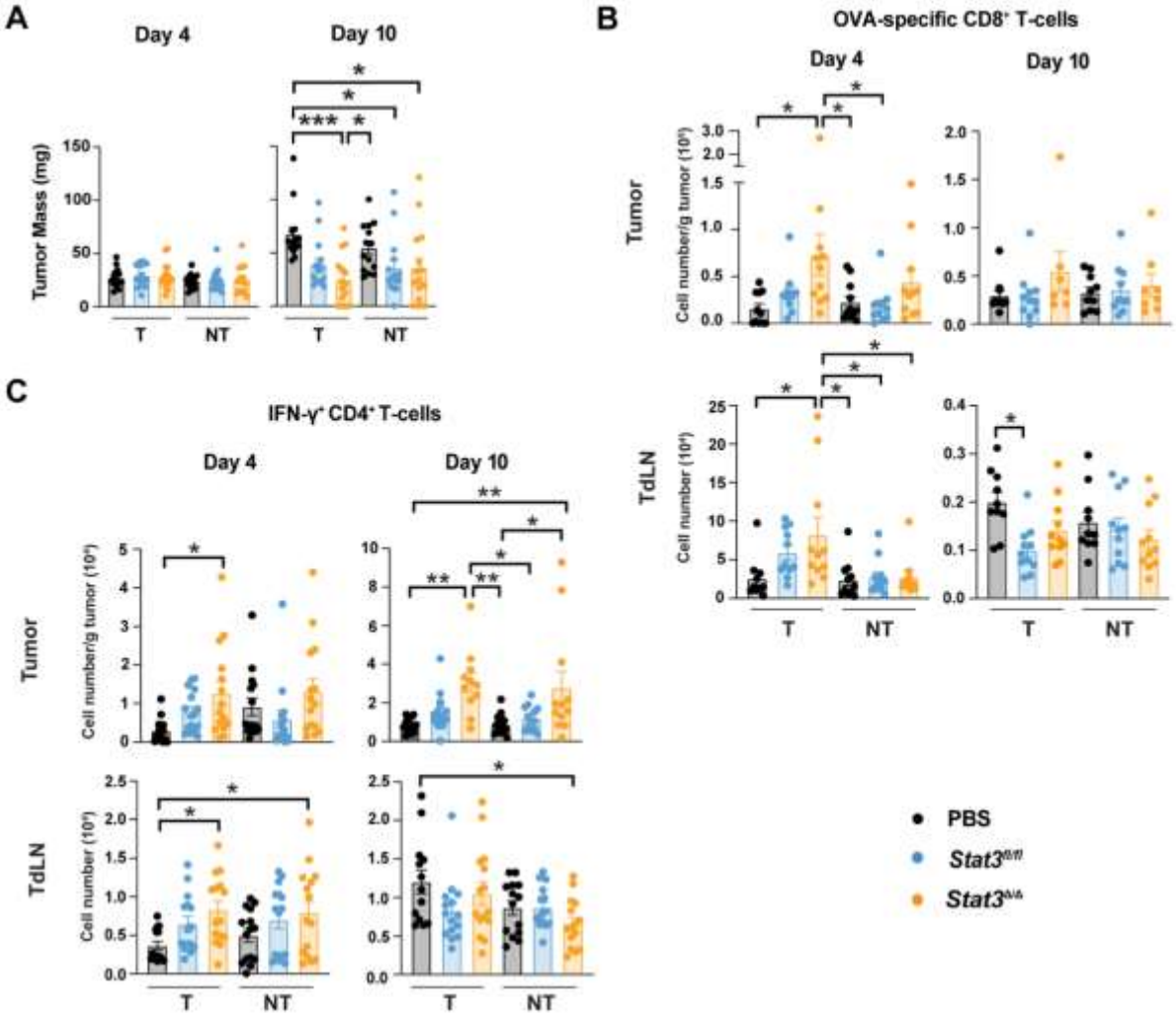
A
Day 4
Day 10
Tumor Mass (mg)
T
NT
B
OVA-specific CD8+ T-cells
Tumor
TdLN
Cell number/g tumor (10⁴)
Cell number (10⁴)
C
IFN-γ+ CD4+ T-cells
PBS
Stat3fl/fl
Stat3Δ/Δ

Figure 3.6. STAT3 inhibits cDC1 vaccine-induced anti-tumor T cell responses. Mice bearing bilateral PyMT-OVA tumors were vaccinated i.t. 7 days after tumor injection with PBS, *Stat3*$^{fl/fl}$ cDC1s, or *Stat3*$^{\Delta/\Delta}$ cDC1s. Following 4 days and 10 days after vaccination, tumors and TdLNs were analyzed by flow cytometry. (A) Tumor mass combined from 3 independent experiments. For day 4, *n* = 15 (PBS, *Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$ NT), *n* = 16 (*Stat3*$^{\Delta/\Delta}$ T). For day 10, *n* = 14 (PBS), *n* = 15 (*Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$). (B) Total numbers of OVA-specific CD8$^+$ T cells, identified as CD45$^+$ CD3$^+$ CD4$^-$ CD8$^+$ SIINFEKL/H2-Kb pentamer$^+$ cells. Data are combined from 2 independent experiments. For day 4 tumors, *n* = 11; for day 4 TdLNs, *n* = 10 (PBS T, *Stat3*$^{\Delta/\Delta}$ NT), *n* = 11 (*Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$ T, PBS NT); for day 10 tumors, *n* = 10 (PBS, *Stat3*$^{fl/fl}$ NT), *n* = 11 (*Stat3*$^{fl/fl}$ T), n = 7 (*Stat3*$^{\Delta/\Delta}$ T), *n* = 8 (*Stat3*$^{\Delta/\Delta}$ NT); for day 10 TdLNs, *n* = 10 (PBS), *n* = 11 (*Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$). (C) Total numbers of Th1 cells, identified as CD45$^+$ CD3$^+$ CD8$^-$ CD4$^+$ IFN-γ^+ cells. Data are combined from 3 independent experiments. For day 4 tumors, *n* = 15 (PBS, *Stat3*$^{fl/fl}$ NT, *Stat3*$^{\Delta/\Delta}$), *n* = 16 (*Stat3*$^{fl/fl}$ T); for day 4 TdLNs, *n* = 15; for day 10 tumors, *n* = 14 (PBS), *n* = 15 (*Stat3*$^{fl/fl}$ T), *n* = 13 (*Stat3*$^{fl/fl}$ NT), *n* = 11 (*Stat3*$^{\Delta/\Delta}$ T), *n* = 12 (*Stat3*$^{\Delta/\Delta}$ NT); for day 10 TdLNs, *n* =13 (PBS T), *n* = 14 (PBS NT), *n* = 15 (*Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$). (A – C) Date are shown as mean ± SEM and each symbol represents an individual biological replicate. Data were analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$.

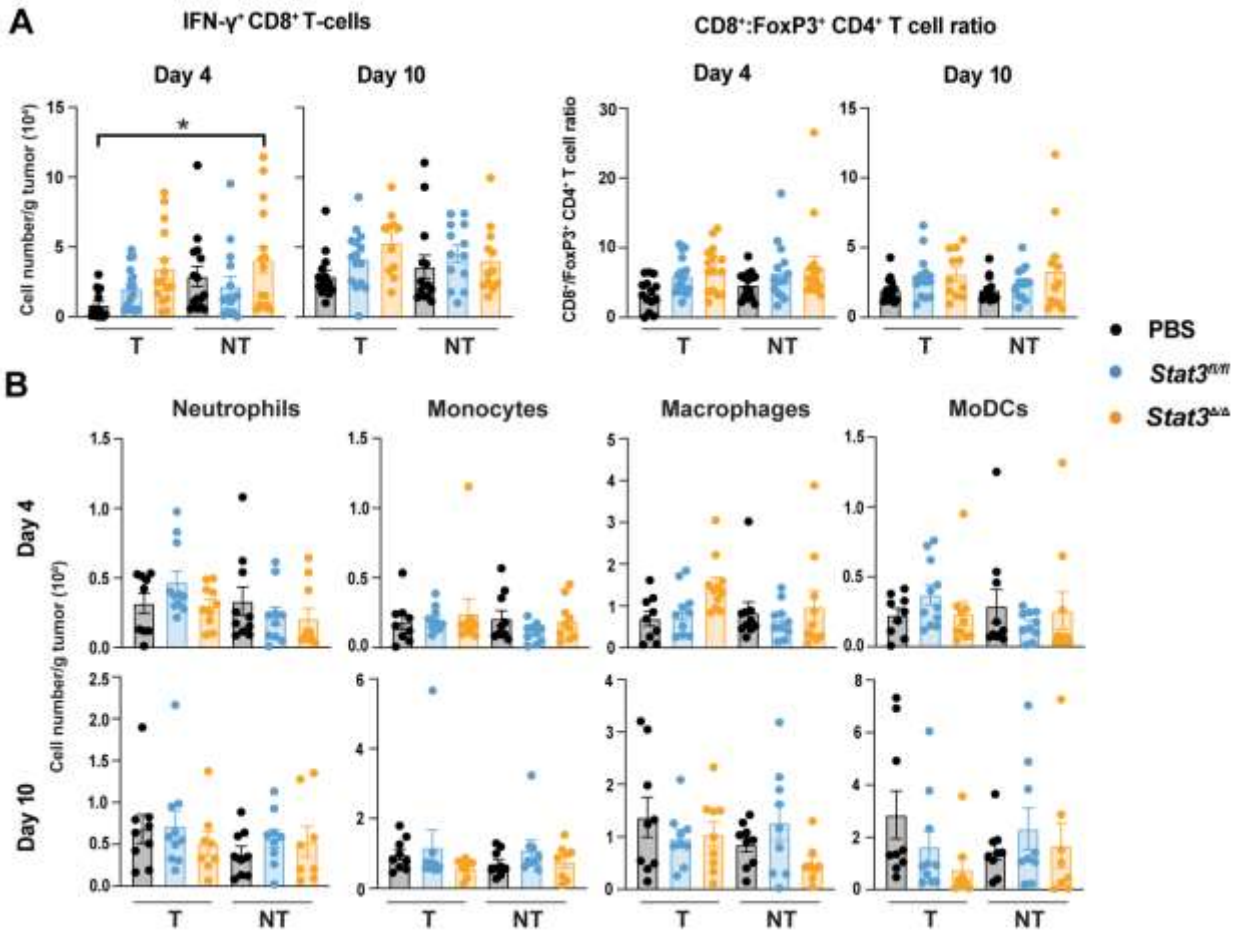

Figure 3.7. cDC1 vaccine-induced T cell responses are inhibited by STAT3 but myeloid responses are unaltered. Mice bearing bilateral PyMT-OVA tumors were vaccinated i.t. 7 days after tumor injection, with PBS, *Stat3*$^{fl/fl}$ cDC1s, or *Stat3*$^{\Delta/\Delta}$ cDC1s. Following 4 days and 10 days after vaccination tumors were analyzed by flow cytometry. (A) Total numbers of IFN-γ$^+$ CD8$^+$ T cells, identified as CD45$^+$ CD3$^+$ CD4$^-$ CD8$^+$ IFN-γ$^+$ cells (left), and the ratio of CD8$^+$:FoxP3+ CD4$^+$ T cells, identified as CD45$^+$ CD3$^+$ CD4$^-$ CD8$^+$ cells and CD45$^+$ CD3$^+$ CD8$^-$ CD4$^+$ Foxp3$^+$ cells, respectively (right). Data are combined from 3 independent experiments. For day 4, n = 15 (PBS, *Stat3*$^{fl/fl}$ NT, *Stat3*$^{\Delta/\Delta}$), n = 16 (*Stat3*$^{fl/fl}$ T); for day 10, n = 14 (PBS), n = 15 (*Stat3*$^{fl/fl}$ T), n = 13 (*Stat3*$^{fl/fl}$ NT), n = 11 (*Stat3*$^{\Delta/\Delta}$ T), n = 12 (*Stat3*$^{\Delta/\Delta}$ NT). (B) Total numbers of neutrophils (CD45$^+$ CD11b$^+$ Ly6G$^+$), monocytes (CD45$^+$ CD11b$^+$ Ly6G$^-$, Ly6C^{hi}, F4/80^{lo}), macrophages (CD45$^+$ CD11b$^+$ Ly6G$^-$, Ly6C^{lo}, F4/80^{hi}, CD11c$^-$ MHC II$^-$), and moDCs (CD45$^+$ CD11b$^+$ Ly6G$^-$, Ly6C^{lo}, F4/80^{hi}, CD11c$^+$ MHC II^{hi}). Data are from 2 independent experiments. For day 4, n = 9 (PBS T), n = 11 (*Stat3*$^{fl/fl}$ T), n = 10 (PBS NT, *Stat3*$^{fl/fl}$ NT, (*Stat3*$^{\Delta/\Delta}$); for day 10, n = 9 (PBS, *Stat3*$^{fl/fl}$ NT), n = 10 (*Stat3*$^{fl/fl}$ T), n = 8 (*Stat3*$^{\Delta/\Delta}$). (A and B) Date are shown as mean ± SEM and each symbol represents an individual biological replicate. Data were analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$.

3.2.5 – IL-10 receptor signaling inhibits cDC1 vaccine efficacy to a similar degree as STAT3

The results above demonstrate that STAT3 inhibits cDC1 vaccine-induced anti-tumor immunity. However, the extracellular factors capable of eliciting STAT3 activity in vaccine-derived cDC1s remain unknown. PyMT-OVA cells express many STAT3-activating cytokines, including IL-10, IL-6, leukemia inhibitory factor (LIF), and G-CSF (Figue 3.4A). cDC1s express low amounts of the receptors for LIF and G-CSF compared to other immune cells, suggesting they may be refractory to these signals in the TME [230]. However, cDC1s express high amounts of the transcripts for the unique receptor subunits for IL-6 (*Il6ra*) and IL-10 (*Il10rb*) [230]. Previously, others have shown that IL-10 suppresses the ability of a macrophage cell line to induce of IFN-γ^+ in $CD4^+$ T cells [231]. Furthermore, IL-10 inhibits moDC-mediated induction $CD8^+$ T cell proliferation and cytotoxicity [232]. As these responses parallel roles identified for STAT3 in cDC1 vaccination, as described above, it was conceivable that IL-10 signaling in cDC1s inhibits vaccine efficacy.

To test this hypothesis, cDC1s were generated from the bone marrow of *Il10rb*$^{-/-}$ mice, which lack the beta receptor subunit critical for IL-10 receptor signaling [233]. *Il10rb*-deficiency did not alter cDC1 development in culture compared to *Stat3*$^{fl/fl}$ or *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 3.8A). To determine whether IL-10Rβ regulated cDC1 development in vivo, the lungs, liver, and spleen of *Il10rb*$^{-/-}$ mice was analyzed by flow cytometry for the presence of cDC subsets. Total cDCs, cDC1s, and cDC2s were present at similar frequencies in *Il10rb*$^{-/-}$ mice relative to WT mice (Figure

3.8B). Therefore, IL-10Rβ is dispensable for cDC1 development in culture and the homeostatic maintenance of both cDC subsets in vivo.

To determine whether IL-10 signaling inhibits cDC1 vaccine efficacy, *Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$, or *Il10rb*$^{-/-}$ cDC1s were used to vaccinate mice bearing bilateral PyMT-OVA tumors and tumor growth was measured over time. While *Stat3*$^{fl/fl}$ cDC1 vaccination delayed tumor growth of vaccine-treated and distal tumors compared to PBS-treated mice, vaccination with *Stat3*$^{\Delta/\Delta}$ cDC1s further enhanced inhibition of bilateral tumor growth, consistent with prior results (Figure 3.8C). Moreover, vaccination with *Il10rb*$^{-/-}$ cDC1s resulted in a similar reduction in bilateral tumor growth compared to *Stat3*$^{\Delta/\Delta}$ cDC1 vaccination (Figure 3.8C). Therefore, these data imply IL-10 signaling through STAT3 inhibits cDC1 vaccine efficacy.

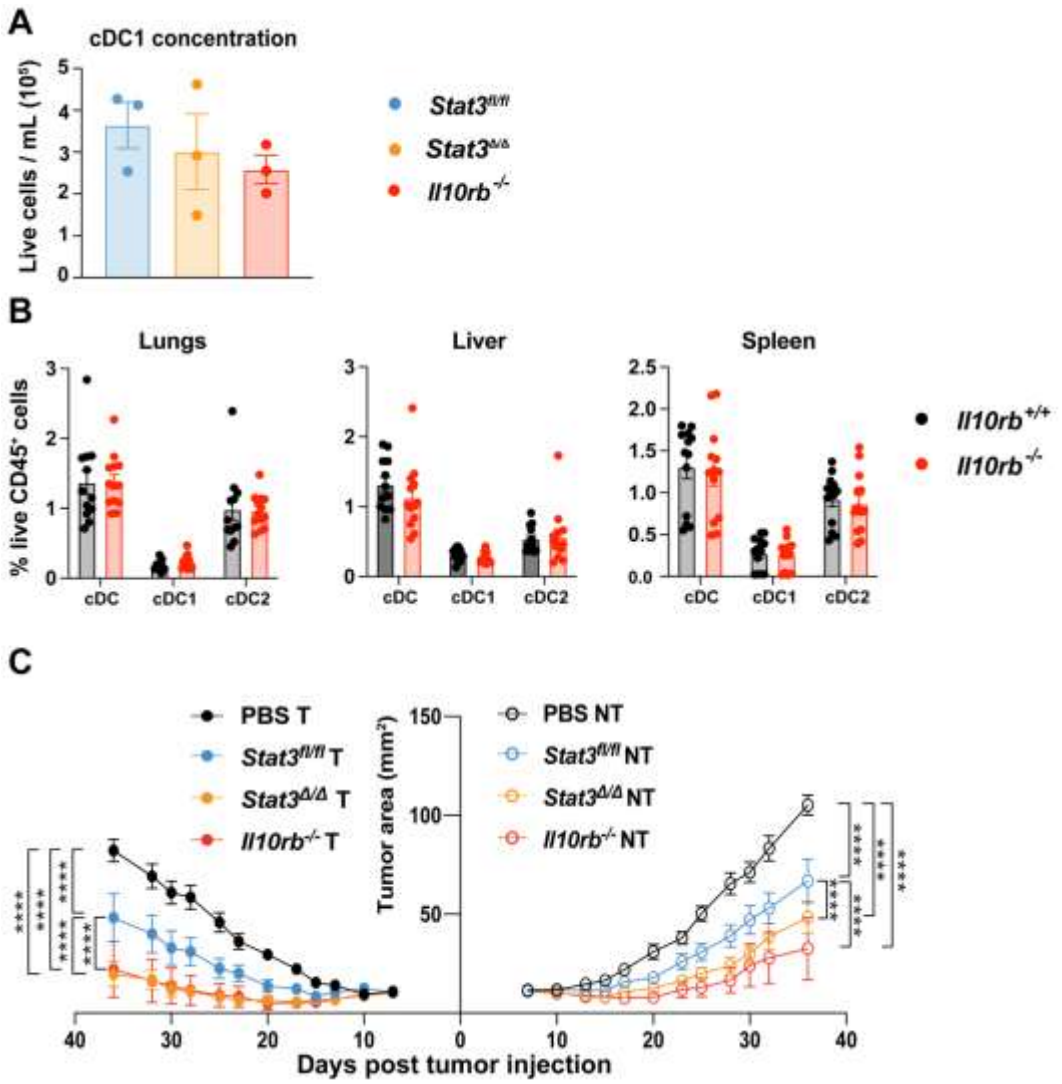

Figure 3.8. IL-10 receptor signaling inhibits cDC1 vaccine efficacy to a similar degree as STAT3. (A) cDC1 concentration on day 17 in cultures utilizing bone marrow from *Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$, or *Il10rb*$^{-/-}$ mice, as assessed by hemacytometer counts and flow cytometry. Data are combined from 3 independent experiments, n = 3. (B) Frequency of cDCs (CD45$^+$ CD3$^+$ CD19$^-$, F4/80$^-$, CD11c$^+$ MHC II^{hi}), cDC1s (CD45$^+$ CD3$^+$ CD19$^-$, F4/80$^-$, CD11c$^+$ MHC II^{hi} XCR1$^+$ CD172α^-), and cDC2s (CD45$^+$ CD3$^+$ CD19$^-$, F4/80$^-$, CD11c$^+$ MHC II^{hi} XCR1$^-$ CD172α^+) in the indicated organs of *Il10rb*$^{+/+}$ and *Il10rb*$^{-/-}$ mice. Data are combined from 2 independent experiments, n = 12 (*Il10rb*$^{+/+}$), n = 13 (*Il10rb*$^{-/-}$). (C) Mean tumor area of treated and non-treated tumors in mice bearing bilateral PyMT-OVA tumors, vaccinated i.t. 7 days after tumor injection, with PBS, *Stat3*$^{fl/fl}$ cDC1s, *Stat3*$^{\Delta/\Delta}$ cDC1s, or *Il10rb*$^{-/-}$ cDC1s. Data are representative of 2 independent experiments, n = 7 (PBS, *Il10rb*$^{-/-}$), n = 9 (*Stat3*$^{fl/fl}$, *Stat3*$^{\Delta/\Delta}$). (A – C) Data are shown as mean ± SEM. (A and B) Each symbol represents an individual biological replicate. Data were analyzed by Welch's T test (B), one-way (A) or two-way (B) ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. **** $p < 0.0001$.

3.3 – Discussion

cDC1s are required for the induction of $CD8^+$ T cell-mediated immunity against tumors and pathogens. However, the cytokine-mediated signaling factors that regulate cDC1 function are largely unknown. Herein, we have shown that IL-10 inhibits poly I:C-induced cDC1 maturation in a STAT3-dependent manner. In addition, we demonstrated that STAT3 and IL-10Rβ inhibit cDC1 vaccine efficacy in a murine model of breast cancer. STAT3 inhibited cDC1 CD86 expression in the TME after vaccination and the induction of tumor antigen-specific $CD8^+$ T cells and Th1 cells. CD80 and CD86 have been shown to induce T cell proliferation in chronic viral infection and in tumors after immune checkpoint blockade (ICB) [234, 235]. Therefore, the increased CD86 expression on *Stat3*$^{\Delta/\Delta}$ cDC1s in the TME post vaccination may contribute to the increase in tumor antigen-specific $CD8^+$ T cells. In addition, cDC1 production of CXCL10 is essential for the recruitment of effector $CD8^+$ T cells to TME [105, 108]. We demonstrated that induction of CXCL10 in cDC1s by poly I:C is inhibited by co-treatment with IL-10 in a STAT3-dependent manner. Thus, the *Stat3*$^{\Delta/\Delta}$ cDC1 vaccine may increase $CD8^+$ T cell abundance in the TME through increased expression of T cell chemoattractants. Taken together, these data imply IL-10 and STAT3 negatively regulate cDC1 vaccine-induced T cell-mediated anti-tumor immunity through a mechanism that involves inhibition of cDC1 maturation (Figures 3.9 and 3.10).

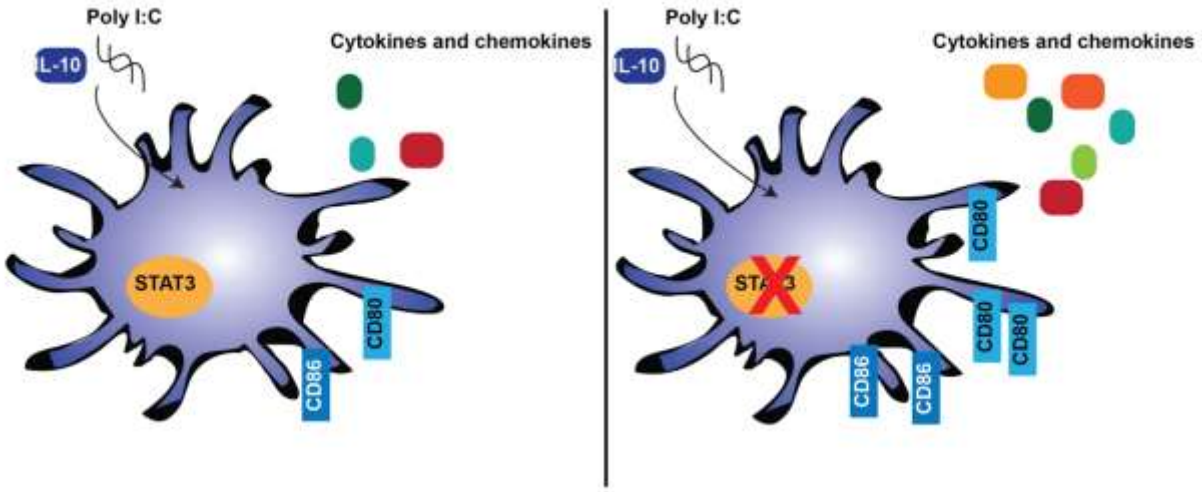

Figure 3.9. Schematic of the role of STAT3 in cDC1 maturation. Pictured left, STAT3-sufficient cDC1s co-treated with IL-10 and poly I:C have limited expression of costimulatory markers, inflammatory cytokines, and chemokines. Pictured right, STAT3-deficient cDC1s exposed to IL-10 and poly I:C show enhanced expression of costimulatory markers, inflammatory cytokines, and chemokines relative to WT cDC1s.

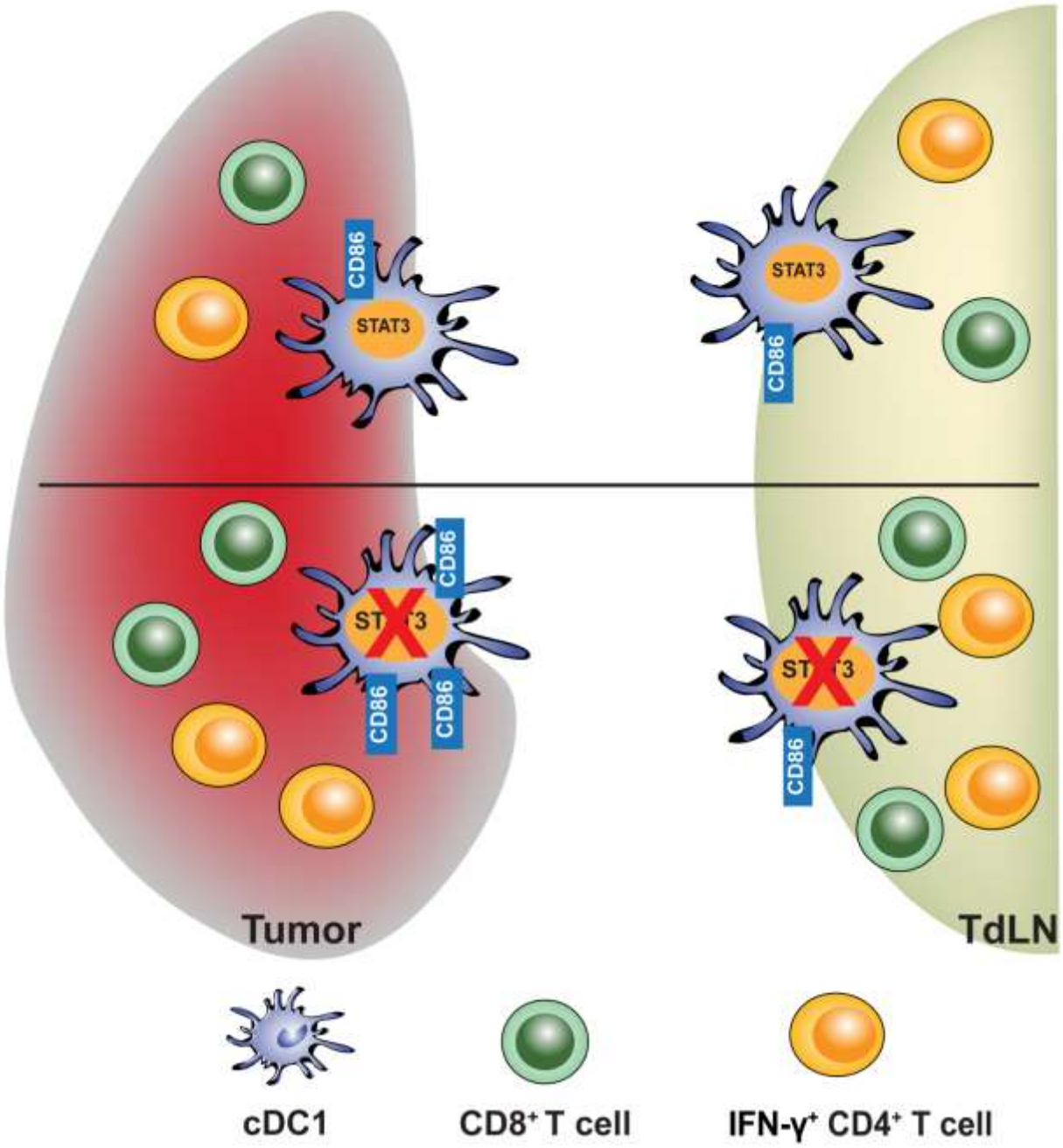

Figure 3.10. Schematic of the role of STAT3 in cDC1 vaccination. Pictured top, vaccination with STAT3-sufficient cDC1s results in limited induction of T cell-mediated anti-tumor immunity. Pictured bottom, vaccination with STAT3-deficient cDC1s results in significant increases in tumor antigen-specific $CD8^+$ T cells and IFN-γ^+ $CD4^+$ T cells compared to STAT3-sufficient cDC1s.

Others have shown that cDC1-mediated anti-tumor immunity in breast cancer is restrained by suppressing cDC1 development [224]. Breast tumors restrict cDC1 development through their expression of G-CSF, which signals in DC progenitors in the bone marrow to inhibit cDC1 differentiation [224]. Although there may be tumor cell-derived G-CSF in our model, our vaccine strategy allowed us to avoid effects on cDC1 development by injecting equal numbers of cDC1s in every group. Furthermore, although G-CSF is a STAT3-activating cytokine, cDC1s express low amounts of *Csf3r*, suggesting they are refractory to this signal [230].

Previously, it has been shown that STAT3 suppresses inflammatory responses in other myeloid subsets [132]. For example, STAT3 deletion in bone marrow-derived macrophages (BMDMs) or GM-DCs renders them hyperactive to TLR stimulation, as assessed by increased inflammatory cytokine secretion post TLR agonist exposure [214, 223]. In the case of BMDMs, autocrine IL-6 signaling in response to TLR activation results in STAT3-dependent downregulation of UBC13, an important enzyme that mediates NF-κB activation [214]. In contrast, we found that *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s respond similarly to TLR activation, as evidenced by comparable induction of costimulatory marker expression and cytokine secretion after poly I:C treatment. Only after cDC1s were exposed to IL-10 and poly I:C was an inhibitory role for STAT3 in cDC1 maturation identified. These data suggest that although numerous STAT3 activating cytokines are induced by poly I:C in cDC1s, they are not sufficient to elicit STAT3-mediated anti-inflammatory effects in an autocrine manner [236, 237]. Therefore, STAT3 appears to use different anti-

inflammatory mechanisms in different immune cell types, as has been suggested previously by others investigating effects of IL-10 signaling [208].

Although our results demonstrate IL-10 signaling inhibits cDC1 maturation and induction of anti-tumor immunity, others have shown that IL-10 stimulates the proliferation and anti-tumor activity of $CD8^+$ T cell in the TME [236, 237]. Interestingly, one mechanism through which IL-10 was shown to promote $CD8^+$ T cell function in the TME was through inhibition of DC IFN-γ secretion [238]. In ICB-treated tumors, IFN-γ production from DCs can result in activation-induced cell death of tumor-infiltrating $CD8^+$ T cells, inhibiting therapeutic efficacy [238]. However, treatment with tumor-targeted IL-10 inhibited DC IFN-γ production and promoted $CD8^+$ T cell survival and anti-tumor immunity [238]. Therefore, IL-10-mediated inhibition of DC function may be required to fine-tune $CD8^+$ T cell activation in certain contexts and lead to favorable outcomes regarding anti-tumor immunity.

Chapter 4 – STAT3 inhibits IFN signaling in cDC1s

4.1 – Background and rationale

Previously, we have shown that STAT3 mediates the inhibitory effect of IL-10 on poly I:C-induced maturation in cDC1s. Poly I:C and other TLR agonists activate a number of signaling pathways [239]. These include the NF-κB and IRF pathways, as well the mitogen-activated protein kinase cascade (MAPK) [239]. TLR agonist-mediated activation of these pathways results in the expression of inflammatory cytokines, chemokines, and other factors. Many of the cytokines can signal in an autocrine fashion to induce secondary and tertiary responses, such as ISG induction by IFNs. Previously, others have shown that IL-10 has cell type-specific roles in inhibiting TLR activation and downstream signaling [208]. For example, macrophages and splenic cDCs induce a similar set of genes in response to TLR4 activation but differ greatly in which genes are inhibited by IL-10 [208]. Specifically, in macrophages, NF-κB signaling appears to be inhibited, whereas in cDCs, IRF signaling is the likely target of IL-10 – mediated inhibition [208]. cDC1s have now been delineated as a distinct cDC subset, responsible for inducing $CD8^+$ T cell-mediated immunity against pathogens and tumors. Although in prior studies we have demonstrated that STAT3 inhibits cDC1 maturation and cDC1-elicited anti-tumor immunity, the mechanism by which STAT3 performs these roles remains unclear.

4.2 – Results

4.2.1 – RNA sequencing identifies IFN signaling as the primary target of STAT3-mediated inhibition of poly I:C-induced maturation in cDC1s

To determine global STAT3-mediated gene regulation of cDC1s, RNA sequencing was performed on *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated for 6 hours with PBS, IL-10, poly I:C, or IL-10 + poly I:C. Immunoblotting confirmed STAT3 depletion in *Stat3*$^{\Delta/\Delta}$ cDC1s and reduced phospho-STAT3 accumulation in response to IL-10 treatment, as previously described (Figures 3.2A and 4.1A). Principle component analysis (PCA) of the transcript expression data revealed 4 distinct clusters corresponding to each treatment condition in *Stat3*$^{fl/fl}$ cDC1s (Figure 4.1B). *Stat3*$^{\Delta/\Delta}$ cDC1s, however, displayed only two distinct clusters (Figure 4.1B). One cluster comprised the PBS and IL-10-treated *Stat3*$^{\Delta/\Delta}$ cDC1s, located proximal to the PBS-treated *Stat3*$^{fl/fl}$ cDC1s (Figure 4.1B). The second cluster contained the poly I:C and IL-10 + poly I:C-treated *Stat3*$^{\Delta/\Delta}$ cDC1s and was located close to poly I:C-treated *Stat3*$^{fl/fl}$ cDC1s (Figure 4.1B). These results imply that IL-10 and IL-10 + poly I:C exposure induce STAT3-dependent transcriptional states, while the expression profile of poly I:C-treated cDC1s is attained independent of STAT3.

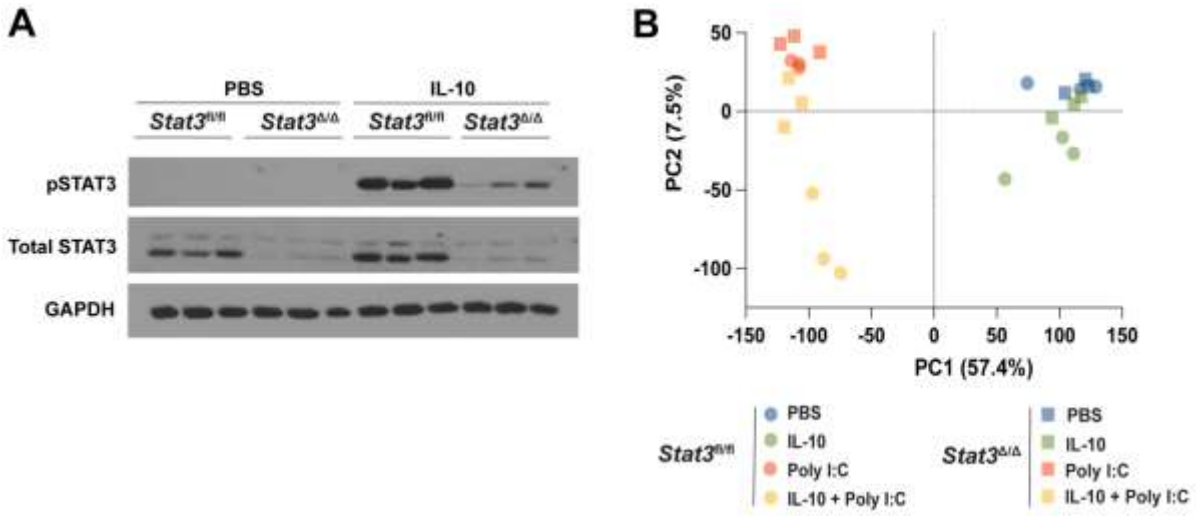

Figure 4.1. RNA sequencing reveals distinct transcriptional states in cDC1s. (A) Immunoblotting for the indicated proteins in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with PBS or IL-10 for 0.5 hours, from 3 independent experiments, *n* = 3. (B) RNA sequencing was performed on the same *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s in (A) after 6 h treatment with PBS, IL-10, poly I:C, or IL-10 + Poly I:C and analyzed by PCA.

To better characterize the transcriptional changes induced by IL-10 and STAT3, the data were analyzed by GSEA and IPA [240, 241]. GSEA with hallmark gene sets indicated enrichment of 10 gene sets in IL-10-treated *Stat3*$^{fl/fl}$ cDC1s compared to PBS-treated *Stat3*$^{fl/fl}$ cDC1s (Figure 4.2A). Notably, the most enriched gene sets were Cholesterol homeostasis and IL6-JAK-STAT3 signaling (Figure 4.2A). Similarly, IPA canonical pathway analysis identified enrichment of the Superpathway of cholesterol biosynthesis as well as the STAT3 pathway, among others (Figure 4.2B). Additionally, IPA upstream regulator analysis indicated IL-1β as the top cytokine mediating the transcriptional changes downstream of IL-10 in *Stat3*$^{fl/fl}$ cDC1s, in accordance with previous results demonstrating IL-1β secretion by IL-10-treated *Stat3*$^{fl/fl}$ cDC1s (Figures 3.3B and 4.2C). Furthermore, IPA upstream regulator analysis for transcription factors predicted multiple STATs, including STAT3, as well as SREBF1, a regulator of the cholesterol biosynthesis pathway, as mediators of the IL-10-induced transcriptional state in *Stat3*$^{fl/fl}$ cDC1s (Figure 4.2C).

In contrast, only the Apoptosis hallmark pathway was found to be enriched in IL-10-treated *Stat3*$^{\Delta/\Delta}$ cDC1s compared to PBS-treated *Stat3*$^{\Delta/\Delta}$ cDC1s using GSEA, and no canonical pathways were found to be enriched when analyzed by IPA (Figures 4.2D). Moreover, IPA identified only STAT1 and IFN-γ in the upstream regulator analysis for transcription factors and cytokines, respectively (Figure 4.2E). Therefore, these data suggest IL-10 – mediated pathway enrichment, as determined by GSEA and IPA, is largely STAT3-dependent.

Finally, when IL-10-treated *Stat3*$^{fl/fl}$ cDC1s and *Stat3*$^{\Delta/\Delta}$ cDC1s were compared directly, the list of gene sets found to be enriched in *Stat3*$^{fl/fl}$ cDC1s closely

resembled those found when comparing IL-10-treated to PBS-treated *Stat3*$^{fl/fl}$ cDC1s, suggesting STAT3 is required for IL-10 – mediated pathway enrichment determined by GSEA (Figures 4.2A and 4.2F). However, while the canonical pathways and upstream regulators identified by IPA when comparing *Stat3*$^{fl/fl}$ cDC1s to *Stat3*$^{\Delta/\Delta}$ cDC1s did differ considerably from those found when analyzing IL-10-treated versus PBS-treated *Stat3*$^{fl/fl}$ cDC1s, there were some findings in common such as the identification of IL-1β as an upstream regulator (Figures 4.2G and 4.2H). Taken together, multiple bioinformatic approaches suggest IL-10 regulates pathways involved in cytokine signaling in cDC1s in a STAT3-dependent manner.

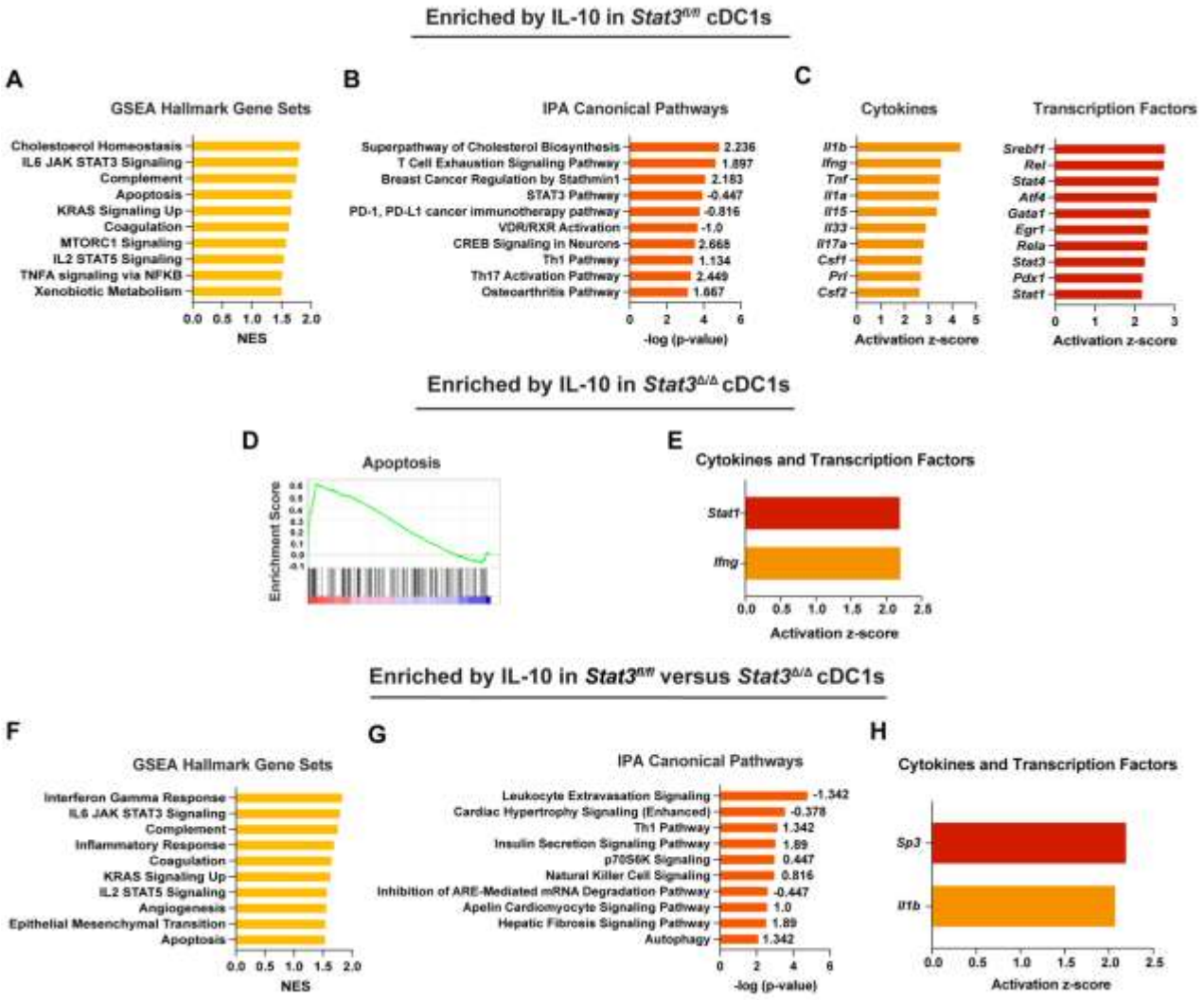
Enriched by IL-10 in Stat3fl/fl cDC1s
A
GSEA Hallmark Gene Sets
Cholesterol Homeostasis
IL6 JAK STAT3 Signaling
Complement
Apoptosis
KRAS Signaling Up
Coagulation
MTORC1 Signaling
IL2 STAT5 Signaling
TNFA signaling via NFKB
Xenobiotic Metabolism
0.0 0.5 1.0 1.5 2.0
NES
B
IPA Canonical Pathways
Superpathway of Cholesterol Biosynthesis 2.236
T Cell Exhaustion Signaling Pathway 1.897
Breast Cancer Regulation by Stathmin1 2.183
STAT3 Pathway -0.447
PD-1, PD-L1 cancer immunotherapy pathway -0.816
VDR/RXR Activation -1.0
CREB Signaling in Neurons 2.668
Th1 Pathway 1.134
Th17 Activation Pathway 2.449
Osteoarthritis Pathway 1.667
0 2 4 6
-log (p-value)
C
Cytokines
Il1b
Ifng
Tnf
Il1a
Il15
Il33
Il17a
Csf1
Prl
Csf2
0 1 2 3 4 5
Activation z-score
Transcription Factors
Srebf1
Rel
Stat4
Atf4
Gata1
Egr1
Rela
Stat3
Pdx1
Stat1
0 1 2 3
Activation z-score
Enriched by IL-10 in Stat3Δ/Δ cDC1s
D
Apoptosis
Enrichment Score
E
Cytokines and Transcription Factors
Stat1
Ifng
0.0 0.5 1.0 1.5 2.0 2.5
Activation z-score
Enriched by IL-10 in Stat3fl/fl versus Stat3Δ/Δ cDC1s
F
GSEA Hallmark Gene Sets
Interferon Gamma Response
IL6 JAK STAT3 Signaling
Complement
Inflammatory Response
Coagulation
KRAS Signaling Up
IL2 STAT5 Signaling
Angiogenesis
Epithelial Mesenchymal Transition
Apoptosis
0.0 0.5 1.0 1.5 2.0
NES
G
IPA Canonical Pathways
Leukocyte Extravasation Signaling -1.342
Cardiac Hypertrophy Signaling (Enhanced) -0.378
Th1 Pathway 1.342
Insulin Secretion Signaling Pathway 1.89
p70S6K Signaling 0.447
Natural Killer Cell Signaling 0.816
Inhibition of ARE-Mediated mRNA Degradation Pathway -0.447
Apelin Cardiomyocyte Signaling Pathway 1.0
Hepatic Fibrosis Signaling Pathway 1.89
Autophagy 1.342
0 2 4 6
-log (p-value)
H
Cytokines and Transcription Factors
Sp3
Il1b
0.0 0.5 1.0 1.5 2.0 2.5
Activation z-score

Figure 4.2. Bioinformatic analyses reveal STAT3-dependent pathways regulated by IL-10 in cDC1s. RNA-sequencing was performed on *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with IL-10 or PBS for 6 hours, from 3 independent experiments, $n = 3$. (A) The ten most enriched pathways in *Stat3*$^{fl/fl}$ cDC1s treated with IL-10 versus PBS, analyzed by GSEA and determined by normalized enrichment score (NES) and (B) IPA. Listed right in (B) are the z-scores for each IPA pathway. A positive z-score indicates predicted activation of the pathway, and a negative z-score indicates predicted inhibition of the pathway. (C) The top ten cytokines (left) and transcription factors (right) identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (A). (D) The GSEA Apoptosis enrichment plot, enriched in *Stat3*$^{\Delta/\Delta}$ cDC1s treated with IL-10 compared to PBS. (E) Cytokines and transcription factors identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (D). (F) The ten most enriched pathways determined by GSEA and (G) IPA when comparing IL-10 – treated *Stat3*$^{fl/fl}$ cDC1s to *Stat3*$^{\Delta/\Delta}$ cDC1s. Listed right in (G) are the z-scores for each IPA pathway. (H) Cytokines and transcription factors identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (G).

Using the same bioinformatic analyses to parse the transcriptional state induced by poly I:C, GSEA indicated enrichment of 21 pathways in poly I:C-treated *Stat3*$^{fl/fl}$ cDC1s compared to PBS-treated *Stat3*$^{fl/fl}$ cDC1s (Figure 4.3A). Many of these pathways involved IFNs, NF-κB, TNF-α, and other inflammatory cytokines (Figure 4.3A). IPA canonical pathway analysis displayed enrichment of pathways involved in cytokine secretion and NF-κB signaling, as well as regulation of translation (Figure 4.3B). In addition, IPA upstream regulator analysis identified primarily inflammatory cytokines and their associated transcription factors (Figure 4.3C). Similarly, when comparing poly I:C-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to PBS-treated *Stat3*$^{\Delta/\Delta}$ cDC1s with GSEA and IPA, primarily inflammatory signaling pathways were found to be enriched (Figures 4.3D and 4.3E). Moreover, similar cytokines and transcription factors were identified by IPA upstream regulator analysis comparing poly I:C-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to PBS-treated *Stat3*$^{\Delta/\Delta}$ cDC1s, as those found when comparing similarly treated *Stat3*$^{fl/fl}$ cDC1s (Figure 4.3F). When directly comparing poly I:C-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to *Stat3*$^{fl/fl}$ cDC1s with GSEA, the Inflammatory response pathway was found to be enriched in the *Stat3*$^{fl/fl}$ cDC1s, but this was the only statistically significant enrichment detected (Figure 4.3G). Likewise, only 3 pathways were found to be enriched when performing comparing poly I:C-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to *Stat3*$^{fl/fl}$ cDC1s by IPA (Figure 4.3H). Therefore, poly I:C induces a broad inflammatory transcriptional program after 6 hours of exposure, dominated by inflammatory cytokine signaling and associated transcription factors, in a largely STAT3-independent manner.

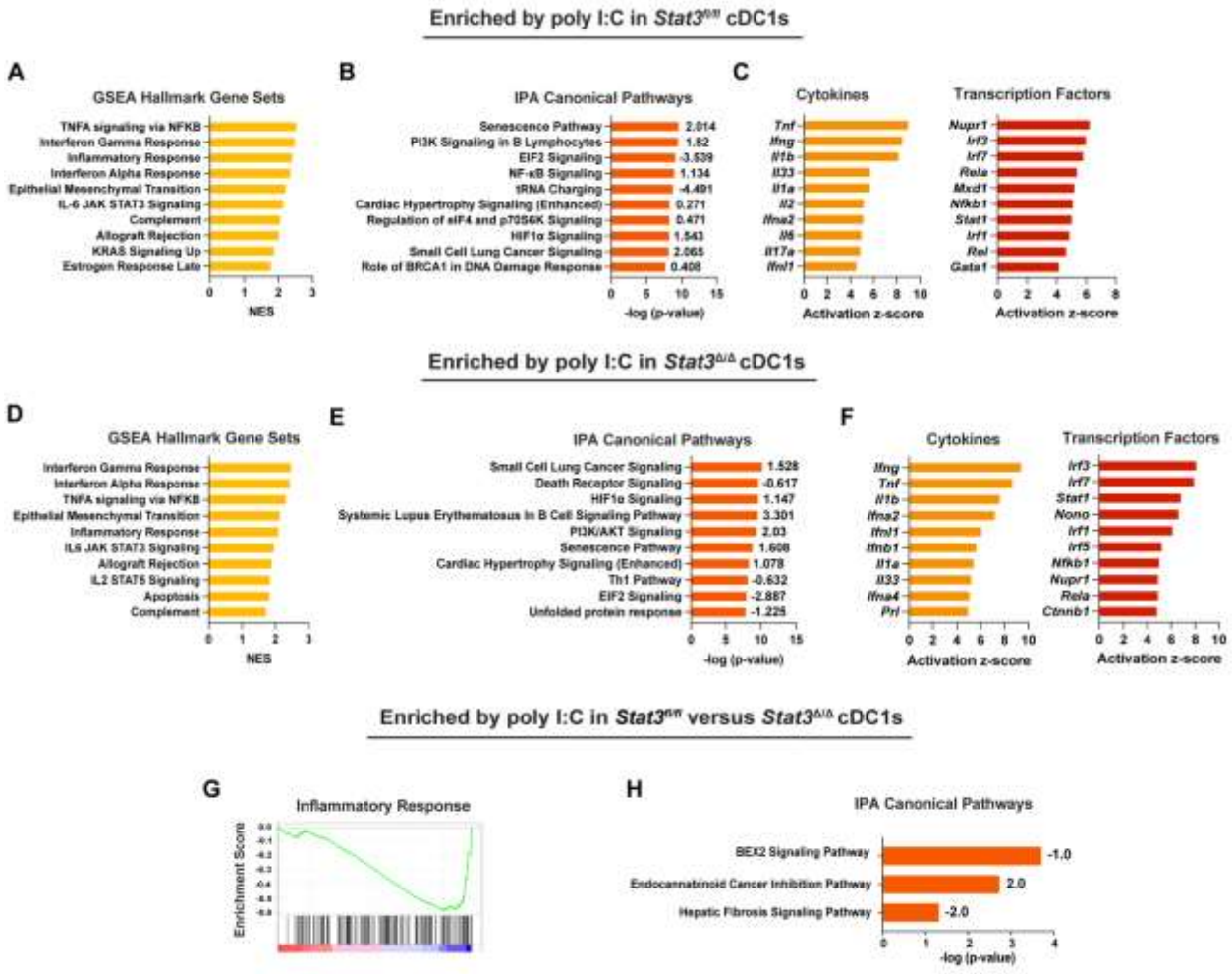

Figure 4.3. Poly I:C induces similar responses in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s. RNA-sequencing was performed on *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with poly I:C or PBS for 6 hours, from 3 independent experiments, $n = 3$. (A) The ten most enriched pathways determined by GSEA and (B) IPA in *Stat3*$^{fl/fl}$ cDC1s treated with poly I:C versus PBS. Listed right in (B) are the z-scores for each IPA pathway. (C) The top ten cytokines (left) and transcription factors (right) identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (B). (D) The ten most enriched pathways determined by GSEA and (E) IPA in *Stat3*$^{\Delta/\Delta}$ cDC1s treated with poly I:C compared to PBS. Listed right in (E) are the z-scores for each IPA pathway. (F) the top ten cytokines (left) and transcription factors (right) identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (E). (G) Pathways enriched when comparing poly I:C-treated *Stat3*$^{\Delta/\Delta}$ to *Stat3*$^{fl/fl}$ cDC1s by GSEA and (H) IPA.

To determine how STAT3 and IL-10 alter the transcriptional state induced by poly I:C, GSEA and IPA were used to compare IL-10 and poly I:C co-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to similarly treated *Stat3*$^{fl/fl}$ cDC1s. GSEA revealed that although many inflammatory pathways are enriched by poly I:C, as previously described, only the Interferon alpha response gene set was enriched when comparing IL-10 and poly I:C co-treated *Stat3*$^{\Delta/\Delta}$ cDC1s to *Stat3*$^{fl/fl}$ cDC1s (Figures 4.3A and 4.4A). In addition, IPA indicated inflammatory pathways such as the Th1 pathway and Dendritic cell maturation were the most enriched pathways in IL-10 and poly I:C co-treated *Stat3*$^{\Delta/\Delta}$ cDC1s compared to *Stat3*$^{fl/fl}$ cDC1s (Figure 4.4B). Of note, type I IFNs promote DC maturation and polarization of Th1 cells [47, 49]. Moreover, IPA upstream regulator analysis filtered for cytokines and transcription factors predicted primarily IFNs and IFN-associated transcription factors, such as IRF3 and STAT1, as mediating the transcriptional differences between in IL-10 and poly I:C co-treated *Stat3*$^{\Delta/\Delta}$ cDC1s compared to *Stat3*$^{fl/fl}$ cDC1s (Figure 4.4C). In summary, bioinformatic analyses demonstrate that STAT3 specifically inhibits poly I:C-induced IFN signaling in IL-10 and poly I:C co-treated cDC1s.

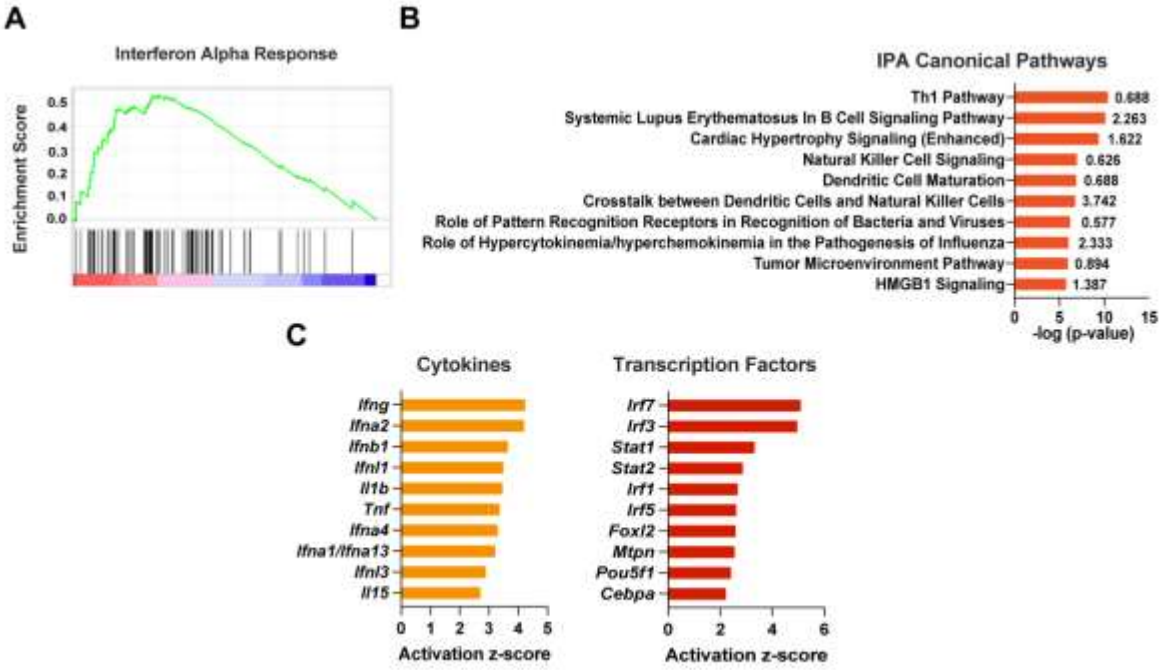

Figure 4.4. IL-10 inhibits poly I:C-induced IFN signaling in cDC1s in a STAT3-dependent manner. RNA-sequencing was performed on *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with IL-10 and poly I:C for 6 hours, from 3 independent experiments, *n* = 3. (A) Pathways enriched in *Stat3*$^{\Delta/\Delta}$ cDC1s compared to *Stat3*$^{fl/fl}$ cDC1s, as determined by GSEA (B) The ten most enriched IPA pathways in *Stat3*$^{\Delta/\Delta}$ cDC1s compared to *Stat3*$^{fl/fl}$ cDC1s. Listed right are the z-scores. (C) The top ten cytokines (left) and transcription factors (right) identified by IPA as potential upstream regulators mediating the transcriptional changes analyzed in (B).

4.2.2 – IFNs, IFN signaling, and ISG expression induced by poly I:C are inhibited by IL-10 in a STAT3-dependent manner

To validate whether IFN signaling is indeed induced by poly I:C and inhibited by IL-10 in a STAT3-dependent manner, as indicated by the pathway analyses, *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C for 6 hours. Immunoblotting revealed comparable phospho-STAT1 accumulation in poly I:C-treated and *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 4.5A). However, while poly I:C-induced phospho-STAT1 accumulation was inhibited by co-treatment with IL-10 in *Stat3*$^{fl/fl}$ cDC1s, poly I:C-responsive phospho-STAT1 accumulation was unaltered by concomitant IL-10 treatment in *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 4.5A). Phospho-STAT1 was undetectable in PBS and IL-10 treated samples, as expected (Figure 4.5A). These results indicate that poly I:C-induced phospho-STAT1 accrual is inhibited by co-treatment with IL-10 in a STAT3-dependent manner. Phospho-STAT3 was induced by IL-10, poly I:C, and IL-10 + poly I:C in *Stat3*$^{fl/fl}$ cDC1s, and to a lesser extent in similarly treated *Stat3*$^{\Delta/\Delta}$ cDC1s (Figure 4.5A). Phospho-STAT3 is likely still detectable in *Stat3*$^{\Delta/\Delta}$ cDC1s due to residual STAT3 that remains after CD11c Cre-mediated deletion of *Stat3* (Figure 4.5A). Total STAT1 abundance was unaltered across all treatment conditions and not effected by deletion of STAT3 (Figure 4.5A). In addition, total STAT3 was decreased in IL-10 and IL-10 + poly I:C-treated *Stat3*$^{fl/fl}$ cDC1s compared to PBS-treated *Stat3*$^{fl/fl}$ cDC1s, but was unaffected by poly I:C alone, suggesting IL-10 negatively regulates STAT3 expression (Figure 4.5A). Taken together, IL-10 – mediated, STAT3-dependent reduction of poly I:C-

induced phospho-STAT1 accumulation suggests inhibition of an autocrine IFN signal.

Next, we sought to determine whether IFN or ISG transcript expression was inhibited in a STAT3-dependent manner upon co-treatment with IL-10 and poly I:C. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C, for 6 hours and analyzed by RT-qPCR. *Cxcl10* was induced by poly I:C and inhibited by co-treatment with IL-10 in a STAT3-dependent manner (Figure 4.5B). In addition, *Ifnb1* was poly I:C-responsive and trended to be inhibited by IL-10 in STAT3-dependent manner (Figure 4.5B). Similarly, multiplexed cytokine and chemokine analysis of supernatants from *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s revealed induction of many inflammatory factors by poly I:C that were inhibited by co-treatment with IL-10 in a STAT3-dependent manner, including both IFN-β and IFN-γ (Figures 4.5C and 4.5D). IFN-α and IFN-λ were below the limit of detection in all conditions tested. Taken together, immunoblotting, RT-qPCR, and cytokine detection assays indicate poly I:C-induced IFN expression and downstream signaling is inhibited by co-treatment with IL-10 in a STAT3-dependent manner.

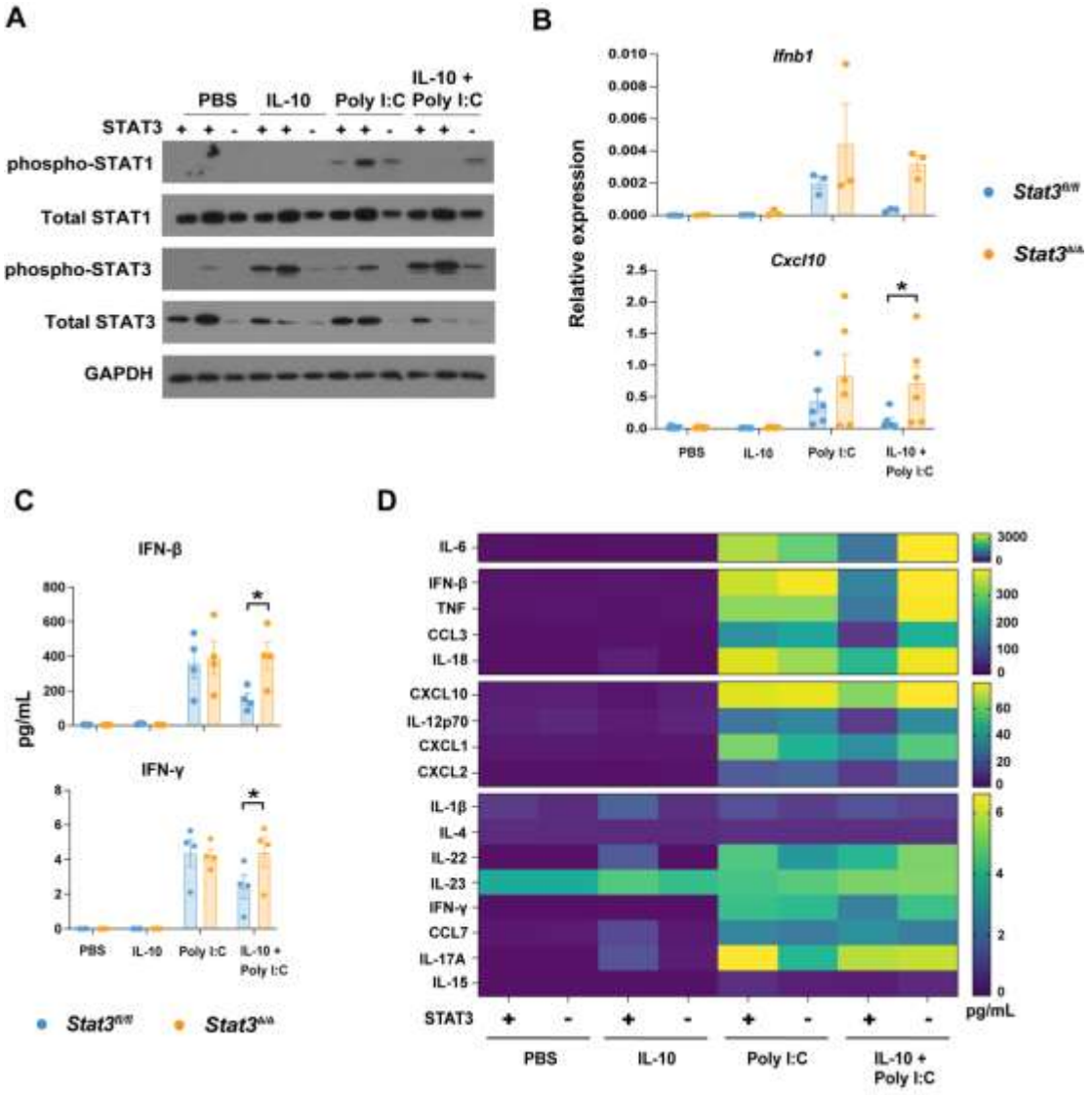

Figure 4.5. Poly I:C-induced IFN expression and signaling are inhibited by co-treatment with IL-10 in a STAT3-dependent manner. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 + poly I:C for 6 hours. (A) Representative immunoblotting for the indicated proteins, from 2 independent experiments, $n = 2$ (*Stat3*$^{fl/fl}$), $n = 1$ (*Stat3*$^{\Delta/\Delta}$). (B) Relative expression of the indicated transcripts, as assessed by RT-qPCR. For *Ifnb1*, data are combined from 3 independent experiments, $n = 3$. For *Cxcl10* data are combined from 2 independent experiments, $n = 6$. (C) IFN abundance in cell culture supernatants determined by Luminex multiplex assay. (D) Mean cytokine and chemokine concentration in cell culture supernatants determined by Luminex multiplex assay. (C and D) Data are combined from 4 independent experiments, $n = 4$. Graphs of individual analytes can be found in the appendix (Figure A2). (B and C) Data are shown as mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. $* p < 0.05$

4.2.3 – Poly I:C-induced IFN signaling is mediated selectively by type I IFNs

Normalized expression values from RNA sequencing analyses indicated poly I:C-mediated induction of *Ifnb1*, and *Ifnl2* is inhibited by concomitant treatment with IL-10 in a STAT3-dependent manner (Figure 4.6A). In addition, analyses of cDC1-produced cytokines demonstrated secretion of IFN-β and IFN-γ in response to poly I:C, which was inhibited by IL-10 via a STAT3-dependent mechanism (Figure 4.5C). Therefore, our data suggest all three IFN types potentially contribute to poly I:C-induced IFN signaling in cDC1s. To determine which IFNs cDC1s are capable of responding to, cDC1s derived from the bone marrow of WT mice were treated with PBS, IFN-β, IFN-γ, or IFN-λ, for 0.5 hours. Immunoblotting revealed IFN-β, and to lesser extent IFN-γ, as inducers of phospho-STAT1 (Figure 4.6B). Although IFN-λ increased phospho-STAT1, this response appeared weaker than that elicited by IFN-β or IFN-γ (Figure 4.6B). Total STAT1 was not altered upon IFN treatment (Figure 4.6B).

Next we assessed IFN-induced ISG expression. *Cxcl10* expression was induced by IFN-β, and to lesser extent by IFN-γ, but was unaltered by IFN-λ, as determined by RT-qPCR (Figure 4.6C). Similarly, surface expression of CD86 was induced by IFN-β, and to lesser extent by IFN-γ, as indicated by flow cytometry (Figure 4.6D). CD40, MHC I, and MHC II were also induced by IFN-β, and although IFN-γ treatment trended to induce these proteins, the increases were not statistically significant (Figure 4.6D). Interestingly, CD80 expression remained unresponsive to all IFN treatments (Figure 4.6D). Furthermore, IFN-λ failed to induce surface expression of any of the costimulatory or antigen presentation molecules tested

(Figure 4.6D). Taken together, IFN signaling and downstream responses were induced in cDC1s by direct stimulation with IFN-β and, to a lesser degree, by IFN-γ treatment. Although IFN-λ could induce a weak phospho-STAT1 signal, it did not affect the expression of any of the genes or proteins assessed, suggesting this signal is not sufficient for ISG induction.

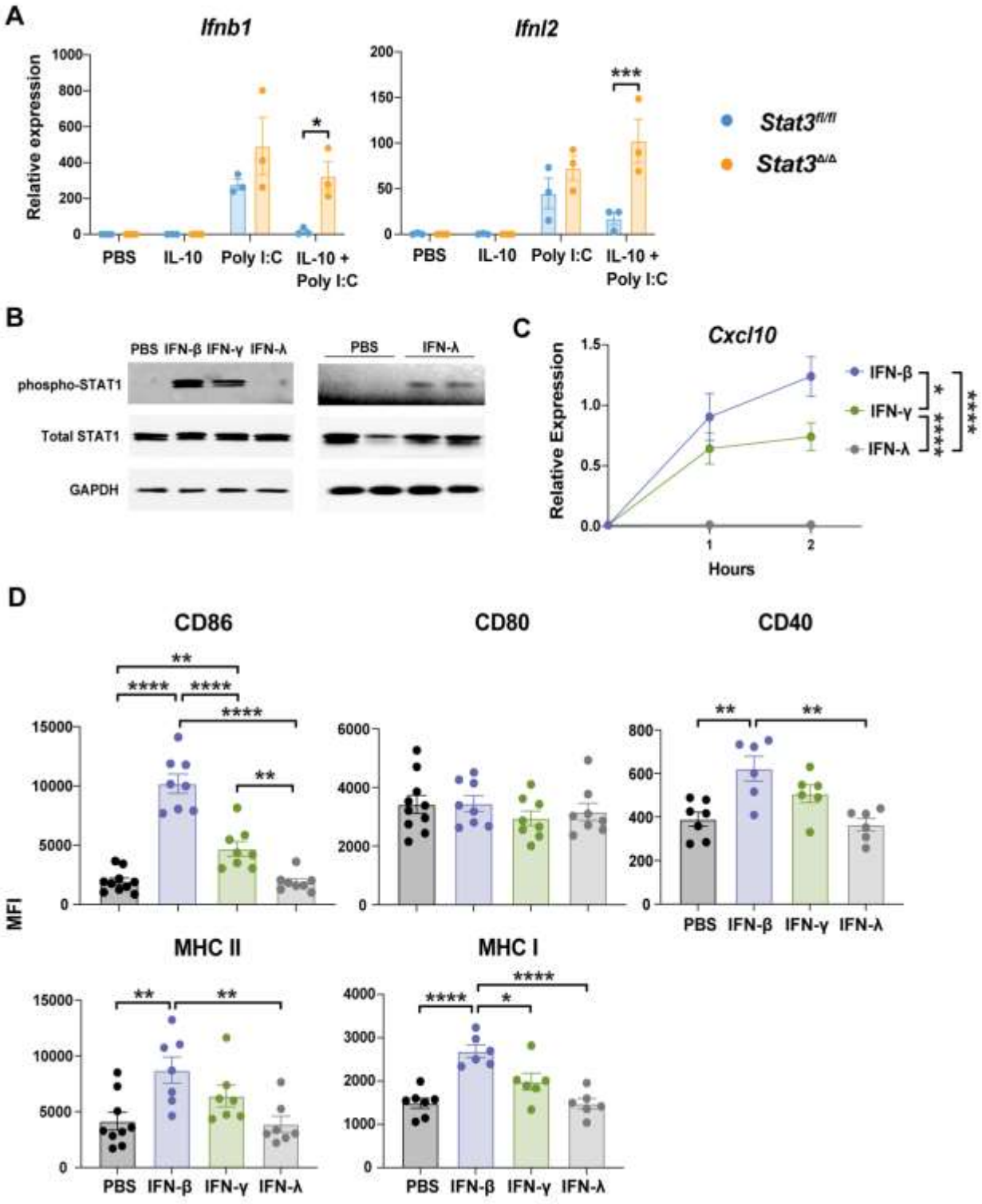
A
Ifnb1
Ifnl2
Relative expression
PBS
IL-10
Poly I:C
IL-10 + Poly I:C
Stat3fl/fl
Stat3Δ/Δ
B
PBS IFN-β IFN-γ IFN-λ
PBS IFN-λ
phospho-STAT1
Total STAT1
GAPDH
C
Cxcl10
Relative Expression
Hours
IFN-β
IFN-γ
IFN-λ
D
CD86
CD80
CD40
MHC II
MHC I
MFI
PBS IFN-β IFN-γ IFN-λ

Figure 4.6. IFN-β and IFN-γ, but not IFN-λ, induce ISG expression in cDC1s. (A). Relative expression of the indicated transcripts in *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C for 6 hours. Relative expression was determined by RNA-sequencing combined from three independent experiments, *n* = 3. (B) Immunoblotting for the indicated proteins in WT cDC1s treated with PBS, IFN-β, IFN-γ, or IFN-λ, for 0.5 hours. Pictured left is a representative blot from 2 independent experiments. Picture right are samples combined from 2 independent experiments (*n* = 2). (C) *Cxcl10* expression, determined by RT-qPCR, in WT cDC1s treated with IFN-β, IFN-γ, or IFN-λ for 1 or 2 hours. Data are combined from 3 independent experiments, *n* = 7 (0 hour), *n* = 8 (1 and 2 hour). (D) Surface expression of the indicated proteins, as assessed by flow cytometry, on WT cDC1s treated with IFN-β, IFN-γ, or IFN-λ, for 16 hours. CD86 and CD80 data are combined from 5 independent experiments, *n* = 10 (PBS), *n* = 8 (IFN-β, IFN-γ, and IFN-λ). MHC II data are combined from 5 independent experiments, *n* = 9 (PBS), *n* = 7 (IFN-β, IFN-γ, and IFN-λ). CD40 and MHC I data are combined from 4 independent experiments, *n* = 7 (PBS), *n* = 6 (IFN-β, IFN-γ, and IFN-λ). (A, C, and D) Data shown are the mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by two-way ANOVA (A and C) or one-way ANOVA (D) and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, ∗∗∗∗ $p < 0.0001$.

To determine which IFNs contribute to poly I:C-induced maturation and IFN signaling, cDC1s were generated from the bone marrow of mice that lack essential receptor subunits for type I IFN signaling (*Ifnar1*$^{-/-}$), or type II IFN signaling (*Ifngr1*$^{-/-}$) [242, 243]. Neither *Ifnar1*- nor *Ifngr1*-deficiency altered total live cell concentration at day 9 or day 17, or cDC1 frequency and concentration at day 17 of culture (Figure 4.7). To assess the role of IFN-λ, an IFN-λ blocking antibody was used. Flow cytometry analyses demonstrated that, contrary to WT cDC1s, *Ifnar1*$^{-/-}$ cDC1s do not induce CD40, CD80, CD86, MHC I, or MHC II in response to poly I:C (Figure 4.8A). However, when directly comparing poly I:C-treated WT cDC1s to *Ifnar1*$^{-/-}$ cDC1s, only CD40, CD80, and CD86 were reduced to a statistically significant degree (Figure 4.8A). *Ifngr1*-deficiency or co-treatment with anti–IFN-λ had no effect on poly I:C-induced surface marker expression (Figures 4.8B and 4.8C). Similarly, poly I:C-induced accrual of phospho-STAT1 was inhibited in *Ifnar1*$^{-/-}$ cDC1s but unchanged in *Ifngr1*$^{-/-}$ cDC1s, as assessed by immunoblotting (Figure 4.9A). Total STAT1 remained unaltered by *Ifnar1*- or *Ifngr1*-deficiency in poly I:C-treated cDC1s (Figure 4.9A). Moreover, induction of *Cxcl10* by poly I:C was determined to be *Ifnar1*-dependent but *Ifngr1*-independent by RT-qPCR (Figure 4.9B). In summary, poly I:C-induced maturation and IFN signaling in cDC1s is mediated by type I IFNs while IFN-γ and IFN-λ are dispensable.

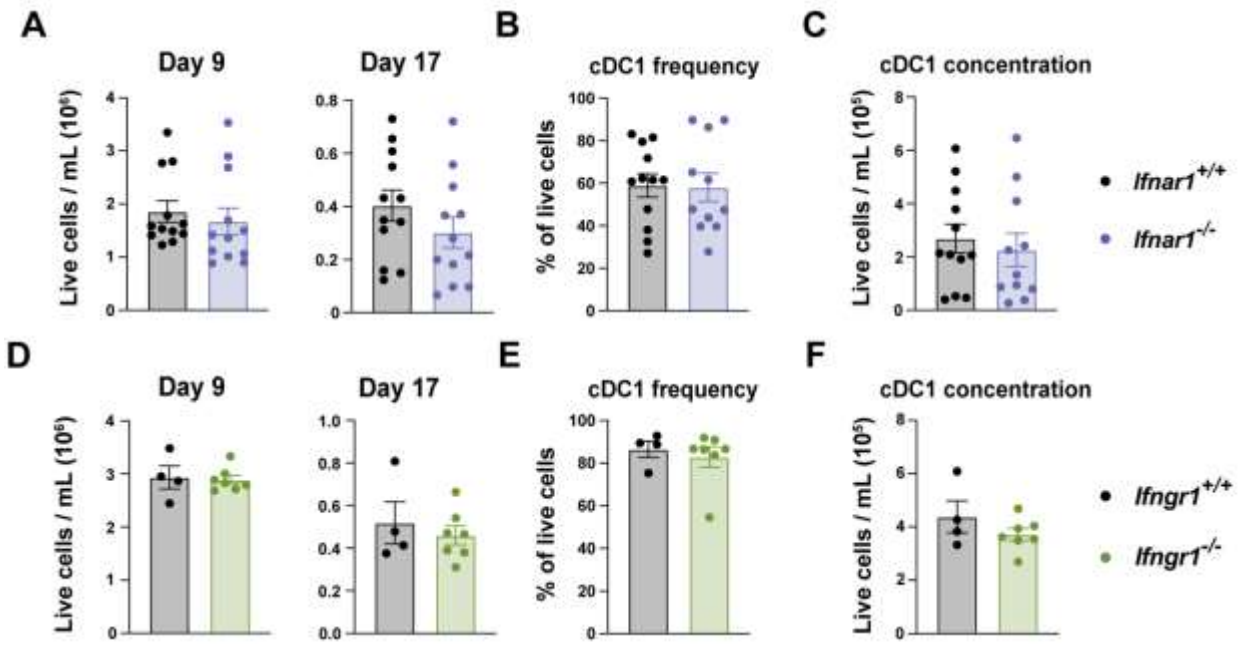

Figure 4.7. *Ifnar1*- or *Ifngr1*-deficiency does not alter cDC1 expansion in culture. cDC1s were derived in culture from the bone marrow of *Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$, or WT mice. (A) Bulk live cell concentration in suspension on the indicated day of culture, assessed by hemacytometer counts. Data are combined from 4 independent experiments, n = 12. (B) cDC1 frequency of live cells on d 17 of culture, as assessed by flow cytometry. (C) cDC1 live cell concentration in suspension on d 17 of culture, as assessed by multiplying bulk live cell concentration on d 17, determined in (A), by cDC1 frequency of live cells, determined in (B). (B, C) Data are combined from 4 independent experiments, n = 12 (*Ifnar1*$^{+/+}$), n = 11 (*Ifnar1*$^{-/-}$). (D) Bulk live cell concentration in suspension on the indicated day of culture, assessed by hemacytometer counts. (E) cDC1 frequency of live cells on d 17 of culture, as assessed by flow cytometry. (F) cDC1 live cell concentration in suspension on d 17 of culture, as assessed by multiplying bulk live cell concentration on d 17, determined in (D), by cDC1 frequency of live cells, determined in (E). (D – F) Data are combined from 3 independent experiments, n = 4 (*Ifngr1*$^{+/+}$), n = 7 (*Ifngr1*$^{-/-}$). (A – F) Data are shown as mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by Welch's T test. Results were considered significant when $p < 0.05$.

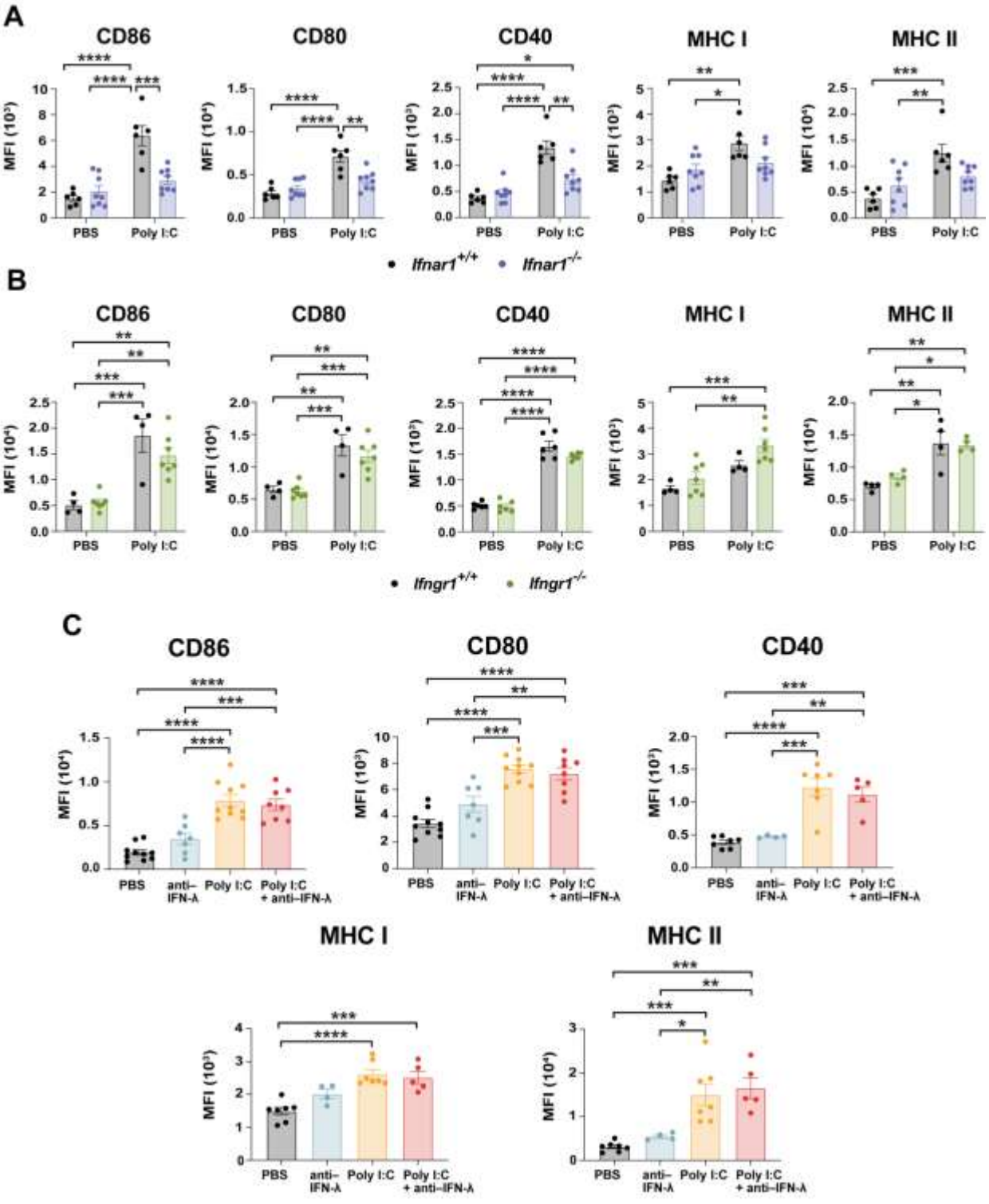
A
CD86
CD80
CD40
MHC I
MHC II
MFI (10³)
MFI (10⁴)
PBS
Poly I:C
Ifnar1+/+
Ifnar1-/-
B
Ifngr1+/+
Ifngr1-/-
C
anti–IFN-λ
Poly I:C + anti–IFN-λ

Figure 4.8. *Ifnar1* is required for poly I:C-induced cDC1 maturation, *Ifngr1* and IFN-λ are dispensable. *Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$ and WT cDC1s were incubated in the presence of PBS or poly I:C, and in some cases anti–IFN-λ with or without poly I:C, for 16 hours. Surface expression of the indicated proteins was determined by flow cytometry. (A) Data are combined from 3 independent experiments, $n = 6$ (*Ifnar1*$^{+/+}$), $n = 8$ (*Ifnar1*$^{-/-}$). (B) For CD86, CD80, and MHC I, data are combined from 3 independent experiments, $n = 4$ (*Ifngr1*$^{+/+}$), $n = 7$ (*Ifngr1*$^{-/-}$). For CD40 and MHC II, data are combined from 2 independent experiments, $n = 6$ (CD40), $n = 4$ (MHC II). (C) For CD86 and CD80, data are combined from 4 independent experiments, $n = 10$ (PBS, poly I:C), $n = 8$ (poly I:C + anti–IFN-λ), $n = 7$ (anti–IFN-λ). For CD40, MHC I, and MHC II, data are combined from 3 independent experiments, $n = 7$ (PBS, poly I:C), $n = 5$ (poly I:C + anti–IFN-λ), $n = 4$ (anti–IFN-λ). (A – C) Data are shown as mean ± SEM. Each symbol represents an individual biological replicate. Results were analyzed by two-way ANOVA (A and B) or one-way ANOVA (C) and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, ∗∗∗∗ $p < 0.0001$.

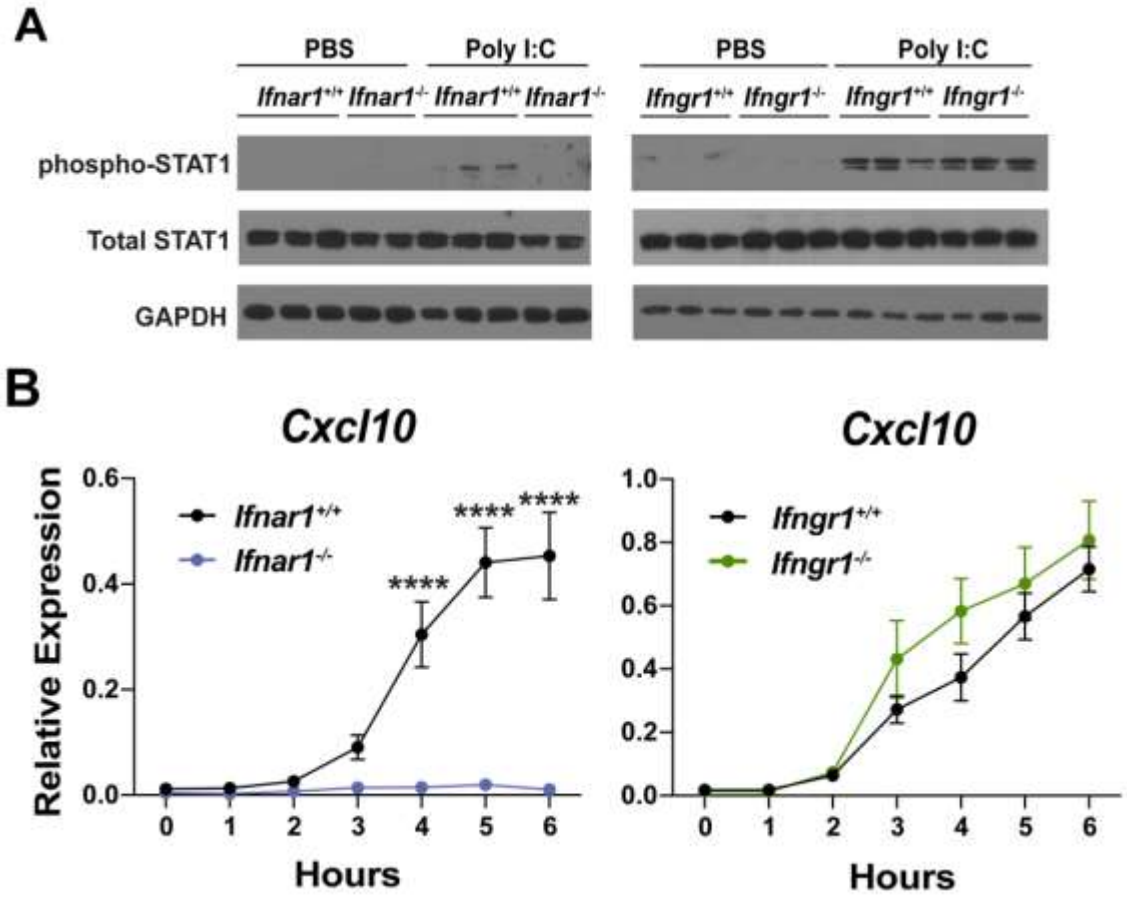

Figure 4.9. Phospho-STAT1 accumulation and *Cxcl10* expression induced by poly I:C are *Ifnar1*-dependent and *Ifngr1*-independent. *Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$ and WT cDC1s were treated with poly I:C for 6 hours. (A) Representative immunoblotting for the indicated proteins from 2 independent experiments, $n = 3$ (*Ifnar1*$^{+/+}$, *Ifngr1*$^{+/+}$, *Ifngr1*$^{-/-}$), $n = 2$ (*Ifnar1*$^{-/-}$). (B) *Cxcl10* expression as determined by RT-qPCR. Data are combined from 3 independent experiments, $n = 4$ (*Ifnar1*$^{+/+}$, *Ifngr1*$^{+/+}$), $n = 7$ (*Ifnar1*$^{-/-}$, *Ifngr1*$^{-/-}$). Data are shown as mean ± SEM. Results were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. **** $p < 0.0001$.

4.2.4 – STAT3 does not directly inhibit IFN-induced phospho-STAT1 or Cxcl10 transcript expression

Although STAT3 inhibited poly I:C-induced IFN-β and IFN-γ expression, it was possible that STAT3 could also inhibit the downstream signaling of these cytokines for the purpose of inhibiting ISG expression. To test this possibility *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were pretreated with PBS or IL-10 for 4 hours followed by exposure to either IFN-β or IFN-γ. As previously seen, IFN-β induced phospho-STAT1 greater than IFN-γ, as assessed by immunoblot, however, this signal was not altered by IL-10 pretreatment or the presence of STAT3 (Figure 4.10A). Total STAT1 abundance was comparable across all conditions (Figure 4.10A). Similarly, *Cxcl10* induction by IFN-β or IFN-γ was not changed by IL-10 pretreatment or STAT3 status (Figure 4.10B). Therefore, STAT3 is unable to inhibit signaling downstream of IFN-β, or IFN-γ under these conditions.

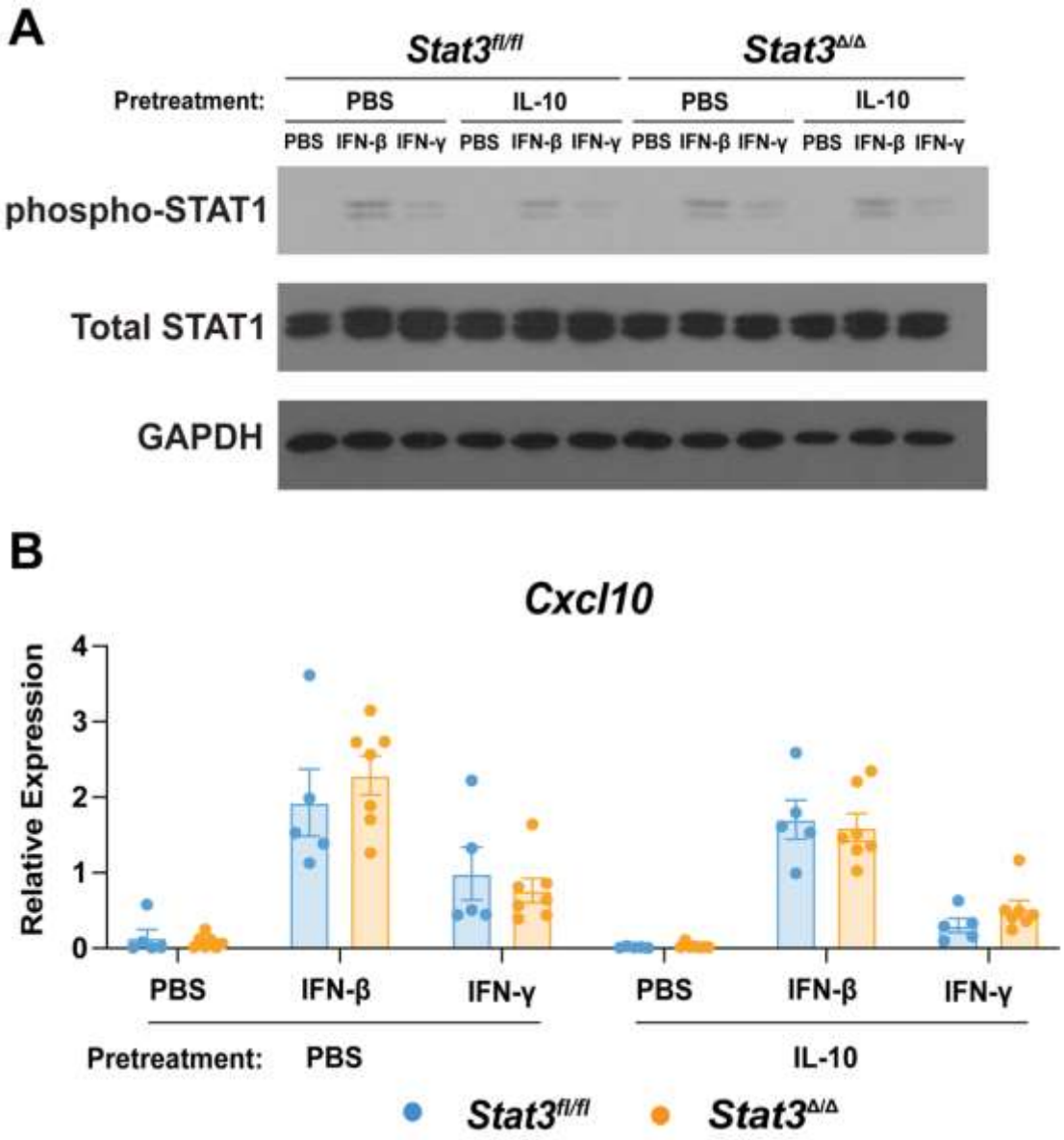

Figure 4.10. STAT3 does not directly inhibit IFN-induced phospho-STAT1 or *Cxcl10* expression. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were pretreated with PBS or IL-10 for 4 hours. Following pretreatment, *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were further exposed to PBS, IFN-β, or IFN-γ for 0.5 hours (A), or 2 hours (B). (A) Representative immunoblotting for the indicated proteins from 2 independent experiments. (B) *Cxcl10* expression, determined by RT-qPCR, combined from 2 independent experiments, $n = 5$ (*Stat3*$^{fl/fl}$), $n = 7$ (*Stat3*$^{\Delta/\Delta}$). Data are shown as mean ± SEM. Each symbol represents an individual biological replicate. Results were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$.

4.2.5 – Selective depletion of STAT3 in cDC1s in vivo does not alter murine melanoma tumor growth

Given that STAT3 inhibits poly I:C-induced type I IFN signaling in cDC1s in vitro, we hypothesized STAT3 inhibits this pathway in tumor-associated cDC1s and thus regulates tumor growth. Previously, type I IFN signaling in cDC1s was shown to be important for anti-tumor $CD8^+$ T cell priming and tumor control of B16 murine melanoma tumors [110]. Therefore, we hypothesized that STAT3 could inhibit these anti-tumor functions of cDC1s. To test this hypothesis, *Stat3*$^{fl/fl}$ mice were bred to *Xcr1*$^{cre/+}$ mice to generate cDC1-specific STAT3 knock out mice (*Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$) and WT controls (*Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$) [92]. B16-OVA cells were injected into these strains and tumor growth was tracked over time. No effect on tumor growth or mouse survival was detected due to STAT3-depletion in cDC1s (Figure 5.11A). We next assessed whether cDC1-intrinsic STAT3 was important in the context of ICB, which relies on type I and II IFNs as well as cDC1s [244]. To test this, B16-OVA tumors were implanted into both *Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$ and *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice and anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4) or control antibodies were administered intraperitoneally (i.p.) two times per week, starting on the fourth day of tumor growth. Although i.p. anti-CLTA4 restrained tumor growth and promoted mouse survival, cDC1-specific knockout of STAT3 did not alter these responses (Figure 4.11B). These results indicate cDC1-specific STAT3 depletion does not alter tumor growth of B16-OVA tumors in the absence or presence of anti-CTLA4 ICB.

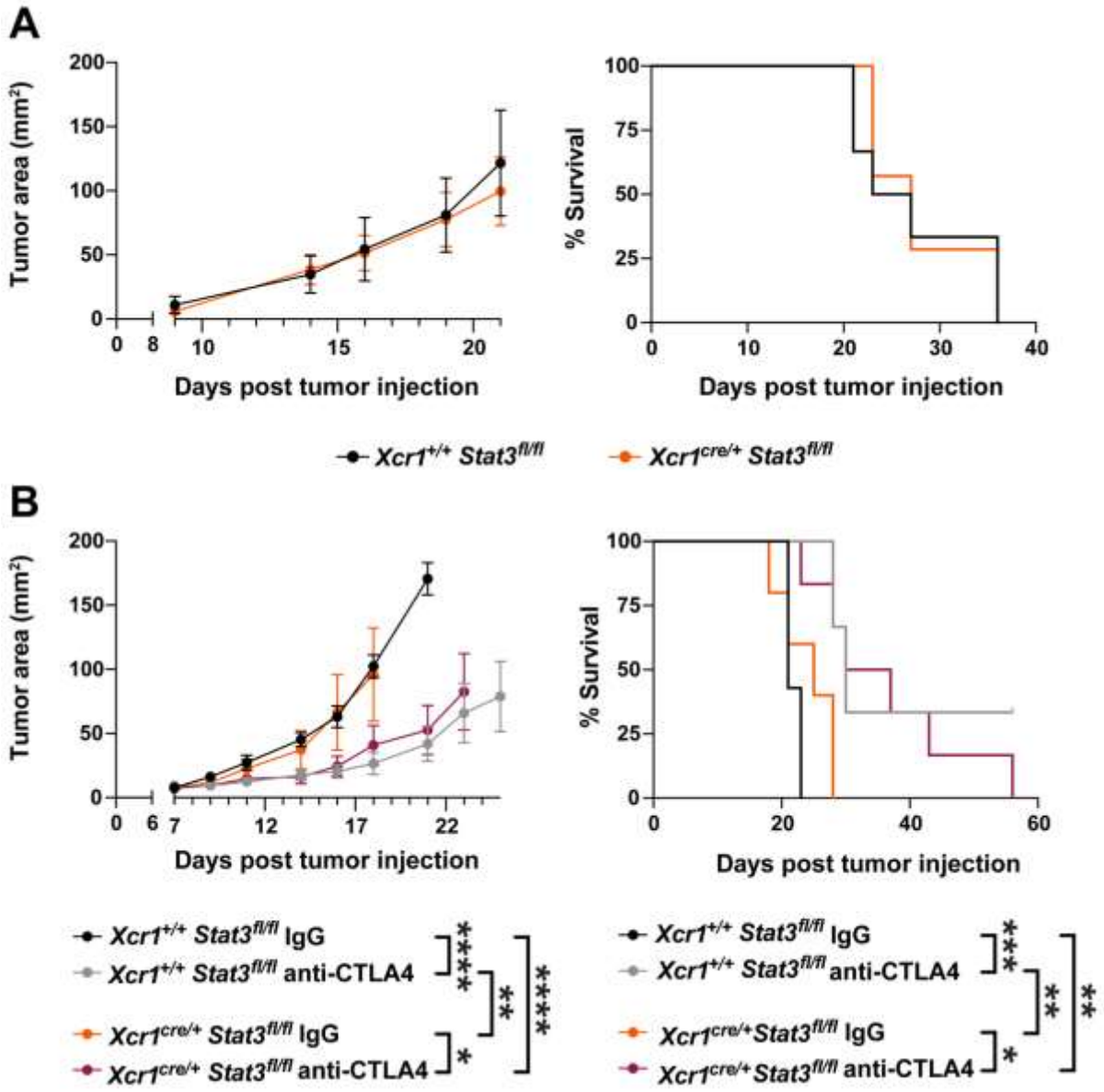

Figure 4.11. Selective depletion of STAT3 in cDC1s in vivo does not alter murine melanoma tumor growth. B16-OVA cells were injected subcutaneously into *Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$ and *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice and tumor growth and mouse survival were tracked over time. In some cases, mice were also treated i.p. with anti-CTLA4 or IgG 2-times weekly for the first 3 weeks of the experiment. (A, B) Tumor area (left) and mouse survival (right). (A) Data are from 1 experiment, n = 6 (*Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$), n = 7 (*Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$). (B) Data are from 1 experiment, n = 7 (*Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$ IgG), n = 6 (*Xcr1*$^{+/+}$ *Stat3*$^{fl/fl}$ anti-CTLA4, *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ anti-CTLA4), n = 5 (*Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ IgG). (A, B) Tumor area data (left) are shown as mean ± SEM and were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Survival data (right) were analyzed by log-rank (Mantel-Cox) test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, ∗∗∗∗ $p < 0.0001$.

4.3 – Discussion

We previously demonstrated that STAT3 mediates the inhibitory effect of IL-10 on poly I:C-induced maturation in cDC1s. Furthermore, both STAT3 and IL-10Rβ inhibited cDC1 vaccine efficacy in murine breast cancer. However, the mechanism through which STAT3 inhibited cDC1 function remained unknown. RNA-sequencing and bioinformatic analyses revealed that although poly I:C induced many inflammatory pathways in cDC1s, IFN-signaling was the primary target of STAT3-dependent inhibition elicited by IL-10. In vitro studies with cDC1s confirmed that poly I:C-induced IFN and ISG expression, at the transcript and protein level, were inhibited by concomitant treatment with IL-10 in a STAT3-dependent manner. Furthermore, poly I:C-induced ISG expression in cDC1s was dependent upon type I IFN signaling, while type II and type III IFNs were dispensable. Taken together, these data support that STAT3 inhibits poly I:C-induced type I IFN expression and downstream signaling in cDC1s, upon concomitant treatment with IL-10 (Figure 4.12).

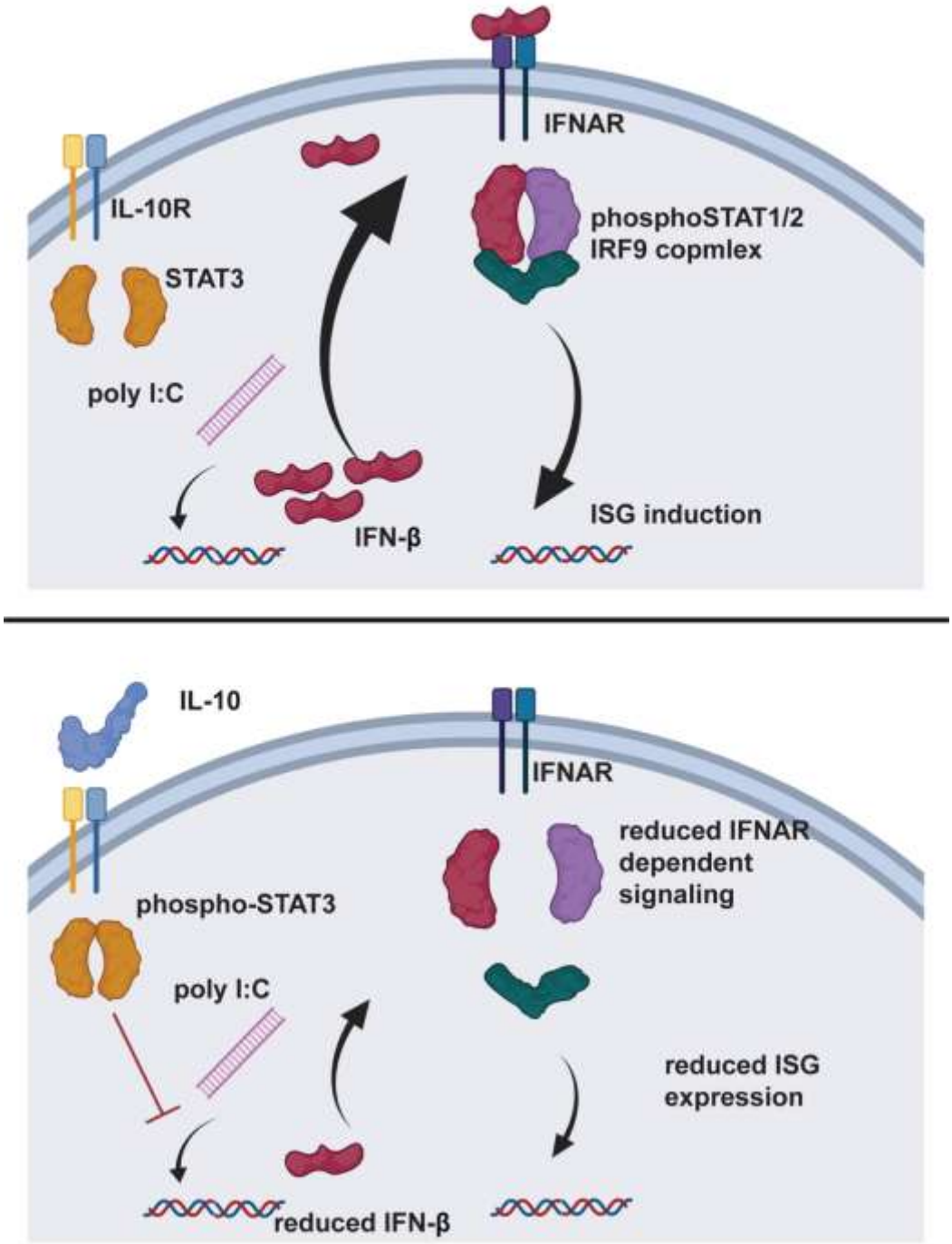

Figure 4.12. Schematic of the role of STAT3 in inhibiting poly I:C-induced type I IFN expression and signaling. Pictured top, treatment with poly I:C induces IFN-β which signals in autocrine fashion to induce ISG expression. Pictured bottom, treatment with IL-10 and poly I:C results in STAT3-dependent inhibition of IFN-β expression. Reduced IFN-β expression is correlated with decreased phospho-STAT1 accumulation and reduced ISG expression. Figure made using Biorender.com

Previously, our lab and others have described mechanisms of IL-10 and STAT3-mediated inhibition of inflammatory signaling in other immune cell populations [212, 214]. However, it has been shown that the anti-inflammatory transcriptional program induced by IL-10 differs based on cell-type [208]. Specifically, RNA-sequencing and bioinformatic analyses demonstrated that IL-10 selectively inhibits the NF-κB pathway in macrophages and the IRF and IFN pathway in splenic cDCs [208]. Our data expand on these findings by showing that in cDC1s specifically, STAT3 is critical for IL-10 – mediated inhibition of type I IFN expression and autocrine signaling. In keeping with the findings that demonstrate different functions for STAT3 depending on cell type, the expression of *Ube2n* which was found to be an important target of STAT3-mediated anti-inflammatory signaling in macrophages was not identified as a differentially expressed gene in our analysis of STAT3-dependent genes in cDC1s (data not shown) [214].

Although these data highlight differences in cDC1s versus other cell types regarding the role of the IL-10 and STAT3, there are also similarities. For example, STAT3 appears to be essential for mediating the anti-inflammatory role of IL-10 in most phagocytic cell populations [204, 223]. In addition, in an early report investigating TLR4-elicited signaling in peritoneal macrophages, lipopolysaccharide (LPS)-induced CXCL10 expression at the protein and transcript level was found to be inhibited by co-treatment with IL-10 [245]. Furthermore, IL-10 was unable to inhibit *Cxcl10* expression induced by direct treatment with IFN-β or IFN-γ, similar to our findings in cDC1s [245]. Taken together, these results suggest that in the context of concomitant exposure to IL-10 and a TLR agonist 1) there is induced a STAT3-

dependent negative regulator of IFN-β and other TLR agonist-induced genes, or 2) STAT3 itself acts as a negative regulator. Interestingly, evidence for the former has been clearly demonstrated in BMDMs and peritoneal macrophages [213, 246]. However, a negative regulator capable of mediating much of the inhibitory role of STAT3 in any cell type has yet to be described [132, 213, 246].

In corroboration with previous reports in other cell types, we found that CD80 and CD86 expression are differentially regulated [247, 248]. Specifically, cDC1s upregulated CD86, but not CD80, in response to IFN-β treatment. However, poly I:C-induced CD86 and CD80 expression were both *Ifnar1*-dependent. These data suggest that type I IFNs are necessary and sufficient for the induction of CD86, while CD80 induction relies on both type I IFNs and poly I:C-elicited signaling.

Others have shown that cDC1s require *Ifnar1* for effective induction of anti-tumor $CD8^+$ T cells and inhibition of tumor growth in B16 melanoma [110]. In addition, it was demonstrated that $CD11c^+$ cells are the main source of *Ifnb1* in this melanoma model [110]. Despite this, we did not observe any effect of selective STAT3-depletion in cDC1s on tumor growth or mouse survival using the B16 melanoma model. This may be because while cDC1s required *Ifnar1*, they were not the $CD11c^+$ population responsible for tumor-induced *Ifnb1* expression [110]. Furthermore, we demonstrated that STAT3 could not inhibit the downstream signaling induced by direct IFN-β treatment in cDC1s in vitro. Therefore, cDC1-STAT3 depletion may not have altered cDC1 function in B16 melanoma because other DC or myeloid populations are the main source of IFN-β.

Chapter 5 – General discussion and future directions

5.1 – Summary

cDC1s are an essential APC population, required for the induction of $CD8^+$ T cell-mediated immunity against pathogens and tumors [1]. However, much of the work attributing these roles to cDC1s has been done using mice that lack this subset altogether, or through correlating human cDC1 abundance with certain clinical outcomes, such as the overall survival of cancer patients [8, 98]. Therefore, the mechanisms that regulate cDC1 function remain largely unknown.

IL-10 is an anti-inflammatory cytokine that inhibits PAMP-elicited responses in most myeloid cell populations [132]. The role of this inhibitory signaling is clearly demonstrated by the fact that IL-10 – deficient mice develop chronic colon inflammation that mimics human inflammatory bowel disease [205, 249]. Chronic colitis occurs in IL-10 – deficient mice through a mechanism involving abnormally high levels of inflammatory cytokine and chemokine production in phagocytes upon sensing commensal microbiota [206, 207, 250]. Importantly, IL-10 is dependent upon STAT3 for the induction of this anti-inflammatory program [204, 223]. However, there appear to be cell type-specific roles IL-10 performs in order to enact anti-inflammatory signaling [208]. Therefore, now that cDC1s have been delineated as a distinct cDC subset, uniquely involved in activating $CD8^+$ T cell-mediated immunity, determining whether and how IL-10 and STAT3 regulate cDC1 function is warranted.

Herein, we demonstrated that IL-10 inhibits poly I:C-induced cDC1 maturation in a STAT3-dependent manner. Although poly I:C induced many inflammatory

pathways involved with IFNs and NF-κB, among others, type I IFN expression and signaling appeared to be the main target of STAT3-mediated inhibition. In addition, using a cell-based vaccination approach in murine breast cancer, STAT3 and IL-10Rβ were found to inhibit cDC1 vaccine-mediated control of tumor growth. Furthermore, only *Stat3*$^{\Delta/\Delta}$ cDC1s were capable of significantly increasing tumor antigen specific CD8^{+} T cell and Th1 cell amounts in the TME and TdLNs after vaccination. The increases in T cells correlated with increased CD86 expression on *Stat3*$^{\Delta/\Delta}$ cDC1s in the TME after vaccination, compared to control cDC1s. Taken together, these results indicate that IL-10 and STAT3 inhibit type I IFN-dependent cDC1 maturation and induction of T cell-mediated anti-tumor immunity.

5.2. – Determination of STAT3-dependent genes that inhibit *Ifnb1* expression

Our data demonstrate that IL-10 inhibits poly I:C-induced IFN-β and downstream ISG expression, in a STAT3-dependent manner. However, the exact mechanism employed by STAT3 to inhibit the type I IFN expression and signaling remains unknown. Previously, others have shown that IL-10 and STAT3 depend on de novo protein synthesis to inhibit *Ifnb1* and other inflammatory transcripts [245, 246]. Furthermore genome-wide analyses of STAT3 binding and gene expression reveal that STAT3 largely promotes gene expression [213]. Therefore, in cDC1s, STAT3 may quickly induce negative regulators of *Ifnb1* expression, in the presence of both IL-10 and poly I:C. RNA-sequencing and chromatin immunoprecipitation experiments at early timepoints after treatment of *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s treated with IL-10 and poly I:C will be essential for developing a list of candidate genes that could inhibit

poly I:C-induced *Ifnb1* expression. Additionally, STAT3 dependent genes downstream of IL-10 exposure alone could yield additional putative negative regulators of cDC1 function. Furthermore, it will be essential to determine the function of any identified negative regulator, as it could act as a 1) direct transcriptional repressor, 2) a chromatin modifier, or 3) a molecule that otherwise interferes with proinflammatory signaling proteins or transcription factors, either by promoting degradation or inhibitory post-translational modification.

5.3 – Novel roles for STAT3 in cDC1-mediated anti-tumor immunity

Although the inhibitory effect of STAT3 on cDC1 vaccination in the PyMT-OVA model was clearly demonstrated, no such role for STAT3 could be established in the B16-OVA model using *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice. Different tumor types display distinct TMEs with respect to the immune cell types that are recruited and the extracellular signals that are expressed [251]. Therefore, it is possible that there is less IL-10 in the B16-OVA TME compared to that of PyMT-OVA, and as a result there is less STAT3-mediated inhibition of cDC1 function. Furthermore, it is still possible that STAT3 did have a role in inhibiting cDC1 costimulatory marker expression, or T cell recruitment in the B16-OVA model, as these parameters were not assessed in the present study. However, these potential changes, if any, were clearly not enough to significantly alter tumor growth kinetics. In addition, the disparate results between the PyMT and B16 models could be due to the differing amounts of cDC1s in the TME. In the cDC1 vaccination experiments, supraphysiologic amounts of cDC1s were injected directly into the PyMT-OVA TME. In contrast, the experiments

performed using B16-OVA cells in *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice likely had far fewer cDC1s in the TME. Therefore, assessing the role of STAT3 in cDC1s using the vaccine system may provide more readily observable effects as compared to the same alteration in the native cDC1 population.

Interestingly, a recent report demonstrated that human cDC1s are the primary source of IFN-λ in the in the TME of breast cancer patients [101]. In addition, IFN-λ expression in the human breast TME correlated with relapse-free survival [101]. Furthermore, treatment of human tumor suspensions with IFN-λ induced expression of CXCL10 and IL-12p70 which support T cell mediated anti-tumor immunity [101]. Although our results demonstrate that mouse cDC1s do not respond to IFN-λ, *Ifnl2* expression was induced by poly I:C and inhibited by concomitant treatment with IL-10 in a STAT3-dependent manner. Therefore, the improved vaccine efficacy with *Stat3*$^{\Delta/\Delta}$ cDC1s could be due in part to increased *Ifnl2* expression. Similarly, IRF1 was recently found to be important for cDC1 maturation, ISG expression, and induction of anti-tumor immunity in the YUMM1.7 model of murine melanoma [252]. Although *Irf1* expression was not significantly altered between IL-10 and poly I:C-treated *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s, IRF1 was identified by IPA as a potential upstream regulator mediating the transcriptional differences between the two groups. Thus, STAT3 may inhibit cDC1-mediated anti-tumor immunity through inhibition of IRF1 activity.

Recently, it has become apparent that although cDC1s are the main cell type responsible for cross-presentation of tumor antigen to CD8$^+$ T cells, this function is not sufficient to induce the rejection of immunogenic tumors [73]. This was elegantly

demonstrated by rescuing *Batf3*$^{-/-}$ mice from cDC1-deficiency through forced overexpression of *Irf8*. Although the cDC1s from these mice could still cross-present antigen and activate CD8^{+} T cells, they were unable to successfully clear tumors that are normally rejected in a cDC1- and CD8^{+} T cell -dependent manner [73]. Therefore, cDC1s perform cross-presentation – independent functions that promote CD8^{+} T cell mediated immunity. Indeed, cDC1s were later found to be essential for the induction of tumor specific Th1 cells in the TME through direct presentation of tumor antigen on MHC II [92]. Activation and polarization of Th1 cells promoted Th1-mediated enhancement of cDC1 function through activating CD40 signaling in cDC1s [92]. Without both the activation of, and crosstalk with Th1 cells, cDC1s were unable to induce CD8^{+} T cell-mediated rejection of tumors. In addition, these findings in the TME corroborate earlier reports in murine virus infection models, that cDC1s induce and polarize Th1 cells, and furthermore, that cDC1s are required for the transmission of Th1 cell-dependent signals required for optimal CD8^{+} T cell function [82, 89, 90]. Interestingly, in the case of vaccinia virus infection, the formation of cDC1-Th1-CD8^{+} T cell tri-cell clusters were essential for full activation of CD8^{+} T cells [90]. Taken together, these results suggest that cDC1s not only induce both CD8^{+} T cells and Th1 cells, but they also act as a physical platform for the mediation of Th1 cell help of CD8^{+} T cell dependent immunity. Herein, it was demonstrated that STAT3 inhibited cDC1 vaccine induction of both tumor antigen specific CD8^{+} T cells, as well as Th1 cells. Therefore, it is conceivable that STAT3 prevents optimal formation of cDC1-dependent tri-cell clusters and, as a result, inhibits optimal T cell-dependent immunity in the TME. However, imaging studies and investigations into the role of

STAT3 in regulating cDC1 naïve T cell activation will be required to test this hypothesis. Furthermore, it will be important to determine the relative effect of STAT3 in cDC1 activation of naïve $CD8^+$ T cells versus naïve $CD4^+$ T cells. For example, STAT3-mediated inhibition of cDC1 function could have greater consequences for the activation and polarization of Th1 cells, and this, in turn, could inhibit cDC1 activation and optimal function of $CD8^+$ T cells indirectly. Future investigations into the specific role of STAT3 in cDC1-mediated activation of each T cell lineage individually, as well as together, will be important for determining the overall effect of STAT3 in the cDC1-induced adaptive immune response.

Moving forward, much remains to be determined regarding the role of STAT3 in cDC1s in the TME. *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice should provide useful for future studies in other tumor models and treatment contexts. For example, improving cDC1 abundance and activation in the TME with FLT3L and poly I:C or CD40 agonist has shown promise in controlling multiple murine tumor types [102, 106, 253]. In addition, poly I:C and FLT3L are being tested in human cancer patients in combination with radiotherapy or ICB [254]. Our results suggest that STAT3 activity in cDC1s may inhibit the efficacy of these therapies. Likewise, combination therapy with a STAT3 inhibitor has the potential to further improve the efficacy of these treatments. Furthermore, investigations into combining indirect STAT3 inhibition with ICB have led to multiple clinical trials [255]. Perhaps these therapies benefit from the improved cDC1 function in the TME described herein. Nonetheless, we were unable reveal in an inhibitory role for STAT3 in native cDC1s in the context of anti-CTLA4 therapy in murine melanoma. Previously, others have shown ICB requires IFN-γ – dependent signaling

in cDC1s. Therefore, STAT3 activity in cDC1s may not have inhibited anti-CTLA-4 therapy because IL-10 and STAT3 inhibited primarily IFN-β and not IFN-γ signaling in cDC1s. Taken together, many ongoing investigations into novel tumor immunotherapies are predicted or designed to involve the functions of cDC1s. Understanding whether and how STAT3 regulates cDC1 functions in these contexts will increase our knowledge of tumor immune regulation and potentially lead to new treatments for the immunotherapy of cancer.

5.4 – STAT3 and cDC1s in immune tolerance

Our results demonstrating the role for IL-10 and STAT3 in inhibiting cDC1 maturation and function in the TME support that this pathway is detrimental for the induction of anti-tumor immunity. However, others have described contexts where IL-10 mediated inhibition of DC function in the TME supports optimal T cell function [238]. Specifically, IL-10 – mediated inhibition of DC IFN-γ production prevented activation induced cell death of tumor infiltrating $CD8^+$ T cells. This suggests that IL-10, and by extension, STAT3-mediated inhibition of cDC1 activation, may be important for fine-tuning $CD8^+$ T cell activation. This regulatory response could be important for the resolution of inflammation and promotion of immune homeostasis at steady-state. STAT3 signaling in $CD11c^+$ cells is essential to prevent chronic colitis [223]. Furthermore, IL-10 is required to tolerate constant sensing of the gut microbiome by phagocytes in the colon without resulting in chronic inflammation [206, 207, 250]. Therefore, it is possible that cDC1s in the colon sense microbial products and have their maturation inhibited by IL-10 and STAT3, in order to prevent inappropriate

activation. However, future studies utilizing *Xcr1*$^{cre/+}$ *Stat3*$^{fl/fl}$ mice to delineate the role of cDC1s in the colon are required to determine the relative contribution of STAT3-mediated immunosuppression in this cell type.

Previously, our lab and others have demonstrated STAT3-dependent mechanisms of immunosuppression in other myeloid cells [212, 214]. For example, in macrophages, STAT3 activity downstream of LPS-induced autocrine IL-6 signaling inhibits UBC13 (encoded by *Ube2n*) expression, an important mediator of NF-κB activity [214]. Although poly I:C induced abundant IL-6 expression in cDC1s, our data do not support that STAT3-deficient cDC1s responded differently to poly I:C exposure, as assessed by transcriptome analyses, surface marker expression, or cytokine secretion, suggesting autocrine IL-6 may not play an inhibitory role in poly I:C-exposed cDC1s. Furthermore, *Ube2n* was not identified as a STAT3 target gene by RNA-sequencing when comparing STAT3-sufficient to -deficient cDC1s (data not shown). Likewise, *Sbno2* and *Etv3*, STAT3-dependent immunosuppressive factors induced by IL-10 in macrophages were not differentially expressed when comparing *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s in our RNA-sequencing analyses (data not shown) [212]. Taken together, our data are consistent with the notion that IL-10 and STAT3-mediated immunosuppression utilizes cell type-specific mechanisms, as has been previously reported [208].

In addition to suppressing the inflammatory activities of cDC1s, STAT3 may be involved with how cDC1s actively promote immune tolerance. For example, at steady-state, cDC1s undergo homeostatic maturation which promotes the induction of self-antigen specific Treg cells, as well as the cross-tolerance of $CD8^+$ T cells [93-95].

These active tolerance mechanisms are distinct from mechanisms that restrain inflammatory signaling, as we have described with IL-10 and STAT3. Interestingly, it has been demonstrated that the ability of steady-state DCs to tolerize $CD8^+$ T cells is dependent upon MHC II-dependent interactions with Treg cells [64, 65, 94]. Therefore, cDC1s may act as platforms not only for the transmission of Th1 cell-dependent signals for the enhancement of $CD8^+$ T cell function, but also Treg cell-dependent signals which inhibit the function of $CD8^+$ T cells. Further study into whether and how STAT3 regulates cDC1 homeostatic maturation, activation of Treg cells, and cross-tolerance of $CD8^+$ T cells is warranted. The results of these studies will be essential in aiding our understanding of how cDC1s promote immune homeostasis.

Appendix

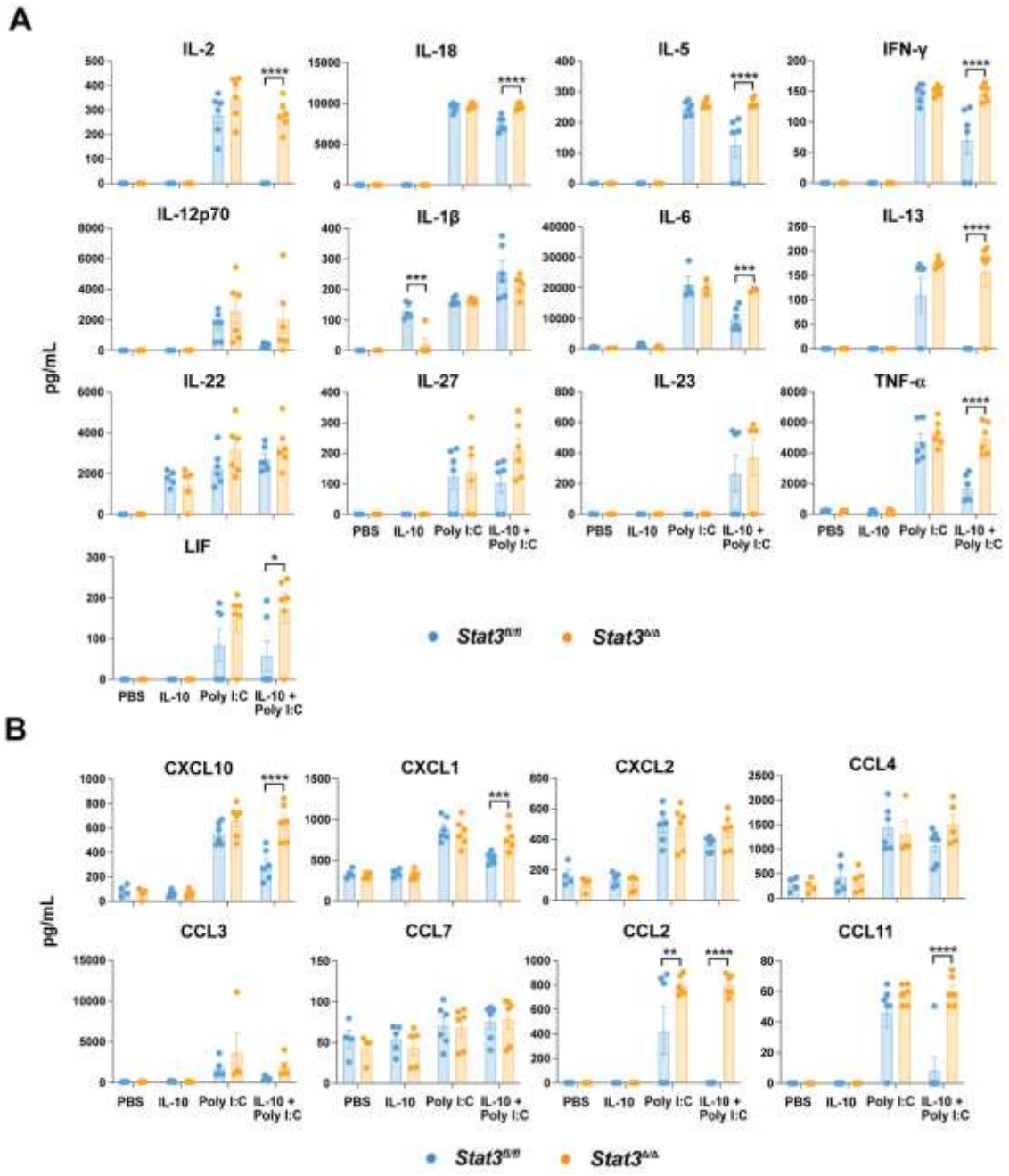

Figure A1. Graphs of individual analytes originally described in Figure 3.3B. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C for 16 hours. Supernatant cytokine (A) and chemokine (B) abundance were determined by Luminex multiplex assay. Data are shown as the mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, ∗∗∗∗ $p < 0.0001$.

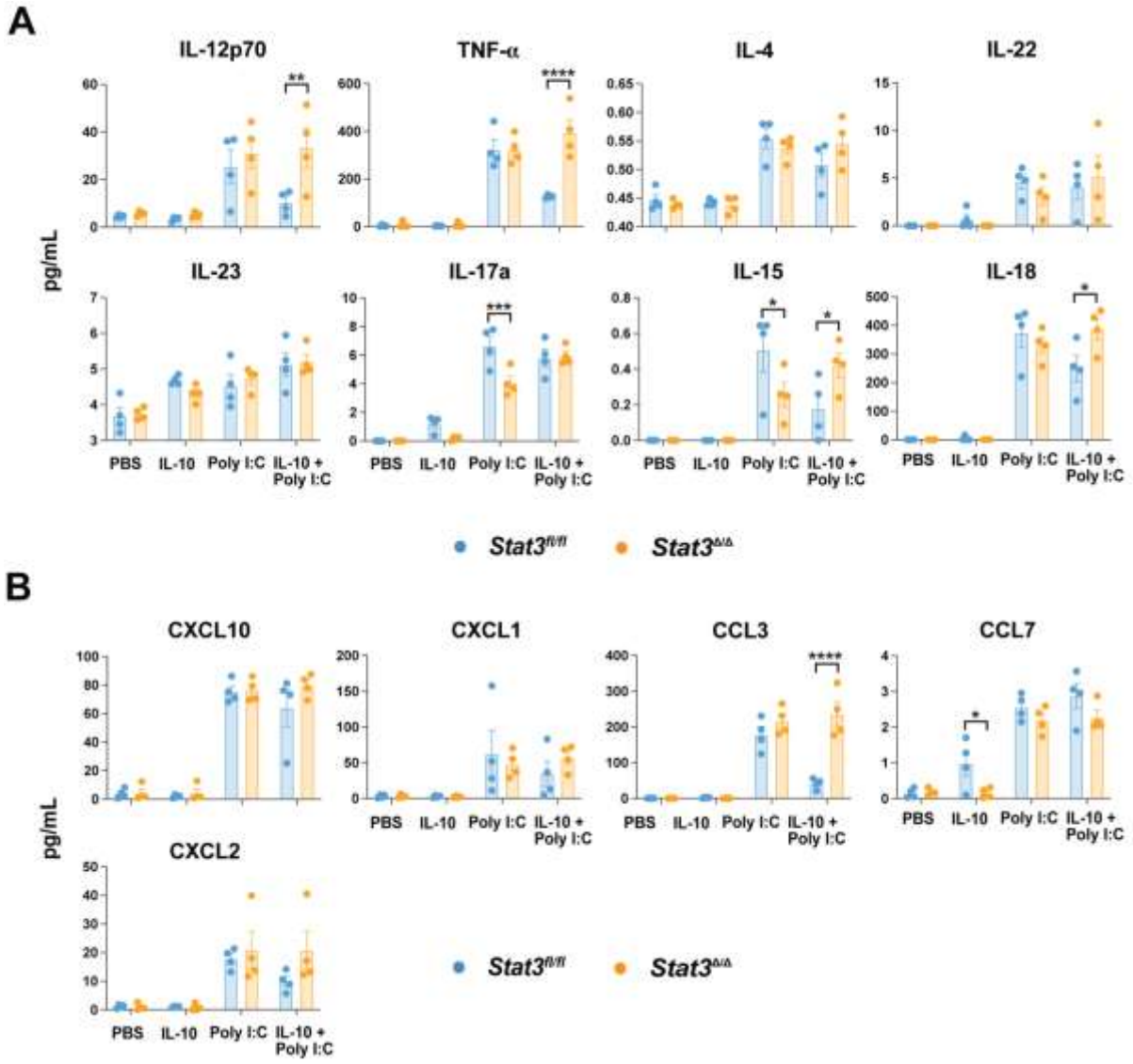

Figure A2. Graphs of individual analytes originally described in Figures 4.5C and 4.5D. *Stat3*$^{fl/fl}$ and *Stat3*$^{\Delta/\Delta}$ cDC1s were treated with PBS, IL-10, poly I:C, or IL-10 and poly I:C for 6 hours. Supernatant cytokine (A) and chemokine (B) abundance were determined by Luminex multiplex assay. Data are shown as the mean ± SEM. Each symbol represents an individual biological replicate. Data were analyzed by two-way ANOVA and Bonferroni's multiple comparison test. Results were considered significant when $p < 0.05$. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$, ∗∗∗∗ $p < 0.0001$.

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Vita

Taylor Thomas Chrisikos was born to Anthony Chrisikos and Deborah Chrisikos. In 2010 he graduated high school at the Christian Brothers Academy in Albany, New York. In 2014 he received his B.S. in Pharmacology and Toxicology from the State University of New York at Buffalo. In 2015 he entered the UT MD Anderson UTHealth Graduate School of Biomedical Sciences, and in 2016 joined the Program of Immunology under the supervision of Dr. Stephanie Watowich.