MANIPULATION OF REGULATORY MYELOID CELL DIFFERENTIATION AND FUNCTION AND ITS THERAPEUTIC IMPLICATIONS

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Organ transplantation is the definitive treatment for end-stage organ diseases that are otherwise untreatable. Modern immunosuppressants have largely subverted acute allograft rejection through non-specific inhibition of T cell activation and proliferation; however, chronic immunosuppression with current therapies carries increased risks of malignancy, infection and drug-associated toxicities. Furthermore, rates of long-term graft failure, mediated at least in part by chronic alloimmune responses, have not improved significantly. New therapies to modify the immune system and promote long-term graft survival are urgently needed.

Myeloid lineage antigen-presenting cells, including dendritic cells (DC) and myeloid-derived suppressor cells (MDSC), are innate immune cells that regulate antigen-specific immunity and are valuable targets to promote immune modulation. In this dissertation, I present data on three pathways that can be modulated to promote expansion and immunosuppressive function of MDSC or downregulate DC immunostimulatory function. Histone deacetylase inhibitors (HDACi) are anti-neoplastic agents that promote tumor cell growth arrest and apoptosis. Recent findings describe novel anti-inflammatory properties. The data presented demonstrate that HDACi promote GM-CSF-mediated MDSC expansion, while impeding DC differentiation and co-stimulatory molecule expression. In addition to modulation of the GM-CSF pathway, new data presented in this thesis demonstrate that the DC-poietin Flt3 ligand (Flt3L) also promotes expansion and activation of MDSC capable of delaying allograft rejection when transferred to heart transplant recipients. STAT3 is a pivotal regulator of Flt3L-driven

myeloid cell expansion as STAT3 inhibition blocks expansion of immunostimulatory DC but further promotes MDSC expansion, without altering their suppressive capacity. Lastly, the immunoregulatory role of newly-described rapamycin-resistant outputs of the mammalian/mechanistic Target of Rapamycin (mTOR) in DC was examined. A novel mTOR signaling pathway that negatively regulates DC expression of anti-inflammatory IL-10 and B7-H1 is described.

In summary, these data describe growth factor and signaling pathways that can be manipulated for the promotion of MDSC expansion and inhibition of DC stimulatory function. Future studies will be required to translate these findings into relevant organ transplantation models and develop their therapeutic potential. Manipulation of myeloid cell development and function is an innovative approach to immune modulation that may lead to new immunosuppressive strategies.

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PREFACE

This dissertation is dedicated to my family and friends who have stood by me throughout the long journey of graduate school. Special thanks is due to my graduate school mentor, Angus Thomson, who was always encouraging no matter the circumstances and pushed me to achieve more than I could have imagined. I am grateful for the support and guidance of Heth Turnquist, who trained me when I started in lab with no immunology experience and has been a valuable mentor ever since. I am truly appreciative of all the friends I have made during my time in lab.

1.0 INTRODUCTION

The mammalian immune system is composed of two distinct, but overlapping, arms termed innate and adaptive, which function in concert to eliminate foreign pathogens. Innate immune cells are the earliest responders to inflammatory insults and include granulocytes (i.e. neutrophils, basophils and eosinophils), macrophages and dendritic cells (DC), while adaptive immune responses are mediated by antigen-specific lymphocytes including B cells and T cells. Innate immune cells are principally composed of myeloid-lineage cells, while lymphoid cells mediate adaptive immunity with the exception of lymphoid-derived innate natural killer (NK) cells. Immunosuppressive agents classically target the effector phase of immunity by impeding T cell function to dampen unwanted inflammatory responses to self (autoimmunity) or immunologic non-self (alloimmunity/transplantation). However, it is becoming increasingly recognized that cells of myeloid origin represent valuable targets in modulating immunity due to their ability to prevent or suppress development of an adaptive immune response. Myeloid-derived suppressor cells (MDSC) are a heterogeneous group of early myeloid progenitor cells that suppress immunity, while DC are fully differentiated myeloid cells at the interface of innate and adaptive immunity that initiate and regulate T cell responses. Understanding the biology of MDSC and DC development allows for opportunities to intervene and suppress unwanted adaptive immune responses.

1.1 MYELOID-DERIVED SUPPRESSOR CELLS ARE INNATE REGULATORS OF IMMUNITY

Myeloid-derived suppressor cell (MDSC) is a new term (1) for a population of myeloid cells that have been recognized to suppress immunity since the 1980's (2-5). There has been a recent resurgence and appreciation of the importance of these non-T and non-NK suppressive cells in the context of immune suppression associated with malignancy. MDSC can represent upwards of 20-40% of nucleated splenocytes in murine tumor models or be elevated at least 5-fold in the peripheral blood of cancer patients (6-8). Aside from cancer, MDSC are found in states of acute and chronic inflammation such as autoimmune uveoretinitis (9), experimental autoimmune encephalomyelitis (10, 11), chronic contact eczema (12), inflammatory bowel disease (13), sepsis (14), viral infection (15) and trauma (16). Additionally, MDSC expand following immunization with complete Freund's adjuvant (17) or vaccinia virus encoding IL-2 (18), suggesting that they may constitute a normal physiologic response to inflammation (19). Thus, enhancing our understanding of MDSC biology will advance our ability to exploit these cells for treatment of inflammatory diseases (20).

1.1.1 Origin and characterization of MDSC

MDSC arise from the bone marrow (BM) under conditions of acute or chronic inflammation and comprise a population of immature myeloid cells and myeloid progenitor cells that are unable to undergo complete differentiation and instead acquire suppressive activity (6). In mice and humans, the phenotype of MDSC is highly heterogeneous, but they are defined by their myeloid origin and suppressive function. Mouse MDSC are generally defined as $CD11b^{+}Gr1^{+}$ (6) but can

express IL-4Rα (CD124) (21), colony stimulating factor 1 receptor (CSF1R, M-CSF receptor, CD115) (22, 23), low/intermediate levels of the macrophage marker F4/80 (24, 25) and the co-stimulatory molecules CD40 (26) and CD80 (B7.1) (27). The phenotype of MDSC in humans is less well-defined compared to murine MDSC. In general, human MDSC are defined as $CD33^{+}HLA\text{-}DR^{-/low}$. They typically express the myeloid marker CD11b but not the lineage markers CD3 (T cells), CD19 (B cells) or CD56 (NK cells) (28).

1.1.2 Granulocytic and monocytic MDSC subsets

More recently, MDSC have been recognized to be composed of two populations based on their morphology and further delineation of their surface antigen expression (29, 30). Granulocytic (also known as polymorphonuclear) and monocytic MDSC are morphologically similar to normal granulocytes and monocytes, respectively, but are functionally distinct due to their ability to suppress immune responses. In the mouse, the Gr1 antibody identifies two populations of cells. $Gr1^{hi}$ cells consist mainly of neutrophils while $Gr1^{int/low}$ cells are predominantly immature myeloid cells (31). Some groups have identified MDSC subsets based on their level of Gr1 expression (32, 33); however, the RB6-8C5 Gr1 antibody clone binds a common epitope shared by the surface antigens Ly6G and Ly6C. Granulocytic MDSC are $CD11b^{+}Ly6G^{+}Ly6C^{-/low}$ while monocytic MDSC are $CD11b^{+}Ly6G^{-}Ly6C^{+}$ (29). Furthermore, granulocytic ($CD49d^{-}$) and monocytic ($CD49d^{+}$) MDSC can be distinguished based on expression of the α4 integrin subunit CD49d (34).

Humans don't have an analogous Gr1 antigen, so characterization of granulocytic and monocytic MDSC subsets is more difficult. Monocytic MDSC are $CD14^{+}$ (35) while granulocytic MDSC are positive for the human granulocyte markers CD15 and CD66b (28, 36).

Beyond morphologic and phenotypic characterization, granulocytic and monocytic MDSC have functionally distinct mechanisms of T cell suppression, which will be described in further detail below. The phenotypes of mouse and human MDSC are summarized in Table 1.1.

Table 1.1 Granulocytic and monocytic MDSC have distinct phenotypes in mice and humans

Species	Granulocytic MDSC	Monocytic MDSC
Mouse	$CD11b^{+}Gr1^{hi}Ly6G^{+}Ly6C^{-/low}CD49d^{-}$	$CD11b^{+}Gr1^{int/low}Ly6G^{-}Ly6C^{+}CD49d^{+}$
Human	$Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}CD14^{-}$ $CD15^{+}CD66b^{+}$	$Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}CD14^{+}CD15^{-}$ $CD66b^{-}$

1.1.3 Regulation of MDSC expansion

Under conditions of normal myelopoiesis, hematopoietic stem cells undergo successive differentiation into increasingly restricted progenitor cells that finally give rise to terminally differentiated macrophages, DC, granulocytes, megakaryocytes and mast cells (Figure 1.1). For reasons that are not well understood, under conditions of inflammation, immature myeloid cells and myeloid progenitors cannot undergo full differentiation into mature myeloid cells and are activated to express immunoregulatory factors. This situation is particularly well-described in the setting of cancer (6, 7).

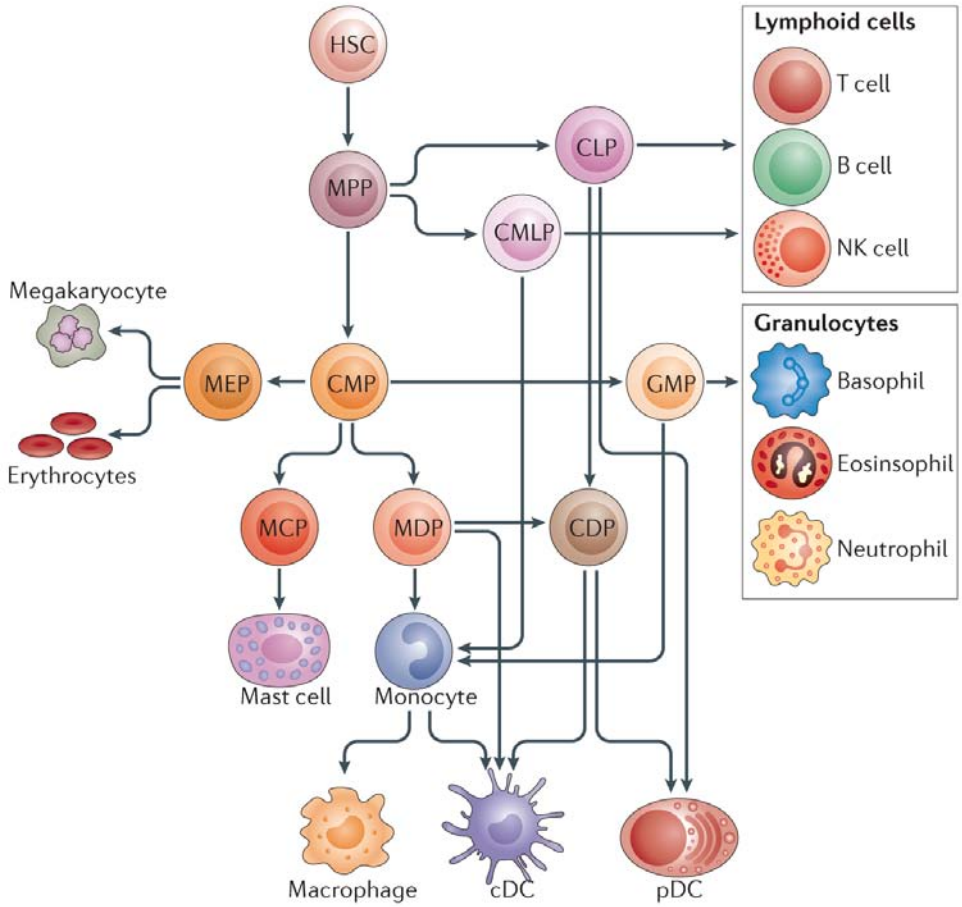

Figure 1.1 Myeloid cell differentiation under normal physiological conditions

Myeloid cells originate from haematopoietic stem cells (HSCs) and multipotent progenitor cells (MPPs). The figure illustrates the network of progenitor cells that gives rise to the various haematopoietic cell lineages. cDC, conventional DC; CDP, common DC progenitor; CLP, common lymphoid progenitor; CMLP, common myelolymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; GMP, granulocyte and macrophage progenitor; MCP, mast cell progenitor; MDP, macrophage and DC progenitor; MEP, megakaryocyte and erythroid progenitor; NK, natural killer; pDC, plasmacytoid DC. From Gabrilovich DI, Ostrand-Rosenberg S and Bronte V. *Nat Rev Immunol*. 2012;12(4):253-68. (7)

MDSC expansion is driven by cytokines and growth factors released by tumor cells and BM stromal cells in response to inflammation that promote myelopoiesis (Figure 1.2). Stem cell factor (SCF) (36), granulocyte-colony stimulating factor (G-CSF) (37), macrophage-colony stimulating factor (M-CSF) (38) and granulocyte macrophage-colony stimulating factor (GM-CSF) (39-41) are all produced by tumors and drive the expansion of MDSC. Factors mediating

the expansion of MDSC in non-malignant inflammation are comparatively understudied. However, colony stimulating factors, including G-CSF, M-CSF and GM-CSF, are involved in the pathogenesis of autoimmune diseases (42), suggesting that these growth factors may also mediate expansion of MDSC in inflammatory settings other than cancer. In addition to these myelopoiesis-promoting growth factors, cytokines such as IL-1β (43) and IL-6 (44), prostaglandins (45, 46) and vascular endothelial growth factor (VEGF) (47, 48) have been reported to expand MDSC. While this is not an exhaustive list of factors involved in MDSC expansion, it highlights the diversity of factors implicated in MDSC expansion.

Many of the factors that promote the expansion of MDSC activate signal transducer and activator of transcription (STAT) 3. This transcription factor is considered the major regulator of MDSC expansion, due to its ability to promote survival and proliferation of myeloid cells, while preventing their differentiation through expression of pro-survival and cell cycle regulators, such as B cell lymphoma-extra large (Bcl-xL), c-myc, cyclin D1 or survivin (6, 49). Addition of tumor-conditioned medium to BM DC cultures supplemented with GM-CSF and IL-4 enhances STAT3 activation and promotes immature myeloid cell proliferation while blocking DC differentiation (50). A similar state of STAT3 hyperactivity is seen in human MDSC where $CD14^{+}HLA\text{-}DR^{-}$ MDSC in the peripheral blood of melanoma patients demonstrate activated STAT3 (51). Inhibition of STAT3 in tumor-bearing mice reduces the incidence of MDSC while promoting DC differentiation (52), and genetic ablation of STAT3 in hematopoietic cells in tumor-bearing mice enhances DC function (53).

The mechanisms by which STAT3 promotes MDSC expansion are multifactorial and not fully understood. STAT3 activation promotes the expression of S100 calcium binding proteins, S100A8 and S100A9, which prevent differentiation of myeloid progenitors into DC and

macrophages and result in MDSC expansion in tumor-bearing mice (54). The mechanism by which S100 proteins promote MDSC expansion is not known (49). STAT3 activation additionally reduces protein kinase C (PKC) βII expression, which prevents DC progenitors from differentiating into DC (55). CCAAT-enhancer-binding proteins (C/EBP) are a family of transcription factors that are involved in granulopoiesis. C/EBPα is required for steady-state granulopoiesis, while C/EBPβ is necessary for "emergency," or demand-driven, granulopoiesis following infection (56). Interestingly, C/EBPβ mRNA transcription is upregulated in granulocyte and macrophage progenitors by G-CSF or GM-CSF *in vivo* (56). G-CSF acts through STAT3 to upregulate C/EBPβ, and STAT3 and C/EBPβ regulate the cell cycle protein c-myc (57), which is required in the hematopoietic lineage for MDSC expansion in tumor-bearing mice (58). The Gr^{hi} subset, which is typically associated with the granulocytic subset of MDSC, is more sensitive than the $Gr1^{int}$ (monocytic) subset of MDSC to hemizygous ablation of C/EBPβ (58). In the absence of C/EBPβ, there is an increase in DC and macrophages in spleens of tumor-bearing mice (58) suggesting that immature myeloid cells can undergo normal differentiation in cancer.

Aside from STAT3, several other pathways are involved in MDSC expansion. Tumor-derived supernatants stimulate extracellular-signal-related kinase (Erk), at least in part due to the presence of GM-CSF, to expand MDSC (59). MDSC expanded by polymicrobial sepsis required the Toll-like receptor (TLR) signaling adaptor molecule myeloid differentiation primary response gene 88 (MyD88), but this is not mediated through TLR4 (14). Alternatively, trauma-induced MDSC require STAT6 for expansion (60), and STAT5 is involved in MDSC survival (49, 61). Lastly, prostaglandins and downstream cyclooxygenase (COX) 2 are emerging as important regulators of MDSC expansion. Obermajer et al (62) have demonstrated that

prostaglandin E2 (PGE2) prevents DC differentiation in human peripheral blood mononuclear cell (PBMC) cultures supplemented with GM-CSF and IL-4, resulting in expansion of MDSC through activation of COX2. Other factors that also stimulate COX2 have similar effects, including lipopolysaccharide (LPS; a TLR4 agonist) and IL-1β (62). Similar results have been reported in mouse BM cell cultures, and tumor-bearing mice lacking the PGE2 receptor have reduced MDSC expansion (45). It is clear from the current literature that MDSC expansion is multifactorial and encompasses signaling pathways primarily including STAT3 but also other STAT family members, mitogen-activated protein kinases (e.g. Erk) and COX2. In addition, the primary signaling pathway responsible for MDSC expansion may depend on the specific model system, where different soluble factors predominating in the model dictate the primary signaling pathway required for expansion.

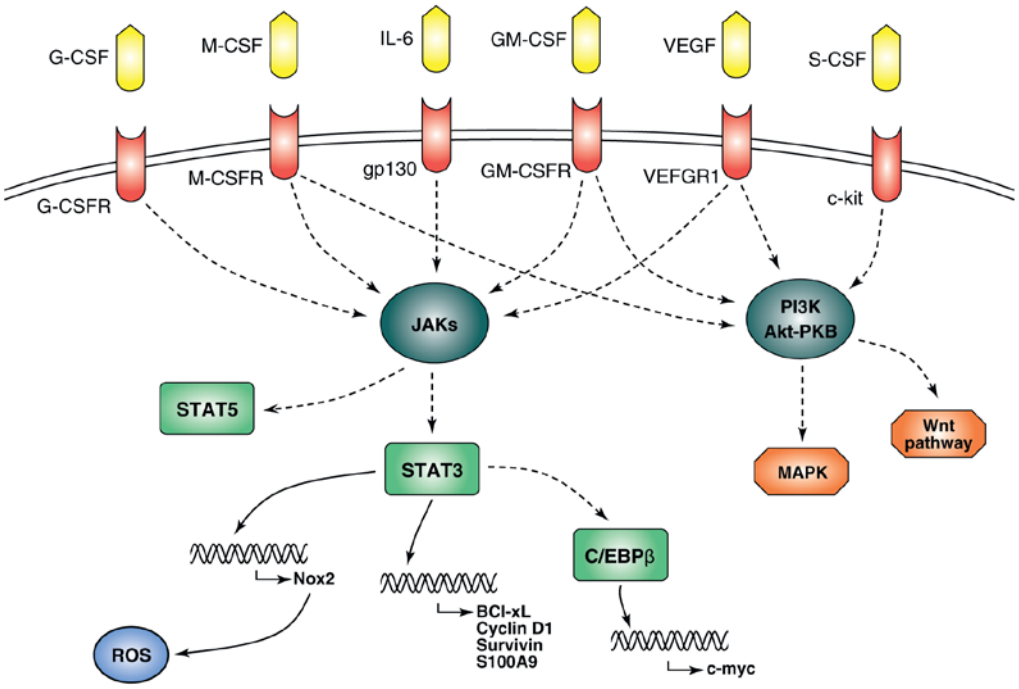

Figure 1.2 Schematics of possible signaling pathways involved in MDSC expansion

Various cytokines produced by tumors or bone marrow stroma in response to chronic infections or inflammation activate several signal transduction pathways that result in activation of Stat3 and Stat5. Stat3 regulates transcription of subunits of Nox2 that results in increased production of ROS [reactive oxygen species], as well as upregulation of a number of anti-apoptotic proteins and possibly CEBPβ that, in turn, up-regulate c-myc. Altogether, these proteins contribute to the proliferation and survival of immature myeloid cells and prevent their differentiation to mature cells. This is manifest in expansion of cells with the MDSC phenotype. From Condamine T and Gabrilovich DI. *Trends Immunol*. 2011:32(1):19-25. (49)

1.1.4 Suppressive mechanisms of MDSC and signals for their activation

It is currently believed that MDSC require factors that not only induce their expansion but also factors that activate their suppressive function (6). MDSC employ numerous mechanisms to suppress T cell proliferation including amino acid depletion, modulation of oxidative stress and secretion of immunosuppressive cytokines (Figure 1.3). L-arginine is a substrate of arginase 1 and inducible nitric oxide synthase (iNOS; NOS2), which are expressed by MDSC. Depletion of L-arginine reduces mRNA and protein expression of the T cell receptor (TCR) signaling complex ζ chain, resulting in reduced T cell proliferation (63, 64). Several studies have

demonstrated reduction of the ζ chain by MDSC (12, 64, 65). IL-4 (60, 66) and IL-13 (60, 64) induce MDSC arginase 1 expression downstream of IL-4 receptor α (IL-4Rα) (67), and STAT6 is required for this activation of arginase 1 (60, 67). Additionally, PGE2 signals through COX2 to promote MDSC arginase 1 expression (68).

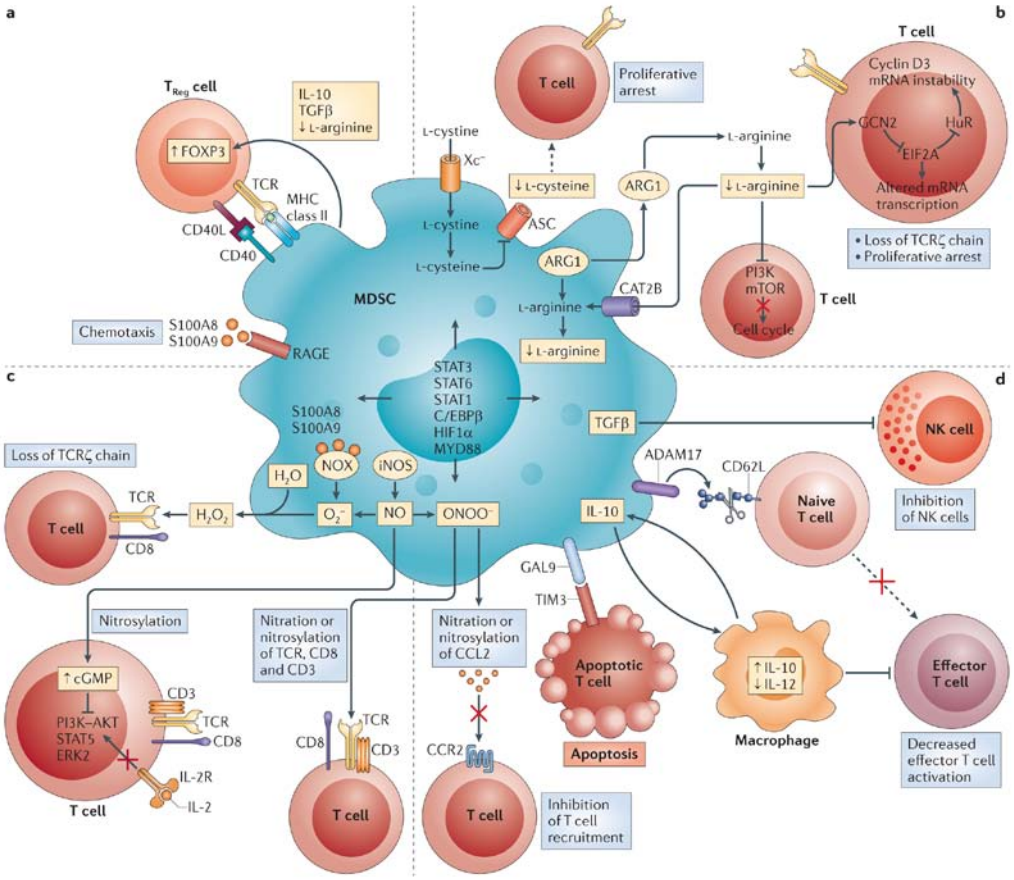

Figure 1.3 Mechanisms of MDSC-dependent inhibition of T cell activation and proliferation

Myeloid-derived suppressor cells (MDSC) can inhibit efficient antitumour T cell responses through a number of mechanisms. (A) Tumour-associated MDSCs induce the development of regulatory T (Treg) cells or expand existing Treg cell populations. The calcium-binding proteins S100A8 and S100A9 are involved in the chemotaxis of MDSCs and other myeloid cells; these effects are mediated in part through the activation of receptor for advanced glycation end-products (RAGE). At the same time, S100A8 and S100A9 along with gp91phox are part of the NADPH oxidase (NOX) complex that is responsible for the increased production of reactive oxygen species (ROS) by MDSCs. (B) Tumour-associated myeloid cells deprive T cells of amino acids that are essential for their growth and differentiation. (C) Tumour-associated myeloid cells release oxidizing molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Peroxynitrite causes nitration and nitrosylation of components of the T cell receptor (TCR) signaling complex, and H_2O_2 causes the loss of the TCR ζ-chain, thereby inhibiting T cell activation through the TCR. (D) Tumour-associated myeloid cells can also interfere with T cell migration and viability. The metalloproteinase ADAM17 (disintegrin and metalloproteinase domain-containing protein 17) cleaves CD62L, which is necessary for T cell migration to draining lymph nodes, and galectin 9 (GAL9) can engage T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on T cells to induce apoptosis. As the induction of the immunosuppressive pathways that are depicted in the figure is regulated by common transcription factors, these pathways can operate in more than one myeloid cell type. ARG1, arginase 1; ASC, asc-type amino acid transporter; CAT2B, cationic amino acid transporter 2 isoform 1 (L-arginine transporter); CCL2, CC-chemokine ligand 2; CCR2, CC-chemokine receptor 2; C/EBPβ, CCAAT/enhancer-binding protein-β; EIF2A, eukaryotic translation initiation factor 2A; ERK2, extracellular signal-regulated kinase 2; FOXP3, forkhead box P3; HIF1α, hypoxia-inducible factor 1α; HuR, Hu-antigen R (also known as ELAVL1); IL, interleukin; IL-2R, IL-2 receptor; iNOS, inducible nitric oxide synthase; mTOR, mammalian target of rapamycin; MYD88, myeloid differentiation primary-response protein 88; NK, natural killer; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; Xc^-, cystine-glutamate transporter. From Gabrilovich DI, Ostrand-Rosenberg S and Bronte V. *Nat Rev Immunol*. 2012;12(4):253-68. (7)

iNOS expressed by MDSC produces nitric oxide (NO), which in turns reacts with superoxide and other reactive oxygen species (ROS) produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to create reactive nitrogen species (RNS), including peroxynitrite. MDSC expression of NADPH oxidase subunits $p47^{phox}$ and $gp91^{phox}$ is regulated by STAT3 (69). Chalmin et al (59) have demonstrated that STAT3 is required for activation of MDSC suppressive activity downstream of TLR2 and MyD88 when stimulated with tumor-derived exosomes containing heat shock protein 72 (Hsp72). Production of NO by iNOS is stimulated by IFNϒ activation of STAT1 (10, 30, 66, 70). C/EBPβ has been shown to regulate both arginase 1 and iNOS expression by MDSC (58). Interestingly, the complement anaphylotoxin C5a stimulates MDSC to increase ROS and RNS production (71). NO inhibits IL-2 receptor signaling in T cells (72) and induces T cell apoptosis (10). RNS in the tumor microenvironment, presumably released by MDSC, result in chemokine C-C motif ligand 2 (CCL2) nitration and reduction of T cell recruitment (73). Furthermore, nitration of tyrosines in the TCR-CD8 complex by MDSC prevents $CD8^+$ T cells from binding to cognate peptide-major histocompatibility complex (MHC) complexes on antigen-presenting cells (APC) (74, 75). Substrate-specific mechanisms of suppression may exist for granulocytic and monocytic MDSC. Granulocytic MDSC preferentially produce high levels of superoxide and ROS but low levels of NO, and monocytic MDSC make large amounts of NO but relatively low levels of ROS (29, 30, 76). However, this demarcation may not always hold true, as granulocytic MDSC expressed iNOS in a *Trypanosoma cruzi* infection model (70).

In addition to arginase 1, iNOS and NADPH oxidase, several other immunomodulatory mechanisms have been described for MDSC. Endotoxin-induced MDSC inhibit T cell proliferation by a heme oxygenase-1 (HO-1)-dependent mechanism (77). Expression of the

costimulatory molecules CD40 (26) and CD80 (27) and the coinhibitory molecule B7-H1 (78) have all been implicated in MDSC-mediated immune regulation through promotion of Treg development and/or function. MDSC have also been reported to secrete the immunoregulatory cytokines IL-10 and transforming growth factor β (TGFβ) (22, 62).

Significant progress has been made identifying the factors and molecular mechanisms responsible for the expansion and activation of MDSC. There is overlap between growth factors and cytokines that expand MDSC and those that activate their suppressive program. Growth factors such as GM-CSF, M-CSF and G-CSF appear to expand MDSC predominantly through STAT3. Inflammatory cytokines including IFNϒ, IL-4 and IL-13 activate MDSC suppressive factors through STAT1 and STAT6. Further study is needed to understand the context in which these generalizations are accurate and to understand signals that block the differentiation of MDSC into fully differentiated myeloid cells.

1.1.5 MDSC regulation of T cells can be antigen-specific or antigen non-specific

The antigen specificity of MDSC appears to be highly dependent on the model, microenvironment and level of activation of target lymphocytes (79). Due to the lack of general immune suppression in cancer patients and the ability of peripheral T cells to respond to non-tumor-associated antigens in tumor-bearing mice, it has been proposed that MDSC are capable of antigen-specific regulation (6, 79). MDSC are capable of inhibiting both $CD4^+$ and $CD8^+$ T cell responses (9, 10, 12, 30, 80). While both granulocytic and monocytic MDSC can suppress antigen-specific $CD8^+$ T cell responses through MDSC subset-specific mechanisms (30), it is not known whether MDSC are capable of antigen-specific $CD4^+$ T cell suppression (6) especially in view of their low or absent expression of MHC class II (19). Of particular importance for

potential cell therapies utilizing these cells, MDSC generated *in vitro* can promote Ag-specific T cell hyporesponsiveness (58). Since tumor-infiltrating human MDSC, but not peripheral blood MDSC, suppress mitogen-activated T cell proliferation non-specifically (81), it has been proposed that antigen-specific regulation by MDSC occurs in peripheral lymphoid organs, while non-specific suppression occurs within the tumor microenvironment (6). In a mouse model of cardiac allograft tolerance induced by donor splenocyte transfusion (DST) and anti-CD40L, suppression of T cells by graft-infiltrating MDSC was non-specific, while BM and splenic monocytes did not suppress (23). Taken together, these findings support the paradigm that MDSC can regulate antigen-specific T cells, but may suppress T cells non-specifically within an allograft.

1.1.6 MDSC in transplantation

The field of tumor immunology has driven much of the progress in our understanding of the biology of MDSC; however, recent interest in the role of MDSC in transplant rejection and tolerance has emerged (19, 20, 82, 83). Significantly, mouse $CD11b^+Gr1^+$ MDSC expand following transplantation of skin (84) or heart (23). DST and anti-CD40L are commonly used to induce experimental donor-specific allograft tolerance. Using this tolerizing regimen in mouse heart transplantation, Garcia et al (23) demonstrated that $CD11b^+Gr1^+CD115^+$ MDSC are required for the induction of tolerance. In this model, MDSC emigrated from the BM in a CCR2-dependent manner and entered the transplanted heart. MDSC isolated from the graft, but not the spleen or BM, suppressed T cell responses non-specifically and promoted Treg development. Tolerance was dependent on MDSC expression of the IFNϒ receptor, STAT1 and iNOS.

In another model of transplant tolerance, costimulation blockade with anti-CD28 monoclonal antibody (mAb) in rat kidney transplantation resulted in MDSC accumulation in the blood and allograft (85). Maintenance of tolerance was dependent on iNOS, and MDSC suppressed effector T cell proliferation and induced apoptosis while sparing Treg. Blood MDSC directed Treg into the graft by downregulating expression of CCL5 following anti-CD28 mAb treatment, resulting in high intragraft levels of CCL5, but low levels in the blood (86).

Although studies of human MDSC in transplantation are limited, $CD14^+$ and $CD14^-$ MDSC subsets have been identified in human renal transplant recipients (87). With their potent immunosuppressive activity, numerous studies have evaluated the therapeutic application of adoptively transferred mouse MDSC generated *in vitro* or *in vivo* in skin transplantation (77, 84), islet transplantation (58, 78) and graft-versus-host disease (GVHD) (64, 88, 89). Although studies regarding the promotion of MDSC expansion and function and the influence of immunosuppressants on MDSC are lacking, these studies highlight the potential therapeutic application of targeting MDSC *in situ* and MDSC cellular therapies.

1.1.7 Generation and transfer of MDSC to regulate immunity

The ability to propagate MDSC *in vitro* is critical for their application as a cellular therapeutic and allows study of their development in the absence of a complex environment such as occurs in tumor-bearing mice. Myeloid cells are typically generated from BM of rodents whereas peripheral monocytes are most commonly used for human cultures. As discussed above, MDSC require factors that induce their activation in addition to their expansion (6). Mouse monocytic MDSC have been generated from BM cells cultured in G-CSF and/or GM-CSF and activated with IL-6 or IL-13 (58, 64). Lechner et al (90) examined the generation of MDSC from human

PBMC by comparing immune factors secreted by human tumor cell lines known to induce MDSC. They found that GM-CSF + IL-6 expanded MDSC with the most potent suppressive capacity, but GM-CSF + IL-1β, PGE2, TNFα or VEGF also induced suppressive MDSC (90). Consistent with these findings, Marigo et al (58) found that human BM-derived MDSC generated in GM-CSF and IL-6 were highly suppressive. Furthermore, addition of PGE2 to GM-CSF and IL-4 human PBMC cultures blocked DC differentiation and led to generation of suppressive MDSC (91). These data suggest that generation of human MDSC *in vitro* is clinically feasible.

Cellular therapy using MDSC has been applied in several mouse models of disease and can modulate antigen-specific immune reactivity (Table 1.2). MDSC adoptive transfer is capable of promoting skin and islet allograft survival and reducing lethality of GVHD in the absence of additional immunosuppression. It is not known if addition of standard immunosuppressants will potentiate the efficacy of adoptively transferred MDSC. Notably, MDSC transfer resulted in antigen-specific hyporesponsiveness (92) or a lack of generalized immunosuppression (58). Improved methods for culturing MDSC *in vitro* and further understanding of factors that regulate their generation *in vitro* and *in vivo* will be critical to advance therapies exploiting MDSC.

Table 1.2 Adoptive transfer of MDSC promotes immune regulation in mouse models

Source of MDSC	Cell Dose and Route	Model	Mechanism and Outcome	Reference
Mouse tumor-bearing splenocytes	3-5x10^6 i.v. 2-3d after T cell transfer	Antigen-specific transgenic $CD8^+$ T cell (OT-I) responses	Antigen-specific $CD8^+$ T cell tolerance but T cells remained responsive to non-specific αCD3 stimulation	(92)
Transplant recipient splenocytes	2x10^5 i.v. on d-1 and d3	MHC class II-mismatched skin allograft	50% long-term survival when transplant-activated MDSC transferred from ILT2 (HLA-G receptor) transgenic mice but not wild-type mice	(84)
Splenocytes from LPS-treated mice	5x10^6 i.v. on d-1	Male to female or MHC class II-mismatched skin allograft	Prolonged allograft survival dependent on heme oxygenase-1	(77)
Mouse tumor-bearing BM	5x10^6 i.v. + 5μg/mouse peptide antigen d1 after T cell transfer	Transgenic T cell induction of diabetes	75% diabetes-free at d30 (antigen-specific) with T cell anergy and induction of Treg	(80)
Tumor-bearing mouse BM	2x10^7 i.v. with T cells	NOD/SCID with transfer of diabetogenic T cells	60% diabetes-free at d100 with reduced lymphocyte infiltration and insulitis	(80)
B6 BM cultures with GM-CSF+G-CSF+IL-13	2 or 6x10^6 i.v. with donor cells	Graft-versus-host disease (B6 to BALB/c)	Cell dose- and arginase-1-dependent improved survival with inhibition of $CD4^+$ and $CD8^+$ T cell responses and maintained graft-versus-leukemia effect	(64)
129SvEv embryonic stem cell line cultured with M2 cytokine cocktail (KL, VEGF, Flt3L and TPO)+M-CSF	2x10^6 i.v. with donor cells, d4 and d10	Graft-versus-host disease (129SvEv to BALB/c)	82% long-term survival	(89)
BALB/c BM cultures with GM-CSF+IL-6 or GM-CSF+G-CSF	10^7 i.v. on d0, 7, 14 and 21	Islet allograft (B6 to BALB/c)	Long-term survival in ~75% (GM-CSF+IL-6 MDSC) or ~40% (GM-CSF+G-CSF MDSC) without generalized immune suppression	(58)
B6 BM cultures with GM-CSF with liver stellate cells (B6, BALB/c or C3H)	2.5x10^6 mixed with islets	Islet allograft (BALB/c to B6)	~45-65% long-term survival, B7-H1-dependent increase in Treg that mediate T cell hyporesponsiveness	(78)

1.2 DENDRITIC CELLS REGULATE ADAPTIVE IMMUNITY

Dendritic cells (DC) were first identified in 1973 by Ralph M. Steinman (93) who shared the Nobel Prize in Physiology or Medicine in 2011 “for his discovery of the dendritic cell and its role in adaptive immunity.” Since their discovery, DC have become a central focus of immunologists for understanding how the body recognizes self from non-self and initiates adaptive immunity. DC are a rare, heterogeneous population of innate APC that initiate and regulate adaptive T cell responses and maintain self tolerance in the normal steady state (94, 95). DC are sentinels stationed throughout the body especially at environment-body interfaces that function to alert the immune system to pathogens and orchestrate adaptive immune clearance of invading microbes (96). However, as directors of T cell reactivity, DC are important in the pathogenesis of immune-mediated disease, including autoimmunity and transplant rejection (97, 98). Increasing our understanding of DC development and function is crucial to harness their powerful immune-directing properties and utilize strategies targeting DC in clinical medicine (94, 95, 99).

1.2.1 DC classification and ontogeny

DC originate from BM hematopoietic stem cells (HSC) and are classified into conventional DC (cDC), plasmacytoid DC (pDC), Langerhans cells (LC) and monocyte-derived DC (Figure 1.4) (100-102). cDC are further divided into migratory DC and lymphoid tissue-resident DC. Migratory DC are sentinels in the periphery that capture antigen and migrate to lymphoid organs where they present antigen to T cells. Lymphoid tissue-resident DC reside within secondary lymphoid organs and capture and present antigen (103). Lymphoid tissue-resident DC are

classified based on their expression of CD4 or CD8. $CD4^+$ lymphoid tissue-resident DC and $CD4^-CD8^-$ double negative DC primarily present antigen to $CD4^+$ T cells, and $CD8^+$ DC cross-present antigen to initiate $CD8^+$ T cell responses (104, 105). pDC are specialized DC that promote anti-viral immunity through the production of type 1 interferons (106). LC reside in the epidermal layer of the skin and are unique due to their local repopulation from skin-resident hematopoietic precursors (107, 108). Monocyte-derived DC are not found during the steady-state but are mobilized during inflammation and differentiate into 'inflammatory DC' (109).

HSC residing in the BM proceed through a series of increasingly restricted stages of differentiation to common myeloid progenitors (CMP), macrophage and DC progenitors and common DC progenitors that ultimately give rise to pDC and precursor DC (pre-DC) (110) (Figure 1.4). Pre-DC exit the BM and enter the circulation where they give rise to cDC (110-112). Fms-like tyrosine kinase 3 (Flt3, CD135) expressed on early hematopoietic precursors is central to the process of steady-state DC repopulation and differentiation (113). In addition to CMP, common lymphoid progenitors are capable of differentiating into DC (114, 115). This is restricted to a portion of CMP or CLP that express Flt3 (116, 117). These studies suggest a high level of plasticity in DC development and the ability of precursors 'committed' to non-myeloid lineages to produce DC.

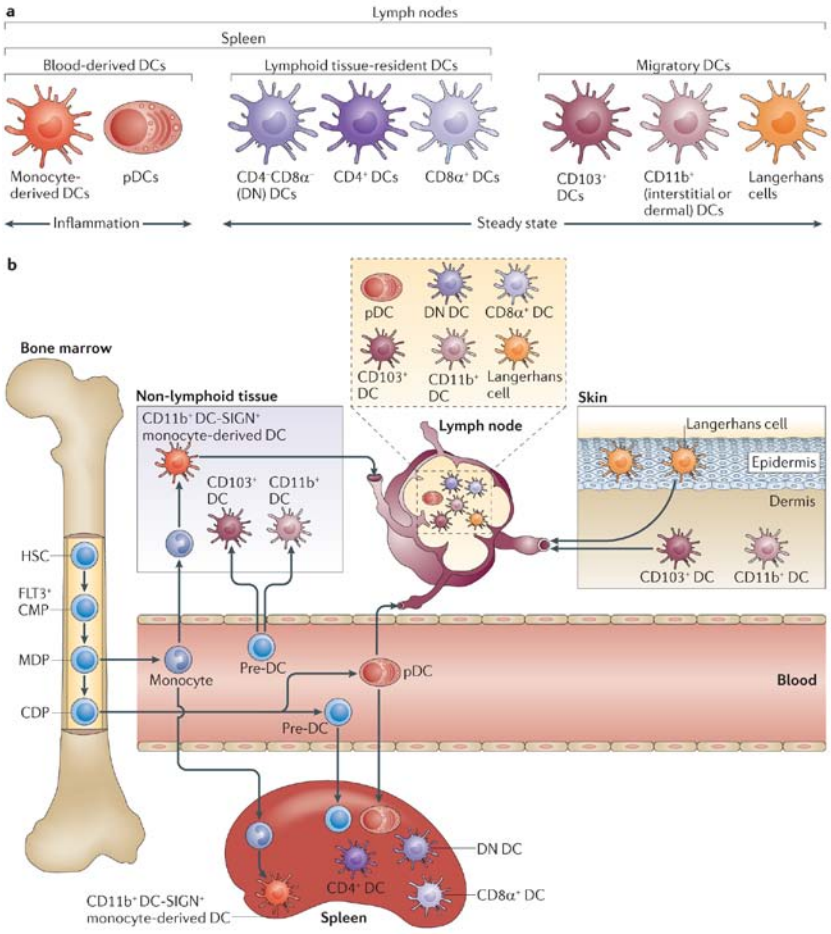

Figure 1.4 Differentiation and trafficking of DC subsets

(A) The figure shows the organization of the dendritic cell (DC) network, and includes the key surface phenotype markers of different DC subsets, which are delineated on the basis of their localization in secondary lymphoid tissues. Gut-associated DCs that express both CD103 and CD11b have been included in the $CD11b^+$ DC subset. Inflammatory monocyte-derived DCs are rapidly recruited to sites of inflammation, whereas other DC subsets are normally present in the steady state. The relationship between inflammatory and steady-state DCs remains an open issue. Moreover, it is unclear whether monocyte-derived DCs can arise through *in situ* proliferation in addition to arriving at tissues via the circulation. (B) In the mouse bone marrow, haematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs), a fraction of which express FMS-like tyrosine kinase 3 (FLT3) and differentiate into more-restricted macrophage and DC progenitors (MDPs). MDPs appear to be the direct precursor to common DC progenitors (CDPs), which give rise to the DC lineages. CDPs produce precursor DCs (pre-DCs) and plasmacytoid DCs (pDCs) that exit the bone marrow and travel through the blood to secondary lymphoid organs and non-haematopoietic tissues. A small proportion of DCs may also be derived from CLPs in the bone marrow and from early T cell progenitors in the thymus. Under steady-state conditions, lymphoid tissue-resident DCs that arise from pre-DCs are the only subsets found in the spleen. This population is comprised of three conventional DC subsets, namely $CD4^+$ DCs, $CD8\alpha^+$ DCs and $CD8\alpha^-CD4^-$ double-negative (DN) DCs. Peripheral lymph nodes contain $CD8\alpha^+$ and $CD8\alpha^-$ DC populations but are also populated by two groups of migratory DCs. Langerhans cells develop in the epidermis and migrate through the basement membrane to the draining lymph nodes via terminal lymphatic vessels that arise in the dermis. The dermal DC population is broadly composed of $CD11b^+$ and $CD103^+$ DCs, and these cells migrate through the lymphatics to the lymph node. Monocytes arrive at tissues from the blood. In response to inflammation, they can develop into monocyte-derived DCs, which adopt many of the characteristics of conventional DCs. DC-SIGN, DC-specific ICAM3-grabbing non-integrin. From Belz GT and Nutt SL. *Nat Rev Immunol*. 2012;12(2):101-13. (101)

In addition to Flt3L-induced signaling, M-CSF and GM-CSF are also involved in DC development. M-CSF promotes differentiation of monocytes, and M-CSF receptor is required for development of LC (118, 119). TGF-β1 is additionally required for LC development in the epidermis (120, 121). Although the GM-CSF receptor is not necessary for homeostatic DC development (113), GM-CSF promotes monocyte differentiation into DC under inflammatory conditions (111, 122). The DC developmental program exhibits further variability under conditions of inflammation, since engagement of TLR expressed on early hematopoietic progenitors can modulate their differentiation (123).

1.2.2 DC coordinate environmental signals to initiate and direct adaptive immunity

Despite being highly heterogeneous, DC subsets share the common ability to perform the professional APC functions of acquiring, processing and presenting antigen to T cells. Immature cDC reside in the periphery, where they act as sentinels by constantly sampling the environment for pathogens and infected or dead cells through a variety of mechanisms, including receptor-mediated endocytosis, macropinocytosis and phagocytosis (124). DC express receptors for the constant region of antibodies (Fc receptors) (125), complement receptors (126), heat shock protein receptors (127, 128), scavenger receptors (129) and C-type lectins (130). In addition, these cells have the ability to constitutively uptake soluble antigen (131). Innate immune cells, including DC, express pattern recognition receptors (PRR) that recognize conserved molecules expressed by microbes called microbe-associated molecular patterns (MAMP; also known as pathogen-association molecular patterns). PRR include surface receptors, most notably TLR (132), and cytoplasmic PRR such as nucleotide oligomerization domain (NOD)-like receptors (NLR) (133) and retinoic acid-inducible gene (RIG)-I-like receptors (RLR) (134). Thus, DC are well-equipped to detect infection or commensal bacterial products (135). In addition to MAMP, DC also respond to inflammation through their expression of cytokine receptors (136).

After recognition of MAMP or in the presence of inflammation, DC undergo signaling events that result in their maturation. DC maturation leads to reduction in antigen uptake, upregulation of antigen presentation in MHC molecules and increased expression of costimulatory molecules (131). Maturation is accompanied by C-C chemokine receptor 7 (CCR7) upregulation, which directs DC to draining lymph nodes (LN), where they interact with and efficiently prime antigen-specific T cells (137, 138). It is now appreciated that DC represent the primary professional APC for priming T cell responses (Figure 1.5) (139).

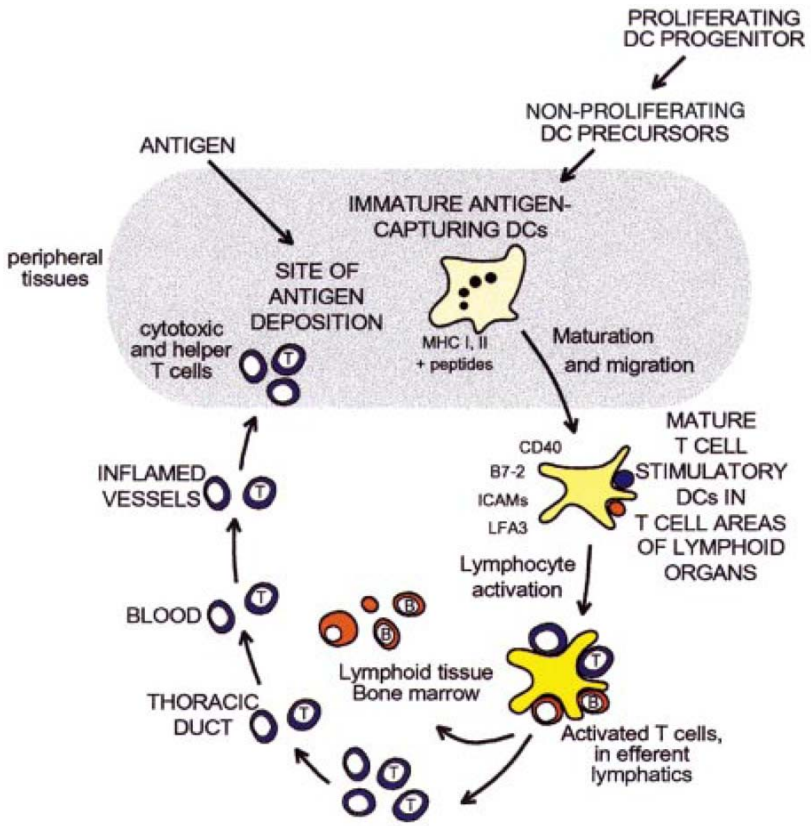

Figure 1.5 Afferent and efferent limbs of immunity that resolve several demands of antigen presentation *in vivo*

Antigens are captured by DCs in peripheral tissues and processed to form MHC-peptide complexes. These immature DCs derive successively from proliferating progenitors and non-proliferating precursors, the latter not being fully committed to form DCs. As a consequence of antigen deposition and inflammation, DCs begin to mature, expressing molecules that will lead to binding and stimulation of T cells in the T-cell areas of lymphoid tissues. If the antigen has also been bound by B cells, then both B and T cells can cluster with DCs, as shown. After activation, T (blue) and B (orange) blasts leave the T-cell area. B blasts move to the lining of the intestine, the bone marrow, and other parts of the lymphoid tissue, such as the medulla of lymph node, with some becoming antibody-secreting plasma cells. T blasts leave the blood at the original site of antigen deposition, recognizing changes in the inflamed blood vessels and responding vigorously to cells that are presenting antigen. This limits the T-cell response to the site of microbial infection. From Banchereau J and Steinman RM. *Nature*. 1998;392(6673): 245-52. (139)

1.2.2.1 DC provide 3 signals to guide T cell activation and differentiation

The ability of DC to activate naïve T cells and direct their differentiation relies on three signals (Figure 1.6). T cells recognize peptide-MHC complexes presented by DC via their antigen-specific TCR (Signal 1). Costimulatory molecules provide signal 2 and further activate signals downstream of the TCR and promote additional effector functions in T cells (140). The majority

of costimulatory molecules belong to either the immunoglobulin or tumor-necrosis factor (TNF) superfamily (141). B7-1 (CD80) and B7-2 (CD86) are classic immunoglobulin superfamily costimulatory ligands expressed by DC that ligate CD28 on T cells (142, 143). Numerous other ligand-receptor pairs have been identified (144, 145).

There is also a regulatory component to signal 2. T cells upregulate the inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) following activation (146). CTLA4 has a higher affinity for B7-1 and B7-2 than CD28 and inhibits T cell activation (147-149). Additionally, coinhibitory ligands that dampen T cell activation are expressed by DC (141). B7-homolog 1 (B7-H1) and B7-DC are examples B7 family coinhibitory molecules that suppress T cell activation through programmed cell death-1 (PD-1) on T cells (150, 151). Thus, costimulatory and coinhibitory molecules finely tune signal 2 to balance the activation and suppression of T cell responses.

Signal 3 is mediated by soluble cytokines released by DC to direct the differentiation of naïve T cells into their T helper lineages (152). IL-12 supports the development of IFNγ-producing Th1 cells (153). Although DC participate in the induction of Th2 responses, the precise mechanisms by which DC perform this function are not fully understood (154). DC induce Th17 differentiation through production of IL-1β, IL-6, TGFβ and IL-23 (155), but they are also capable of limiting T cell activation through production of anti-inflammatory IL-10 (156). Thus, DC provide coordinated stimulatory and inhibitory signals to T cells to regulate antigen-specific activation and differentiation.

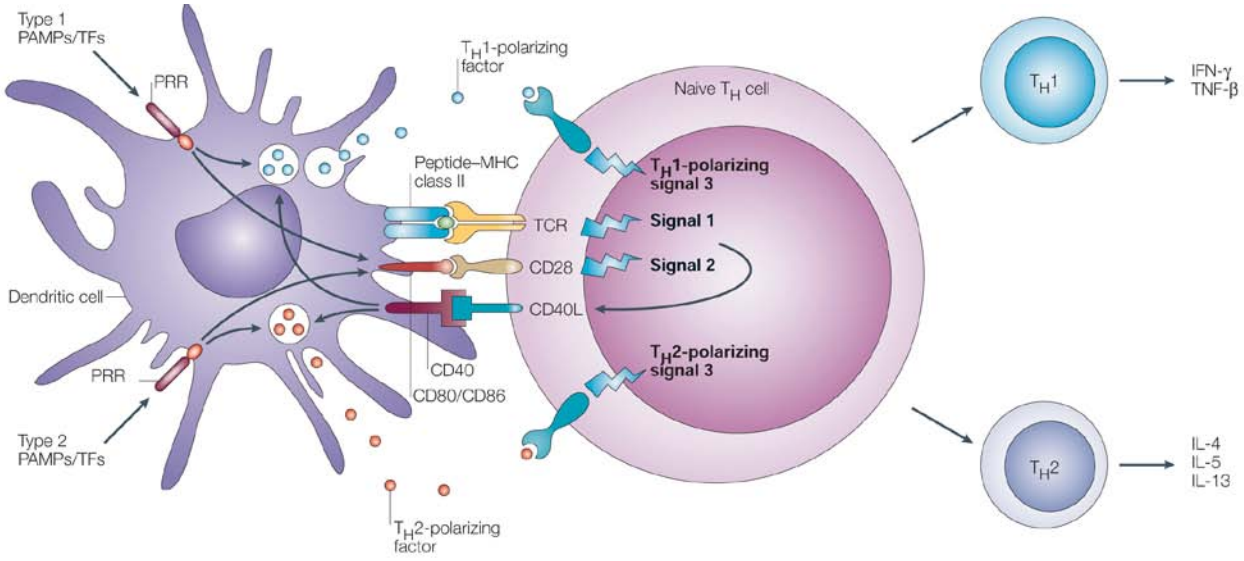

Figure 1.6 T-cell stimulation and T helper 1 (T_H1)/T_H2-cell polarization require three dendritic cell-derived signals

Signal 1 is the antigen-specific signal that is mediated through T-cell receptor (TCR) triggering by MHC class-II-associated peptides processed from pathogens after internalization through specialized pattern recognition receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by dendritic cells (DCs) after ligation of PRRs, such as Toll-like receptors (TLRs) that are specialized to sense infection through recognition of pathogen-associated molecular patterns (PAMPs) or inflammatory tissue factors (TFs). Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2) that promote the development of T_H1 or T_H2 cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or TFs. Type 1 and type 2 PAMPs and TFs can be defined as those that selectively prime DCs for the production of high levels of T_H1-cell-polarizing or T_H2-cell-polarizing factors. Whereas, the profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2. IFN-Ɣ, interferon-Ɣ; TNF-β, tumor-necrosis factor-β. From Kapsenberg ML. *Nat Rev Immunol*. 2003;3(12):984-93. (96)

1.2.3 DC establish and maintain tolerance

During their generation in the thymus, most T cells expressing self-reactive TCR are removed to establish central tolerance (157). However, autoreactive T cells can escape thymic education and be activated during inflammatory responses to a pathogen, but these T cells are controlled through extra-thymic mechanisms to establish peripheral tolerance (98). In addition to being the most potent stimulators of T cells, DC have the ability to regulate T cell responses and are critical for central and peripheral tolerance (158, 159). cDC and pDC reside within the thymic

medulla and participate in T cell selection along with antigen-presenting thymic epithelial cells (157, 160-163). It was postulated (159) that steady-state DC continuously sample the environment and maintain T cell tolerance to antigens encountered in the periphery in the absence of inflammation. Indeed, DC present self antigens to T cells *in vivo* (164). DC exposure to apoptotic, but not necrotic, material maintains their immature state (165) and induces CCR7 expression (166). Thus, DC acquire apoptotic material from the normal turnover of peripheral tissue cells and can maintain tolerance to these antigens (167). This was proven *in situ* by targeting antigen to steady-state DC, resulting in antigen-specific T cell deletion and hyporesponsiveness (168). Furthermore, constitutive deletion of DC in mice results in loss of $CD4^+$ T cell self-tolerance and lethal autoimmunity (169). As a proof-of-principle, studies in human volunteers have demonstrated that subcutaneous injection of immature DC results in antigen-specific inhibition of $CD8^+$ effector T cells (170) and development of $CD8^+$ regulatory T cells (171). Thus, DC are able to regulate antigen-specific immunity in humans and are required for self-tolerance in mice.

1.2.4 mTOR is a ubiquitous regulator of metabolism that controls DC development and function

The mammalian/mechanistic Target of Rapamycin (mTOR) is a serine/threonine kinase that is widely studied due to its evolutionarily conserved role in controlling cell growth and proliferation. Since the discovery that rapamycin (RAPA), an mTOR inhibitor, has powerful immunosuppressive properties, immunologists have begun unraveling the function of mTOR in immune cells. Historically, studies of mTOR biology have focused on its role in T lymphocytes because the anti-proliferative action of RAPA on these cells results in immunosuppression. However, recent advances have demonstrated that mTOR also has important functions in myeloid APC to regulate their differentiation and function (172).

1.2.4.1 mTOR biology

mTOR is a highly-conserved, integrative serine/threonine kinase that is a central regulator of cell growth and metabolism (173). mTOR is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase protein family (173) and is a mammalian homolog of the TOR1 and TOR2 genes first identified in *Saccharomyces cerevisiae* (174). Until recently, it was unknown if a second mTOR gene exists in mammals, but a second mTOR-containing signaling complex was identified in 2004 (175, 176). In mammals, a single mTOR kinase performs the catalytic function of two separate complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) (Figure 1.7). mTORC1 is composed of regulatory associated protein of mTOR (raptor), mammalian lethal with Sec13 protein 8 (mLST8) and proline-rich substrate of Akt of 40 kD (PRAS40). mTORC2 contains rapamycin-insensitive companion of mTOR (rictor), mLST8, mSIN1 and protein associated with rictor (PROTOR) (177). DEP domain-containing mTOR-interacting protein

(DEPTOR) interacts with both complexes and inhibits their activity (178). mTORC1 senses environmental signals, including nutrients, hormonal cues and energy levels, to regulate mRNA translation and protein and lipid synthesis (173, 179). Thus, mTORC1 functions as a central switch between catabolic and anabolic processes (179). Considerably less is known about signals that regulate mTORC2 activity. Rho family GTPases and protein kinase Cα (PKCα) are downstream of mTORC2 and regulate actin organization (175, 176). Together, mTORC1 and mTORC2 are believed to coordinate with one another to regulate the timing and spatial orientation of cell growth and division.

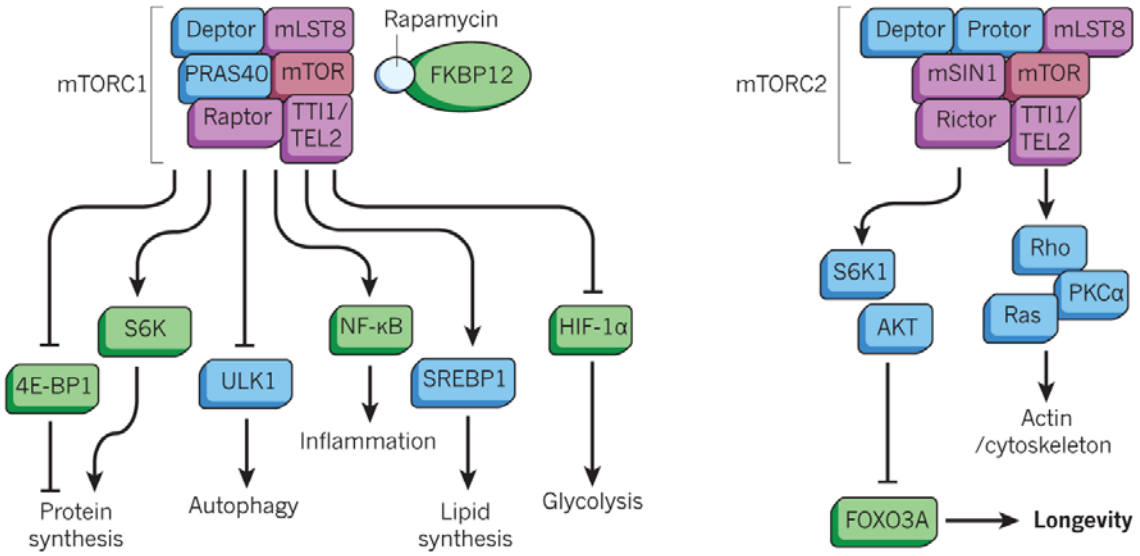

Figure 1.7 The two mTOR complexes have distinct constituent proteins and regulate different downstream processes

Here (figure represents data from studies in mice) mTORC1 comprises deptor, PRAS40, raptor, mLST8, mTOR and TTI1-TEL2. mTORC2 is comprised of deptor, mLST8, protor, rictor, mSIN1, mTOR and TTI1-TEL2. Rapamycin binds to FKBP12 and inhibits mTORC1 by disrupting the interaction between mTOR and raptor. Regulation of lipid synthesis by mTORC1 is thought to occur mainly through sterol-regulatory-element-binding protein transcription factors (shown here as SREBP1) by a mechanism that is not completely understood. mTORC1 negatively regulates autophagy through multiple inputs, including inhibitory phosphorylation of ULK1, preventing formation of the ULK1-ATG13-FIP200 complex (which is required for initiation of autophagy). mTORC1 promotes protein synthesis through activation of the translation initiation promoter S6K and through inhibition of the inhibitory mRNA cap binding 4E-BP1, and regulates glycolysis through HIF-1α. mTORC2 inhibits FOXO3a through S6K1 and AKT, which can lead to increased longevity. The complex also regulates actin cytoskeleton assembly through protein kinase C α (PKCα), Rho GTPases and Ras proteins. From Johnson SC, Rabinovitch PS and Kaeberlein M. *Nature*. 2013;493(7432):338-45. (179)

1.2.4.2 Inhibition of mTOR

The antifungal macrolide rapamycin (RAPA) was first isolated from soil samples from Easter Island in the 1970's (180). RAPA functions as an allosteric inhibitor of mTORC1 that exerts its inhibitory effect by binding the immunophilin FK506-binding protein 1A, 12 kDa (FKBP12) (181). mTORC2 is insensitive to inhibition by RAPA (175, 176); however, prolonged exposure to RAPA can inhibit mTORC2 assembly (182, 183). Despite exhibiting homology with PI3K in the kinase domain, selective active site ATP-competitive mTOR inhibitors (i.e. Torin1, PP242) have been described recently that can inhibit signaling downstream of both mTOR complexes

with a half maximal inhibitory concentration (IC_{50}) less than 10 nM (184-187). The use of ATP-competitive mTOR inhibitors in non-immune cells has revealed the unexpected finding that RAPA does not inhibit mTORC1 completely. Independent of their ability to inhibit mTORC2, ATP-competitive mTOR inhibitors further inhibit cell proliferation and cap-dependent translation, as well as induce autophagy more effectively than RAPA (184, 185).

1.2.4.3 mTOR regulates DC immunobiology

mTOR is a crucial regulator of innate and adaptive immune cell function (172). Much of our knowledge of the function of mTOR comes from the investigation of RAPA, with a primary focus on T cell immunobiology. However, mTOR is emerging as an important regulator of DC homeostasis and function (188-190). RAPA reduces the number of splenic DC under homeostatic conditions and following mobilization with the DC poietin Flt3L (188). Flt3L stimulates mTOR signaling in DC, and deletion of phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K-mTOR pathway, leads to mTOR-dependent expansion of $CD8^+$ and $CD103^+$ cDC subsets (191). Flt3L requires STAT3 activation to promote DC differentiation (192), and mTORC1 is a positive regulator of STAT3 (193-195). Thus, DC development downstream of Flt3L occurs through an Flt3L-mTORC1-STAT3 pathway.

mTOR is also involved in diverse cellular processes in DC required for the generation of effective immune responses (172). RAPA disrupts macropinocytosis and receptor-mediated endocytosis in DC and reduces expression of antigen uptake receptors, an effect that is independent of the phenotypic immaturity of DC treated with RAPA (196, 197). In contrast to the calcineurin inhibitors cyclosporine A and tacrolimus, pre-treatment of DC with RAPA does not affect antigen processing or presentation on MHC class I or class II through classical routes or cross-presentation (198). However, RAPA augments antigen presentation in mouse DC by

stimulating autophagy, which directs antigen into the MHC class II compartment (199). RAPA treatment has been reported to enhance (200) or permit (190) CCR7 upregulation during human and mouse DC maturation, respectively, suggesting that mTORC1 signaling is not required to direct CCL19/21-mediated migration to secondary lymphoid organs. Furthermore, mouse RAPA-treated DC still migrate to secondary lymphoid organs following adoptive transfer (189).

Despite normal migratory ability, RAPA dampens other aspects of phenotypic maturation and dramatically alters cytokine secretion by DC. RAPA prevents IL-4-dependent maturation of DC by decreasing expression of the IL-4 receptor (188). DC treated with RAPA show diminished upregulation of B7 family costimulatory molecules and MHC class II following maturation with various inflammatory stimuli (189, 190, 201, 202). Although RAPA reduces secretion of IL-12p70 following CD40 ligation (190), recent studies have shown that RAPA-treated DC stimulated with LPS secrete increased levels of IL-12p70 and reduced levels of IL-10 (195), particularly by the $CD86^{lo}$ immature DC subset (202). Furthermore, RAPA increases DC expression and secretion of IL-1β due to mTOR inhibition of caspase-1 (201, 203). Despite these paradoxical pro-inflammatory effects on DC cytokine production, DC treated with RAPA are weak stimulators of syngeneic and allogeneic T cells (189, 201, 202) but support regulatory T cells (190). In total, through studies using RAPA, we now appreciate that mTORC1 modulates DC antigen capture and presentation, co-stimulatory molecule expression and cytokine production. Considerably less is known about the function of mTORC2 in immunity. mTORC2 is involved in fate decisions in the differentiation of T cells (204, 205), but its role in innate immune cells, including DC, is not known.

1.2.5 Donor and recipient DC regulate rejection in transplantation

DC are central regulators of adaptive alloimmunity that act to initiate and regulate alloreactive T cell responses. T cell alloimmunity is mediated by three non-mutually exclusive pathways termed the direct, indirect and semi-direct pathways (Figure 1.8) that are initiated when APC migrate to secondary lymphoid organs (SLO) and interact with T cells (206). The direct pathway is mediated by donor APC, especially DC, that are transferred within the allograft during transplantation and present intact allogeneic MHC molecules to recipient T cells (207-209). The indirect pathway is initiated by recipient DC presenting processed donor alloantigen within recipient MHC class II molecules to $CD4^+$ T cells (207). Additionally, indirectly-reactive $CD8^+$ T cells can be cross-primed (210). The semi-direct pathway is mediated by recipient DC that acquire intact donor MHC complexes from donor cells; however, direct evidence that this mechanism of allorecognition occurs *in vivo* to mediate rejection is lacking (209).

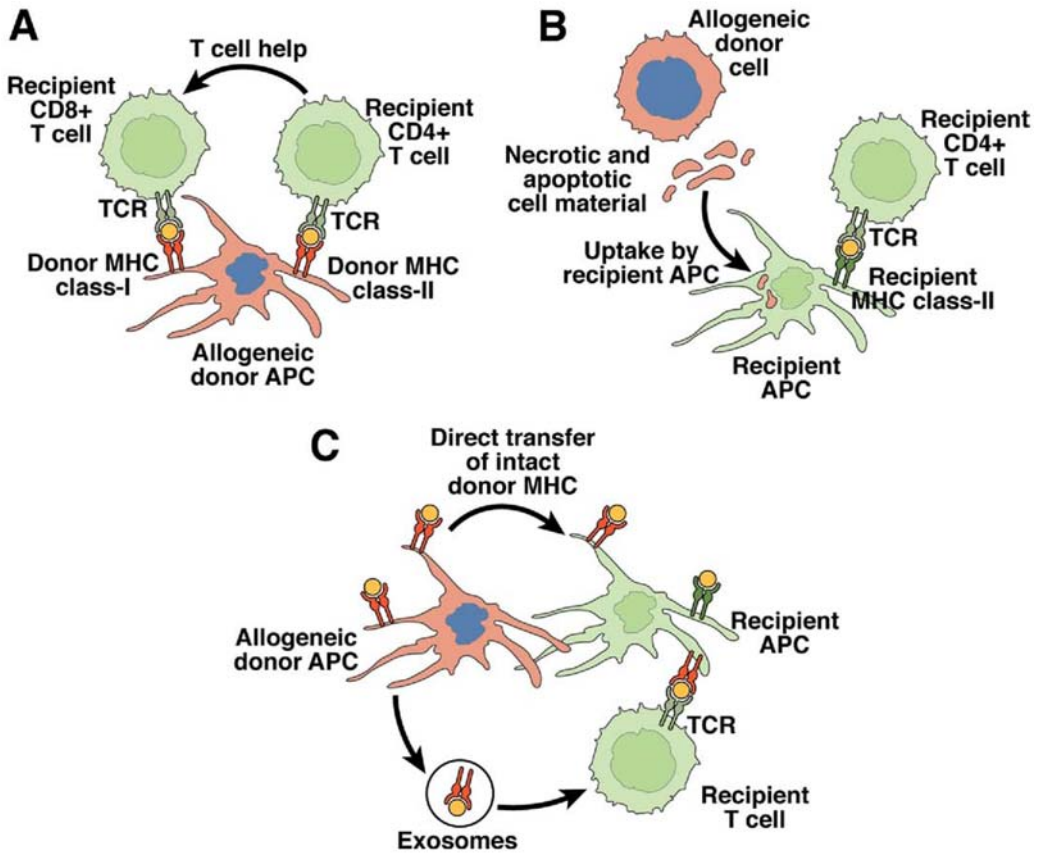

Figure 1.8 Pathways of alloantigen presentation

Three nonmutually exclusive pathways of allorecognition have been described. (A) In the direct pathway, recipient T cells recognize intact allogeneic MHC molecules on the surface of donor APCs. The direct pathway is responsible for the large proportion of T cells that have reactivity against alloantigens due to cross-reactivity of the T-cell receptor (TCR) with self and foreign MHC molecules. (B) In the indirect pathway, recipient APCs trafficking through the allograft phagocytose allogeneic material shed by donor cells (mostly peptides derived from allogeneic MHC molecules) and present it to recipient T cells on recipient MHC molecules. (C) In the semidirect pathway, recipient APCs acquire intact MHC molecules following direct contact with donor APCs and/or through fusion with donor APC-derived exosomes. These chimeric recipient APCs stimulate recipient T cells through direct and indirect pathways. From Sànchez-Fueyo A and Strom TB. *Gastroenterology*. 2011;140(1):51-64. (211)

In transplantation, DC rapidly mature under the influence of endogenous inflammatory mediators released during ischemia/reperfusion injury that act on PRR (207, 212, 213). DC maturation is accompanied by C-C chemokine receptor 7 (CCR7) upregulation, which enhances their migration to secondary lymphoid organs (SLO), where they interact with and efficiently prime alloreactive T cells (137, 138, 206). While mature DC promote alloreactive T cell

responses (214), immature or maturation-resistant DC can suppress allograft rejection and promote transplant tolerance through induction of antigen-specific T cell anergy/deletion and Treg (95, 159, 215, 216). The ability of DC to initiate and regulate alloreactive T cell responses emphasizes the importance of targeting these APC with therapeutic agents and understanding the impact of immunosuppressants on DC immunobiology.

1.3 STATEMENT OF THE PROBLEM

In 2012, 28,052 organ transplants were performed in the United States to provide definitive treatment for end-stage organ disease (Organ Procurement and Transplantation Network). The discovery of pharmacologic immunosuppressive agents capable of inhibiting immune responses against an organ allograft was a medical breakthrough that overcame acute rejection, leading to the birth of the field of organ transplantation in the 1960's. Modern pharmacologic immunosuppressive regimens have played a crucial role in achieving excellent short-term outcomes due to prevention and treatment of acute rejection episodes, but late graft failure ('chronic rejection') remains a significant obstacle (217) that exacerbates the current organ shortage. Additionally, general suppression of the immune system with chemical immunosuppressants carries significant risks, including infections, malignancy and toxicity (218). Clinically-utilized immunosuppressive regimens fail to prevent the development of global vascular narrowing within a transplanted organ or chronic allograft vasculopathy (CAV), an outcome of chronic immune responses against the allograft (219-221). The pathogenesis of CAV is multi-fold and poorly understood; however, anti-graft immune responses are the predominant factor in the development of CAV (222).

Alloantigen-specific T cells are central mediators of acute rejection and CAV (223-225). T cell function is tightly regulated by interactions with myeloid lineage cells, including MDSC and DC. While T cells are classically the primary target of immunosuppressants, DC are emerging as equally important drug targets (226). However, agents capable of boosting MDSC activity have not been investigated. MDSC and DC are potent regulators of antigen-specific T cell immunity and thus represent valuable targets for the prevention of anti-graft immune responses. Understanding the pathways regulating the development and function of MDSC and DC is an important step towards (i) utilizing these cells as cellular therapeutic agents and (ii) targeting them *in situ* with new immunomodulatory agents to skew immunity away from detrimental anti-graft responses to minimize the need for generalized immunosuppression and ultimately establish donor-specific tolerance.

1.4 SPECIFIC AIMS AND HYPOTHESES

The overall hypothesis of this thesis is that DC and MDSC differentiation and function can be manipulated with pharmacologic and biologic agents to favor their immune regulatory properties. Hypotheses directed towards examining three pathways governing the expansion, differentiation and/or function of DC and MDSC will be presented in the following Specific Aims.

1.4.1 Specific Aim 1 (Chapter 2): To assess the effect of histone deacetylase inhibition on MDSC differentiation and function

Histone deacetylase inhibitors (HDACi) alter chromatin accessibility by inhibiting the removal of lysine residues from histone proteins to modulate gene expression. More recently, it has been shown that protein acetylation is an important post-translational modification that changes protein function (227). In addition to their anti-neoplastic properties, HDACi are emerging as anti-inflammatory agents (228) that reduce DC stimulatory capacity (228-232). HDACi inhibit DC differentiation (230, 233), thus we hypothesized that HDACi will augment MDSC expansion by preventing DC differentiation (see also page 41).

1.4.2 Specific Aim 2 (Chapter 3): To determine if Fms-like tyrosine kinase 3 ligand expands and activates MDSC

Fms-like tyrosine kinase 3 (Flt3) and its ligand, Flt3L, promote the development of DC (234-236) through the transcription factor STAT3 (192). The ability of Flt3L to promote MDSC expansion has not been previously examined. STAT3 is a major transcription factor involved in the expansion and activation of MDSC (6, 49) and is activated by Flt3L (192). Therefore, we hypothesized that Flt3L will expand and activate MDSC in a STAT3-dependent manner (see also page 68).

1.4.3 Specific Aim 3 (Chapter 4): To ascertain the function of RAPA-resistant mTOR in DC

mTOR is an integral regulator of APC and T cell function (172, 237) that is the catalytic subunit of two mTOR-containing complexes,- mTORC1 and mTORC2 (175, 176). mTORC1 regulates DC differentiation and promotes their stimulatory function (95, 172, 189). Much of our understanding of mTORC1 function in DC stems from the use of RAPA as an inhibitor of mTOR; however, RAPA spares mTORC2 function (175, 176). Additionally, mTORC1 exerts RAPA-resistant functions in mouse embryonic fibroblasts (184, 185). Due to the recent discovery of RAPA-resistant mTORC1 and mTORC2 outputs and new methods to inhibit these complexes, little is known about the role of RAPA-resistant mTOR in DC function. We hypothesized that RAPA-resistant mTOR has distinct immunoregulatory functions from RAPA-sensitive mTORC1 in DC (see also page 80).

2.0 HISTONE DEACETYLASE INHIBITION FACILITATES GM-CSF-MEDIATED EXPANSION OF MYELOID-DERIVED SUPPRESSOR CELLS IN VITRO AND IN VIVO

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2.1 ABSTRACT

Chromatin-modifying histone deacetylase inhibitors (HDACi) exhibit anti-inflammatory properties that reflect their ability to suppress dendritic cell function and enhance regulatory T cells. The influence of HDACi on myeloid-derived suppressor cells (MDSC), an emerging

regulatory leukocyte population that potently inhibits T cell proliferation, has not been examined. Exposure of GM-CSF-stimulated murine BM cells to HDACi led to a robust expansion of monocytic MDSC ($CD11b^{+}Ly6C^{+}F4/80^{int}CD115^{+}$) that suppressed allogeneic T cell proliferation in a nitric oxide synthase- and heme oxygenase-1-dependent manner with similar potency to control MDSC. The increased yield of MDSC correlated with blocked differentiation of BM cells and an overall increase in hematopoietic stem and progenitor cells ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$). *In vivo*, TSA enhanced the mobilization of splenic HSPC following GM-CSF administration and increased the number of $CD11b^{+}Gr1^{+}$ cells in BM and spleen. Increased numbers of $Gr1^{+}$ cells that suppressed T cell proliferation were recovered from spleens of TSA-treated mice. Overall, HDACi enhance MDSC expansion *in vitro* and *in vivo*, suggesting that acetylation regulates myeloid cell differentiation. These findings establish a clinically applicable approach to augment this rare and potent suppressive immune cell population and support a novel mechanism underlying the anti-inflammatory action of HDACi.

2.2 INTRODUCTION

Histone acetylation classically modulates gene expression, whereby acetyl groups bound to lysine residues of histone proteins relax DNA binding, permitting gene accessibility and transcription. Histone deacetylase inhibitors (HDACi) increase the extent of histone acetylation by inhibiting removal of acetyl groups from histones, resulting in tighter DNA binding and reduction in gene expression (240). Histone acetylation offers a precise regulatory mechanism where only a small proportion of genes are regulated by HDAC inhibition (229). However, new evidence demonstrates non-histone protein acetylation to also be an important post-translational modification, which suggests that acetylation is a more global regulatory mechanism than originally appreciated (227).

Trichostatin A (TSA) is a naturally-occurring antifungal metabolite produced by *Streptomyces* that potently inhibits HDAC activity. TSA and other HDACi are well-known anti-neoplastic agents that modulate gene expression, leading to cell cycle arrest, differentiation, or apoptosis (241). The HDACi suberoylanilide hydroxamic acid (SAHA; Vorinostat) is FDA-approved for the treatment of cutaneous T cell lymphoma. Recently, HDACi have been shown to suppress inflammatory disease (228) and to inhibit experimental graft-versus-host disease (GVHD) (242), systemic lupus erythematosus (243) and colitis (244). One mechanism that correlates with these anti-inflammatory effects of HDACi is the ability of these agents to target dendritic cell (DC) and other myeloid lineage antigen (Ag)-presenting cell (APC) functions (228-232).

DC are professional BM-derived APC with unparalleled ability to stimulate naïve and memory T cells and regulate their function (94, 95, 139). It has been demonstrated recently that HDACi reduce the stimulatory capacity of these potentially potent APC (228-232). Specifically,

HDACi inhibit DC differentiation (230, 233) and reduce the expression of major histocompatibility complex (MHC) gene products, costimulatory molecules and proinflammatory cytokines by these cells, rendering them less immunostimulatory (230). In addition, HDACi increase the expression of indoleamine 2,3-dioxygenase (IDO) (231, 232), reduce production of T helper type 1 (Th1) cell-attracting chemokines, and selectively inhibit the induction of Th1 responses due to reduced bioactive interleukin (IL)-12 secretion (229). While studies of the anti-inflammatory activity of HDACi have focused on conventional myeloid DC (mDC), their influence on myeloid-derived suppressor cells (MDSC), that have emerged recently as important regulators of immune reactivity (6), has not been investigated.

MDSC are a rare, heterogenous population of incompletely differentiated, immature myeloid and myeloid progenitor cells. They expand from BM progenitors under inflammatory conditions, particularly in cancer (6), and in response to granulocyte macrophage-colony stimulating factor (GM-CSF) *ex vivo* (64) and *in vivo* (245). Murine MDSC co-express CD11b and Gr1 and comprise 20-30% and 2-4%, respectively, of normal murine BM and splenocyte populations (6). MDSC potently inhibit T cell proliferation and are therefore regarded as important regulators of immune reactivity (6). They have emerged as potential therapeutic agents based on their ability to suppress GVHD (64) and to mediate experimental transplant tolerance (23). Given the ability of HDACi to impair DC differentiation (230, 233), we hypothesized that TSA might increase the generation of MDSC from GM-CSF-stimulated BM cells by preventing the differentiation of BM cells into mature myeloid cells.

We demonstrate for the first time, the ability of HDACi to enhance the generation of functional MDSC both *in vitro* and *in vivo*. TSA inhibited the differentiation of BM cells stimulated with GM-CSF *in vitro* and augmented hematopoietic stem and progenitor cells

(HSPC) in BM cell cultures correlating with increased numbers of MDSC. The related HDACi SAHA also increased phenotypic MDSC in GM-CSF-exposed BM cultures. TSA administration following GM-CSF delivery enhanced the mobilization of splenic HSPC and augmented the expansion of BM and splenic $CD11b^{+}Gr1^{+}$ cells *in vivo*. Greater numbers of splenic $Gr1^{+}$ cells, with potent ability to suppress allogeneic T cell proliferation, were recovered from mice given TSA and GM-CSF than from those given GM-CSF alone. Taken together, these novel findings demonstrate the ability of HDACi to enhance the expansion of MDSC *in vitro* and *in vivo*, correlating with their ability to expand HSPC and impede BM cell differentiation.

2.3 MATERIALS AND METHODS

Animals. Experiments used 8-12 week old male C57BL/6 (B6; $H2K^{b}$) and BALB/c ($H2K^{d}$) mice from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the specific pathogen-free facility of the University of Pittsburgh School of Medicine, and studies were conducted under an institutional animal care and use committee-approved protocol.

Cell culture and purification. BM cells were differentiated for 7 days (d), as described (202) in recombinant (r) mouse GM-CSF (1000 U/ml; Schering-Plough, Kenilworth, NJ, USA) alone or with r mouse IL-4 (1000 U/ml; R&D Systems, Minneapolis, MN, USA). On d7, non-adherent MDSC were purified by labeling with phycoerythrin (PE)-conjugated anti-Gr1 monoclonal antibody (mAb) and anti-PE immunomagnetic bead purification (Miltenyi Biotec, Auburn, CA, USA) (246). mDC were isolated as described (202). TSA (0.1-10 nM; Sigma, St. Louis, MO, USA) or SAHA (10-500 nM; Selleck, Houston, TX, USA) was added to cultures on d2, 4, and 6.

Flow cytometry. Phenotypes were analyzed as described (188, 202) using fluorochrome-conjugated mAb and streptavidin (eBioscience, San Diego, CA, USA or BD Bioscience, San Jose, CA, USA). HSPC and DC progenitors were analyzed on d4 of BM cell culture (110). A lineage (Lin) mAb cocktail consisting of anti-CD19, -B220, -CD3, -CD4, -CD8, -NK1.1, -Ter119, -CD11b and -CD11c was used to set the Lin^- gate. Anti-CX_3CR1 was purchased from Abcam (Cambridge, MA, USA). Appropriately-conjugated, species and isotype-matched IgG served as controls. Adjusted overall cell population frequency was calculated by multiplying the frequency of the indicated population by the number of events for the gate divided by the total number of events recorded in the live cell singlet gate.

MDSC suppression assay. MDSC suppressor function was ascertained as described (33), with minor modifications. Isolated BM-derived $Gr1^+$ MDSC (B6) were rested overnight and tested for suppressor activity in allogeneic mixed leukocyte reaction (MLR) where $1x10^4$ γ-irradiated DC (B6; 20 Gy) stimulated $CD3^+$ BALB/c T cell responders ($4x10^5$) for 72 hours (h) in the presence of 0-$3x10^4$ MDSC in 96-well, round-bottom plates. MDSC were rested for 16h to allow lipopolysaccharide (LPS) stimulation of DC that were also isolated on d7 and tested as stimulators (data not shown). N^{ω}-Hydroxy-nor-L-arginine (norNOHA; Calbiochem, Gibbstown, NJ, USA; 500 μM), N^G-Methyl-L-arginine (L-NMMA; Sigma; 0.5 mM), or tin protoporphyrin (SnPP; Enzo Life Sciences, Farmingdale, NY, USA; 0.15 mM) were added where indicated. Alternatively, B6 splenic $Gr1^+$ MDSC ($2x10^5$), isolated from treated mice, were tested for their ability to suppress proliferation of $CD3^+$ BALB/c T cell ($2x10^5$) responders stimulated with γ-irradiated B6 mDC ($5x10^4$).

Immunoblot. Immunoblotting was performed as described (202) on lysates from >1x10^6 BM cells or isolated $Gr1^+$ cells using primary mAb to acetyl-histone H4 (Lys8; Cell Signaling Technology), arginase-1 (BD PharMingen), heme oxygenase-1 (Enzo Life Sciences), inducible nitric oxide synthase (Abcam), β-actin (Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Novus Biologicals, Littleton, CO, USA).

Hydrodynamic plasmid transfection of mice and TSA administration. B6 mice were injected with mouse GM-CSF-pcDNA3 (50 μg; Dr. Joyce Solheim, University of Nebraska Medical Center, Omaha, NE, USA) or enhanced green fluorescent protein (eGFP)-pcDNA3 (50 μg; Addgene, Cambridge, MA, USA) expression vectors as described (247). TSA administration (1 mg/kg/d; i.p.) was initiated 1d later (d1) and continued through d5. Spleens and femoral BM were harvested for analysis on d6.

Statistical analyses. Results are expressed as means ± 1 standard deviation (SD). Significant differences between means were determined using a one-tailed Student's 't' test and Prism (GraphPad Software, La Jolla, CA, USA), and $p<0.05$ was considered significant.

2.4 RESULTS

2.4.1 TSA enhances BM cell proliferation in response to GM-CSF and especially GM-CSF + IL-4 stimulation and skews myeloid lineage differentiation

We first examined the influence of TSA on murine BM cell cultures stimulated with either GM-CSF or GM-CSF + IL-4. Under both conditions, addition of TSA (10 nM) led to a significant increase in total cells recovered on d7 (Figure 2.1A). Although TSA modestly increased the

proliferation of BM cells stimulated with GM-CSF alone, addition of TSA to GM-CSF in the presence of IL-4 enhanced cell proliferation approximately 3-4 fold (Figure 2.1A), and in a dose-dependent manner (Figure 2.2A). We next investigated the identity of the BM-derived cells expanded in TSA-treated cultures. Growth in GM-CSF alone led to a higher frequency of mDC ($CD11b^+CD11c^+$) than growth in GM-CSF + IL-4 (Figure 2.1B). Under both conditions, 10 nM TSA reduced the incidence of $CD11b^+CD11c^+$ DC (Figure 2.1B), consistent with impairment of DC differentiation by HDACi (230, 233). As reported previously (248), plasmacytoid DC (pDC; $B220^+CD11c^+$) were not generated to a significant degree in either GM-CSF alone or GM-CSF + IL-4, and this was unchanged in the presence of TSA (Figure 2.1C).

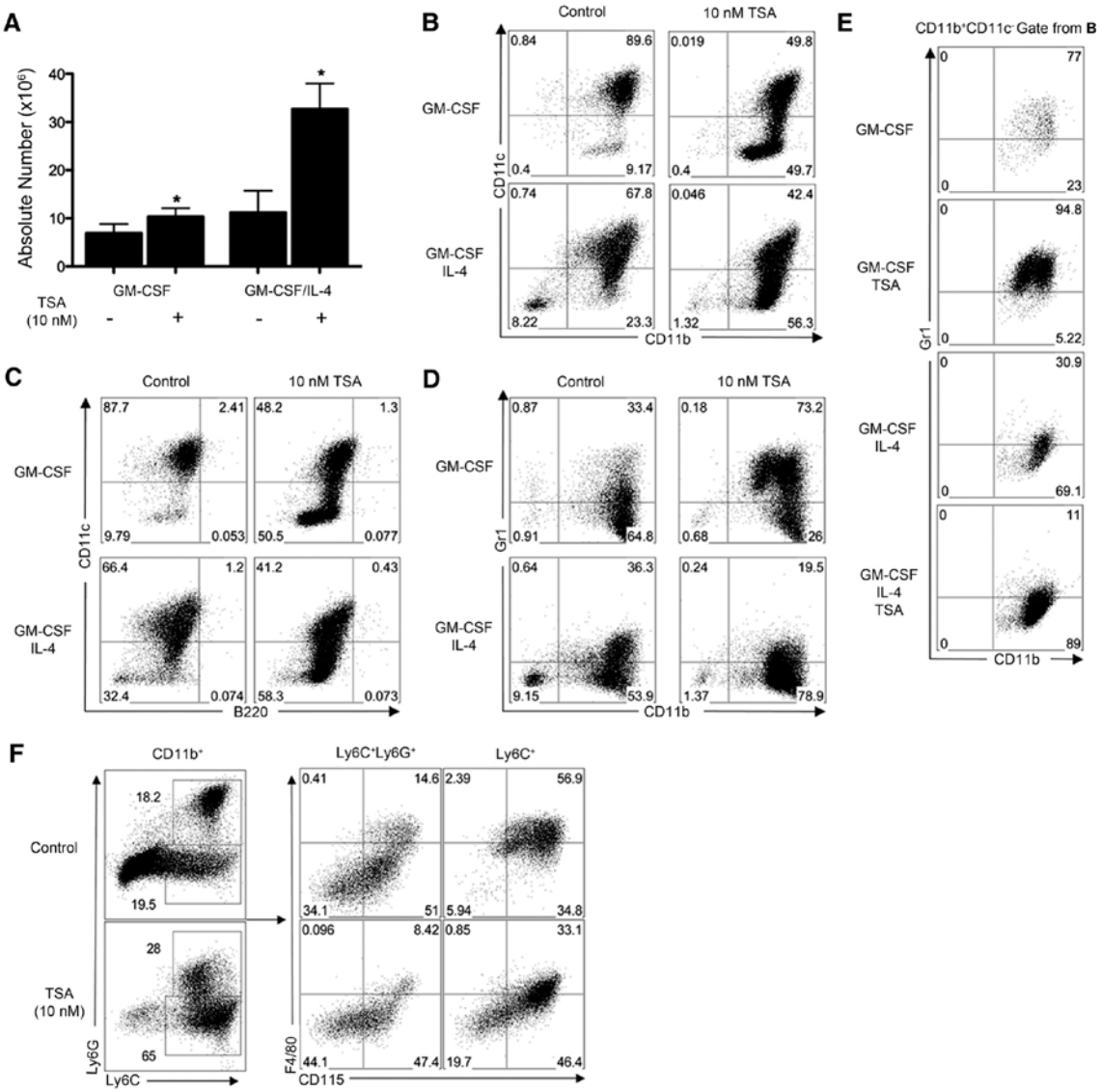

Figure 2.1 TSA enhances the expansion of BM-derived cells stimulated with GM-CSF ± IL-4 and favors the development of CD11b$^+$Gr1$^+$ cells in GM-CSF-stimulated BM cell cultures

(A) C57BL/6 (B6) mouse BM cells were grown in GM-CSF ± IL-4, with or without 10 nM TSA. Addition of TSA to cultures led to enhanced expansion of total cells in GM-CSF and especially GM-CSF + IL-4. Absolute cell number is the cell count per 20 ml culture starting with 3-4x10^6 BM cells. Means + 1SD for n=3 separate experiments are shown. Cell samples were analyzed by flow cytometry for phenotypic (B) myeloid DC (mDC; CD11b$^+$CD11c$^+$), (C) plasmacytoid DC (pDC; B220$^+$CD11c$^+$) or (D) putative myeloid-derived suppressor cells (MDSC; CD11b$^+$Gr1$^+$). (B) TSA reduced the percentage of mDC by half when added to GM-CSF cultures and by approximately one third when added to GM-CSF + IL-4-stimulated cultures. (C) Cultures stimulated with GM-CSF ± IL-4 produced few pDC, and this was unchanged by TSA treatment. (D) TSA led to increased generation of CD11b$^+$Gr1$^+$ cells in GM-CSF-stimulated cultures, and this effect was reversed by addition of IL-4. (E) CD11b$^+$CD11c$^-$ cells were gated and analyzed for Gr1 expression. The majority of these cells expressed Gr1 in the absence of IL-4, while most cells were Gr1$^-$ in the presence of IL-4, regardless of exposure to TSA. (F) Cells from GM-CSF-stimulated cultures were further analyzed for expression of Ly6C and G (Gr1 epitopes), F4/80 and CD115. TSA-exposed cultures showed a 3-fold increase in CD11b$^+$Ly6C$^+$ cells that were mostly F4/80^{int}CD115$^+$. Data are representative of n=2 or more independent experiments. *, $p<0.05$ when compared to untreated control, determined by unpaired Student's 't' test.

There is evidence that GM-CSF expands murine MDSC from BM cells *in vitro* (25, 64) and *in vivo* (245). We found that TSA had contrasting effects on the generation of $CD11b^{+}Gr1^{+}$ presumptive MDSC, depending on whether the BM cells were differentiated in GM-CSF alone or GM-CSF + IL-4 (Figure 2.1D). Specifically, addition of TSA to cultures stimulated with GM-CSF alone led to an increase in the incidence of $CD11b^{+}Gr1^{+}$ cells, from approximately 30% to 70% (Figure 2.1D). Similarly, addition of SAHA, a clinically-utilized HDACi, to GM-CSF-stimulated BM cell cultures led to a dose-dependent increase in total cells (Figure 2.2B) and the frequency of $CD11b^{+}Gr1^{+}$ cells (Figure 2.2C and 2.2D). By contrast, in GM-CSF + IL-4-stimulated cultures, TSA reduced the incidence of $CD11b^{+}Gr1^{+}$ cells but enhanced the incidence of $CD11b^{+}Gr1^{-}$ cells (from approximately 55% to 80%; Figure 2.1D). Within the $CD11b^{+}CD11c^{-}$ gate, TSA enhanced the proportion of $CD11b^{+}Gr1^{+}$ cells in GM-CSF-stimulated cultures and alternatively, $CD11b^{+}Gr1^{-}$ cells when GM-CSF + IL-4 was used (Figure 2.1E). Therefore, TSA differentially alters BM cell myeloid lineage commitment when stimulated with GM-CSF, depending on the presence of IL-4.

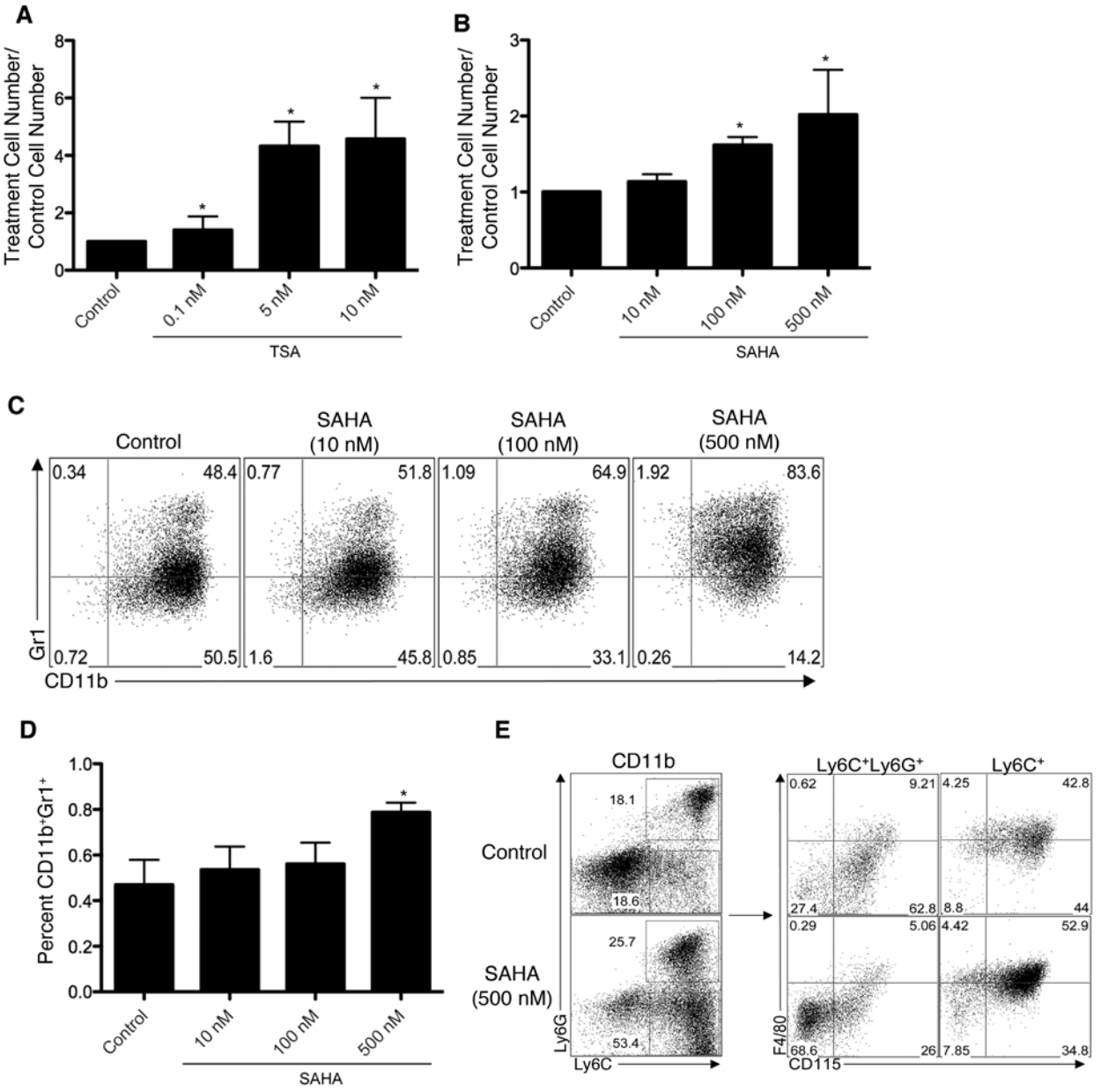

Figure 2.2 TSA and SAHA dose-dependently increase BM cell yield, and SAHA enhances $CD11b^+Gr1^+$ cells in GM-CSF-stimulated BM cell cultures

(A) B6 BM cells were cultured with GM-CSF, IL-4 and increasing concentrations of TSA. Total cells were enumerated on d7. Means + 1SD of n=5 or more independent experiments are shown. (B) B6 BM cells were cultured with GM-CSF and increasing concentrations of SAHA and enumerated on d7. (C, E) BM cell phenotype from (B) was determined by flow cytometry, and the frequency of $CD11b^+Gr1^+$ cells was quantified (D). Data in (B) and (D) are the means + 1SD of n=3 independent experiments, while (C) and (E) are representative of n=3 independent experiments. *, $p<0.05$ when compared to untreated control, determined by unpaired Student's 't' test.

MDSC are divided into granulocytic and monocytic subsets depending on their expression of the Gr1 epitopes, Ly6G and Ly6C, respectively, and further classified according to their expression of CD115 (macrophage colony-stimulating factor receptor) and F4/80 (6). Cells isolated from GM-CSF-stimulated BM cultures were assessed for their expression of these surface markers. Addition of TSA to these cultures increased the frequency of $CD11b^{+}Ly6C^{+}$ cells that were predominantly $F4/80^{int}CD115^{+}$ approximately 3-fold (Figure 2.1F). SAHA demonstrated a comparable effect on GM-CSF-mediated BM cell differentiation, favoring $CD11b^{+}Ly6C^{+}F4/80^{int}CD115^{+}$ cells (Figure 2.2E).

2.4.2 TSA expands hematopoietic stem and progenitor cells correlating with increased growth of myeloid cells

Since TSA could induce differing myeloid lineage cell expansion, depending on the presence of IL-4 (Figure 2.1), we considered that its effect might be to enhance the proliferation of an upstream myeloid progenitor, with divergent differentiation directed by appropriate hematopoietic growth factors. To ascertain the influence of TSA on myeloid progenitors, BM cells cultured in GM-CSF ± IL-4 were harvested on d4 following 2d exposure to TSA (10 nM) and assessed for myeloid lineage progenitors, as demonstrated in Figure 2.3.

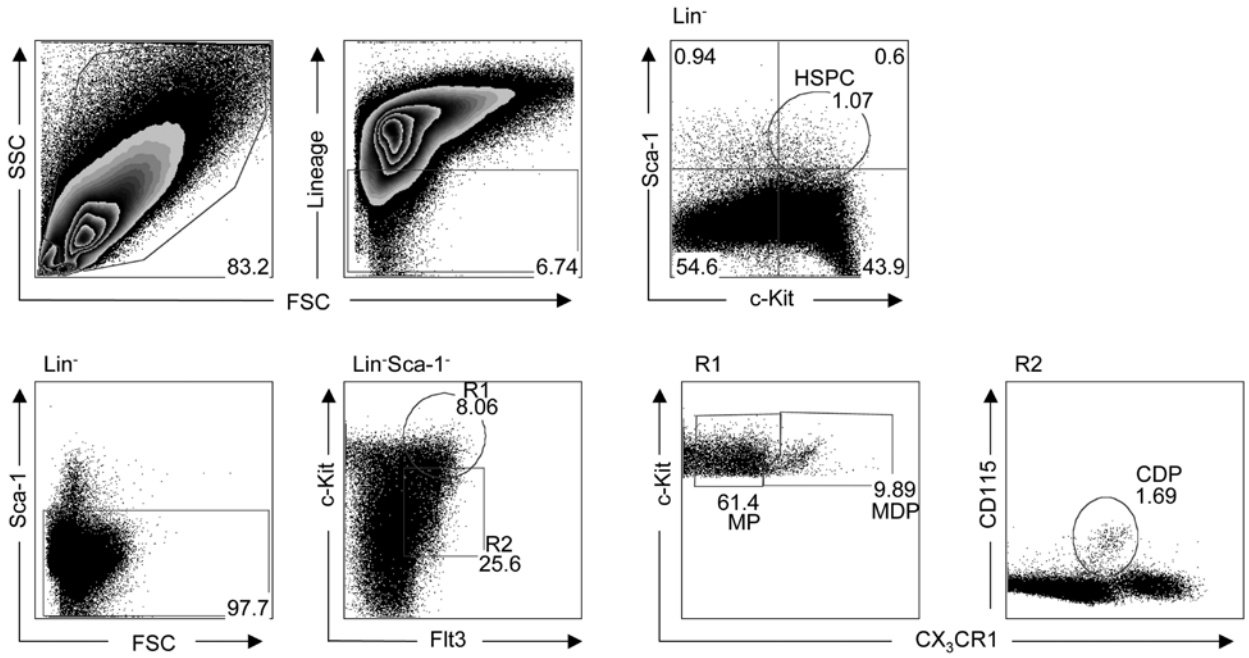

Figure 2.3 Gating strategy used to identify HSPC and myeloid progenitors in BM cultures on d4

The percentage of cells within the gate is indicated. The example shown is from a GM-CSF + IL-4-stimulated culture. Hematopoietic stem and progenitor cells (HSPC; $Lin^-Sca\text{-}1^+c\text{-}Kit^+$), myeloid progenitors (MP; $Lin^-Sca\text{-}1^-c\text{-}Kit^{hi}Flt3^+CX_3CR1^-$), monocyte and dendritic cell progenitors (MDP; $Lin^-Sca\text{-}1^-c\text{-}Kit^{hi}Flt3^+CX_3CR1^+$) and common plasmacytoid and conventional DC progenitors (CDP; $Lin^-Sca\text{-}1^-c\text{-}Kit^{lo}Flt3^+CD115^+CX_3CR1^+$) are shown. Data are representative of n=3 independent experiments.

TSA significantly increased the frequency of hematopoietic stem and progenitor cells (HSPC; $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$), irrespective of the presence of IL-4 (Figure 2.4A and 2.4B), but exerted no significant enhancing effect on the frequency of myeloid progenitors (MP), monocyte and DC progenitors (MDP) or common DC progenitors (CDP). However, cultures containing exogenous IL-4 exhibited an increased frequency of MP compared to those stimulated with GM-CSF alone (Figure 2.4A). In agreement with the increased frequency of HSPC in cultures exposed to TSA, the absolute number of HSPC in these cultures was increased approximately 3-fold, irrespective of the presence of IL-4 (Figure 2.4C). BM cells exposed to GM-CSF and TSA overnight demonstrated enhanced acetylation of histone H4 (Figure 2.4D). These findings indicate that the increased number of myeloid lineage cells induced by TSA (Figure 2.1) correlated with early expansion of HSPC, with the fate of the HSPC being determined by specific exogenous growth factor addition to the cultures.

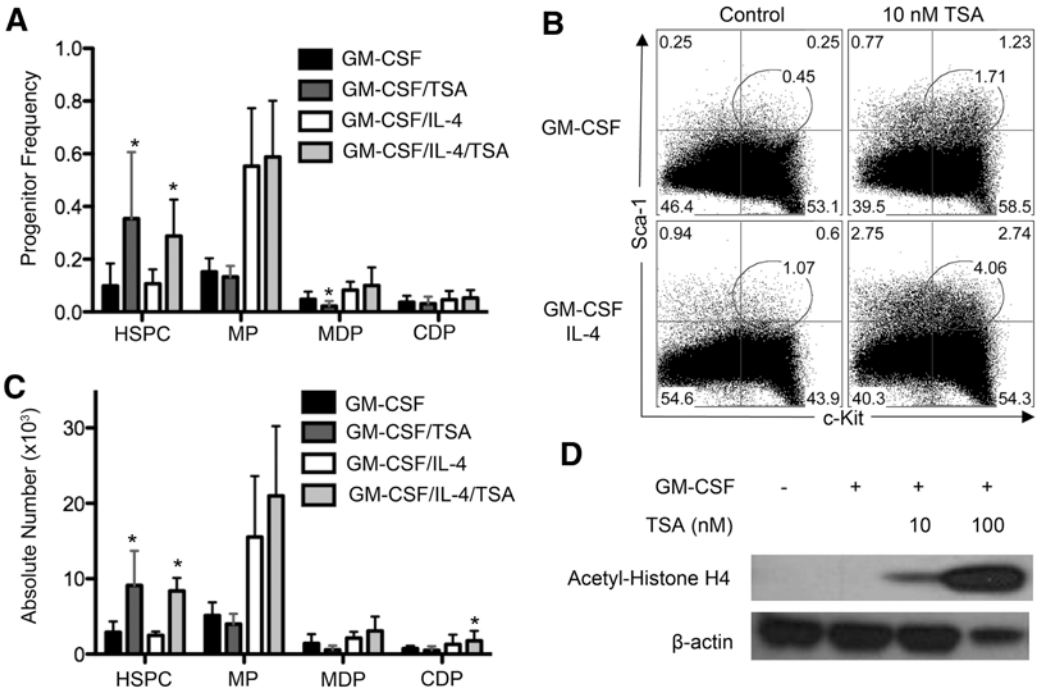

Figure 2.4 Culture of BM cells in TSA leads to an increased frequency and absolute number of hematopoietic stem and progenitor cells

B6 BM cells were harvested on d4 following 2d exposure to TSA (10 nM). (A) The relative frequency of HSPC and specific progenitor cell populations was determined by flow cytometry. HSPC were defined as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$, myeloid progenitors (MP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{hi}Flt3^{+}CX_3CR1^{-}$; monocyte and DC progenitors (MDP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{hi}Flt3^{+}CX_3CR1^{+}$, and common DC progenitors (CDP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{lo}Flt3^{+}CD115^{+}CX_3CR1^{+}$. Data are means + 1SD of n=4 independent experiments. (B) Representative flow cytometry plots demonstrating the expansion of HSPC by TSA. Plots shown are Lin^{-} gated and representative of n=4 independent experiments. (C) The absolute number of HSPC and specific myeloid progenitor populations was determined per plate. Cell number was determined by multiplying the average total number of cells isolated per plate for each treatment by the frequency of each progenitor. Data are means + 1SD of n=3 independent experiments. *, $p<0.05$ when compared to untreated control, determined by unpaired Student's 't' test. (D) BM cells were stimulated with GM-CSF (1000 U/ml) for 2 h prior to treatment with TSA at the indicated concentrations overnight. Acetyl-histone H4 and β-actin were detected by immunoblot. Data are representative of n=2 independent experiments.

2.4.3 Increased numbers of mDC and functional MDSC are generated in the presence of TSA

We next sought to verify the function of presumptive MDSC recovered from TSA-treated, GM-CSF- and GM-CSF + IL-4-stimulated cultures. A >2-fold increase in the number of $Gr1^{+}$ cells recovered by positive bead selection from GM-CSF alone-stimulated cultures was achieved in the presence of TSA (10 nM; Figure 2.5A), verifying the increase in these cells determined by

flow cytometry (Figure 2.1). Cells isolated by Gr1 positive selection were primarily $CD11b^{+}Ly6C^{+}F4/80^{int}CD115^{+}$ (Figure 2.5B). The ability of the presumptive MDSC to suppress alloreactive $CD3^{+}$ T cell proliferation in MLR was determined. $CD11b^{+}Gr1^{+}$ cells differentiated in TSA were as potent in suppressing allogeneic T cell responses on a per cell basis as those from control cultures (Figure 2.5C and Figure 2.6A). MDSC from GM-CSF + IL-4-stimulated cultures (that yielded fewer cells [Figure 2.5A]) were slightly more effective on a per cell basis in suppressing $CD3^{+}$ T cell proliferation than those generated in GM-CSF alone (Figure 2.6B and 2.6C).

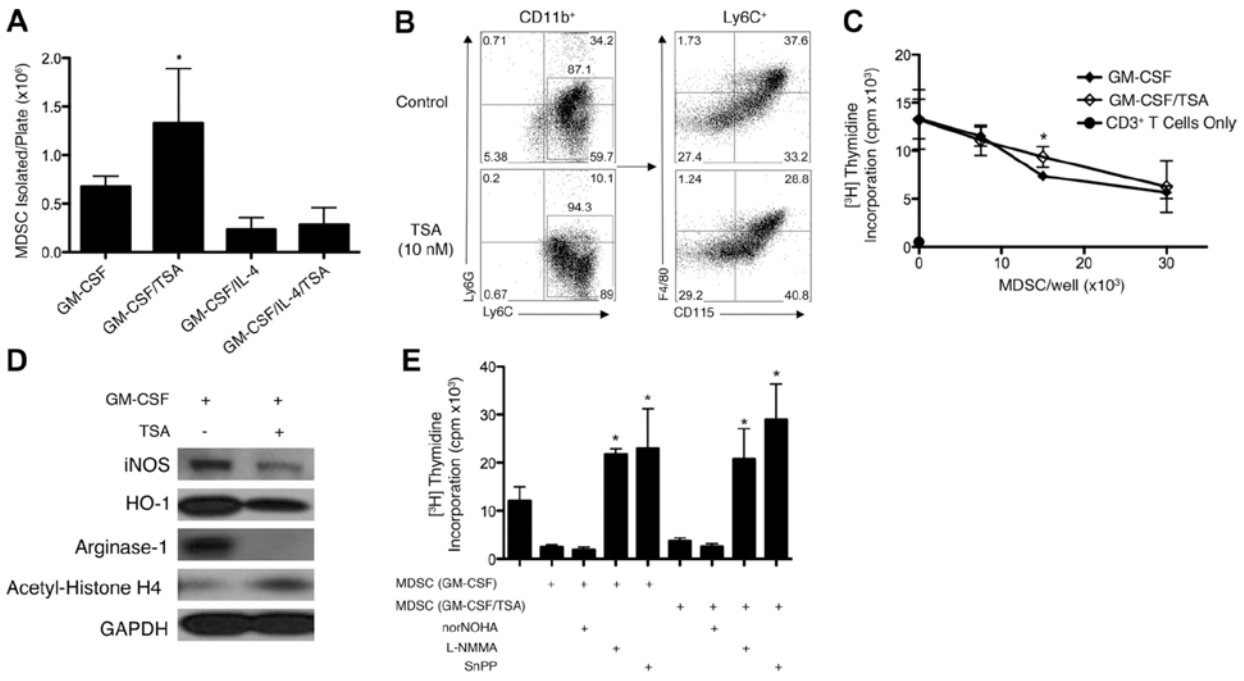

Figure 2.5 Putative MDSC expanded by TSA exhibit suppression of T cell proliferation

(A) MDSC were isolated by $Gr1^+$ immunomagnetic bead selection from 7d cultures in GM-CSF ± IL-4 supplemented with TSA (10 nM). TSA increased the numbers of MDSC from GM-CSF-stimulated cultures. Differentiation of BM cells in GM-CSF with TSA yielded approximately twice as many MDSC as untreated cultures. Significantly more MDSC were isolated from GM-CSF-stimulated cultures than those containing GM-CSF + IL-4. (B) The phenotype of MDSC isolated by Gr1 positive selection was determined by flow cytometry. (C) MDSC (B6) isolated from cultures described in (A) were tested as suppressors in allogeneic MLR at graded ratios from 0 to $3x10^4$ MDSC with $1x10^4$ DC (B6) as stimulators and $4x10^5$ $CD3^+$ T cells (BALB/c) as responders. Addition of TSA to GM-CSF-stimulated cultures did not alter the ability of MDSC to suppress T cell proliferation. (D) Expression of arginase-1, iNOS, and HO-1 were detected by immunoblot of freshly-isolated Gr1 cells from GM-CSF-stimulated BM cell cultures or those containing TSA. Acetyl-histone H4 was also detected by immunoblot, and GAPDH was included as a loading control. (E) MLR were set up as in (C), with $3x10^4$ MDSC. Inhibitors of arginase-1 (norNOHA), iNOS (L-NMMA) and HO-1 (SnPP) were added to cultures on d0. MDSC from GM-CSF- and GM-CSF/TSA-stimulated cultures required both iNOS and HO-1 to suppress T cell proliferation. The bar graph in (A) shows the means + 1SD of n=2 independent experiments. The dot plots in (B) are representative of n=3 independent experiments, and the data in (C), (D), and (E) are representative of n=2 independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant compared to untreated control (*, $p<0.05$) using a Student's 't' test.

MDSC suppress T cell proliferation by local depletion of L-arginine by arginase-1 and inducible nitric oxide synthase (iNOS). NO produced by iNOS also has direct T cell suppressive effects (6). Likewise, the cytoprotective and immunoregulatory enzyme heme oxygenase-1 (HO-1) is a critical mechanism for the T cell suppressive capacity of MDSC (77). Cells isolated by

Gr1 positive selection were immunoblotted to detect expression of arginase-1, iNOS, and HO-1 (Figure 2.5D). $Gr1^+$ cells isolated from TSA-exposed cultures had lower expression of iNOS and HO-1 and did not express arginase-1. As expected, these cells had higher levels of acetylated histone H4 compared to control. Arginase-1 and iNOS are inhibited by the addition of nor-NOHA and L-NMMA, respectively (64). Additionally, HO-1 is selectively inhibited by SnPP. Addition of these inhibitors to MLR containing MDSC from GM-CSF-stimulated cultures demonstrated that iNOS and HO-1 are independently indispensable for the suppressive function of the MDSC (Figure 2.5E). The function of MDSC expanded in the presence of TSA also required these enzymes. Thus, although $Gr1^+$ cells isolated from TSA-exposed cultures displayed reduced levels of iNOS and HO-1, they still displayed potent T cell suppressive function. Taken together, these results indicate that increased numbers of MDSC can be recovered from BM cell cultures containing TSA, and that the suppressive function and mechanism of these cells is similar to those from control cultures.

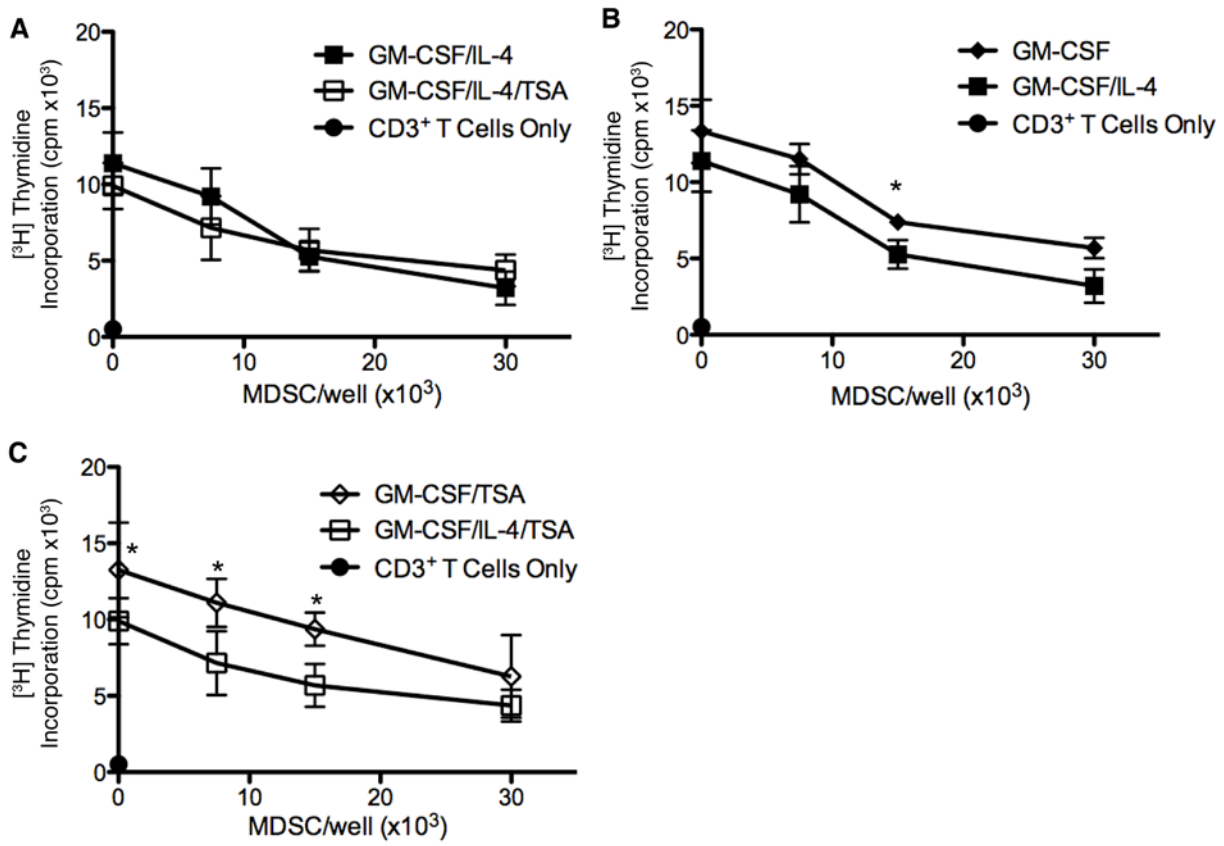

Figure 2.6 MDSC generated from BM cultures stimulated with GM-CSF and IL-4 are moderately more suppressive than those generated in GM-CSF alone

(A) Addition of TSA to GM-CSF + IL-4-stimulated cultures did not alter the ability of MDSC to suppress T cell proliferation. (B)-(C) Comparison of MDSC generated in GM-CSF or GM-CSF + IL-4 alone (B) with those also generated with 10 nM TSA (C). The plots in (A)-(C) are representative of n=2 or more independent experiments. Error bars indicate SD of triplicate wells. Differences were considered significant (*, $p<0.05$) using a Student's 't' test.

Histone deacetylase inhibition during the generation of mDC from BM cells stimulated with GM-CSF + IL-4 led to increased numbers of a homogenous population of $CD11c^+$ cells with reduced granularity compared to those from control cultures (Figure 2.7A and 2.7B). Consistent with previous reports (230, 231), exposure of BM cells stimulated with GM-CSF + IL-4 to increasing concentrations of TSA led to a dose-dependent reduction in several functionally important DC surface molecules, including CD40, CD80, CD86, MHC class II Ag ($I\text{-}A^b$) and the chemokine receptor CCR7 (Figure 2.7C and 2.7D).

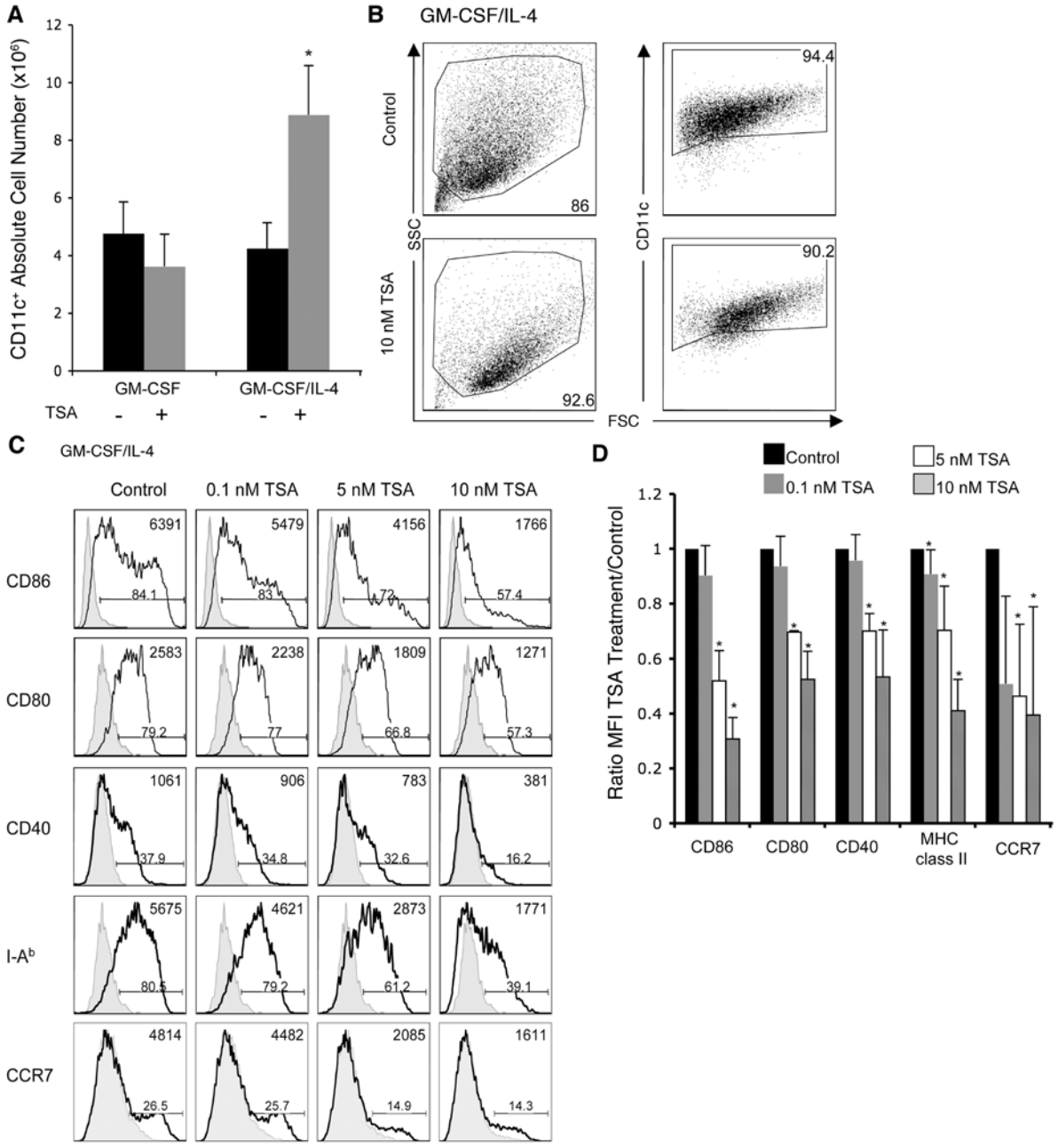

Figure 2.7 TSA enhances expansion of immature DC in GM-CSF + IL-4-stimulated BM cell cultures

(A) B6 BM cells were grown in GM-CSF ± IL-4, with or without 10 nM TSA. mDC were isolated by CD11c purification on d7 and enumerated by trypan blue exclusion. IL-4 was required for TSA-induced expansion of DC, leading to a 2-fold increase compared to cultures without TSA. Absolute cell number is the cell count per 20 ml culture starting from 3-4x10^6 BM cells. (B) mDC generated in BM cell cultures stimulated with GM-CSF + IL-4 and TSA (10 nM) demonstrated reduced size (forward scatter) and granularity (side scatter) compared to control. (C) DC phenotype was determined on d7 by flow cytometry after CD11c immunomagnetic purification. Mean fluorescence intensity (MFI) is shown in the upper right and % positive cells indicated above the horizontal bar. Isotype control is shown in grey. (D) Bar graph representing the ratio of MFI for the treatment group versus the control group for each molecule examined. Data are representative of n=3 or more independent experiments. Data in (A) and (D) are the means + 1SD obtained from n=3 or more independent experiments. Differences from control values were considered significant (*) if $p<0.05$ using an unpaired Student's 't' test.

2.4.4 TSA augments GM-CSF-mediated expansion of $CD11b^+Gr1^+$ BM cells *in vivo*

In order to extend our *in vitro* finding that TSA enhanced MDSC production from BM cells (Figure 2.1 and 2.5), we tested its ability to promote expansion of these myeloid cells *in vivo* (Figure 2.8). In mice given TSA together with GM-CSF, a significant increase in the absolute number of BM cells was observed (Figure 2.8A). TSA increased the absolute number and adjusted frequency of total BM $CD11b^+Gr1^+$ cells significantly compared with mice treated with GM-CSF alone (Figure 2.8B-D). Conversely, $B220^+$ cells, representing a non-myeloid cell population, were reduced significantly (Figure 2.8B and 2.8C). Of the two subsets of $CD11b^+Gr1^+$ cells, TSA exerted a greater effect on the $Gr1^{hi}$ subset (Figure 2.8C and 2.8D).

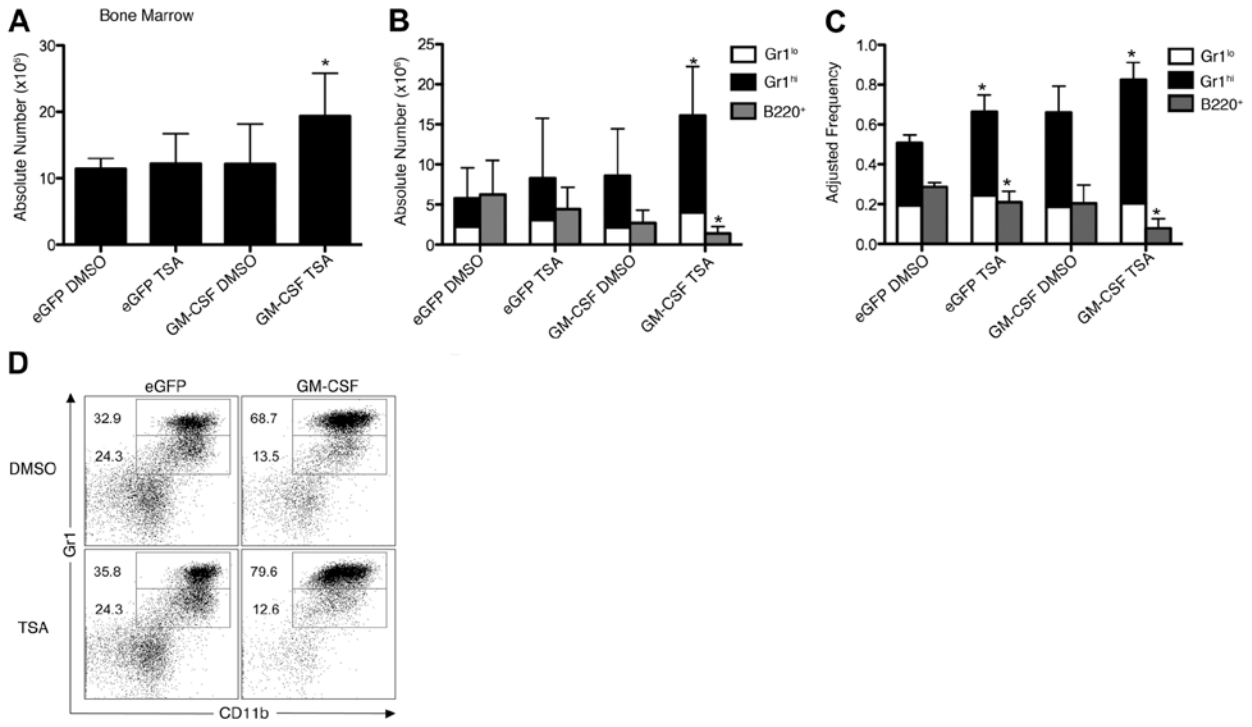

Figure 2.8 TSA enhances GM-CSF-mediated in vivo expansion of CD11b$^+$Gr1$^+$ cells in BM

B6 mice receiving murine GM-CSF plasmid or control eGFP plasmid were treated with TSA (1 mg/kg/d) or vehicle (DMSO) for 5d. (A) Viable BM cells from one femur were enumerated using trypan blue exclusion. The absolute number (B) and adjusted frequency (C) of Gr1^{hi} and Gr1^{lo} subsets of BM CD11b$^+$Gr1$^+$ cells were identified by flow cytometry. (D) Representative flow cytometry plots of CD45$^+$-gated cells (1x10^4 events) demonstrating MDSC gates. The data in (A)-(C) are plotted as means + 1SD for n=3 independent experiments with 2-4 animals per group. Significance values (*, $p<0.05$) were determined by unpaired Student's 't' test for total CD11b$^+$Gr1$^+$ cells and B220$^+$ cells.

2.4.5 TSA enhances GM-CSF-mediated expansion of splenic MDSC *in vivo*, correlating with enhanced mobilization of peripheral HSPC

In BM cell cultures, TSA enhanced the number and frequency of HSPC (Figure 2.4) correlating with an increase in myeloid cells (Figure 2.1, 2.2, 2.5 and 2.7). We ascertained whether this effect of TSA could also be seen *in vivo* following GM-CSF administration (Figure 2.9). Although no significant effect was observed on HSPC residing in the BM (Figure 2.9A and 2.9B), TSA significantly enhanced the absolute number (Figure 2.9C) and frequency (Figure 2.9D and 2.9E) of HSPC in the spleens of mice treated with GM-CSF.

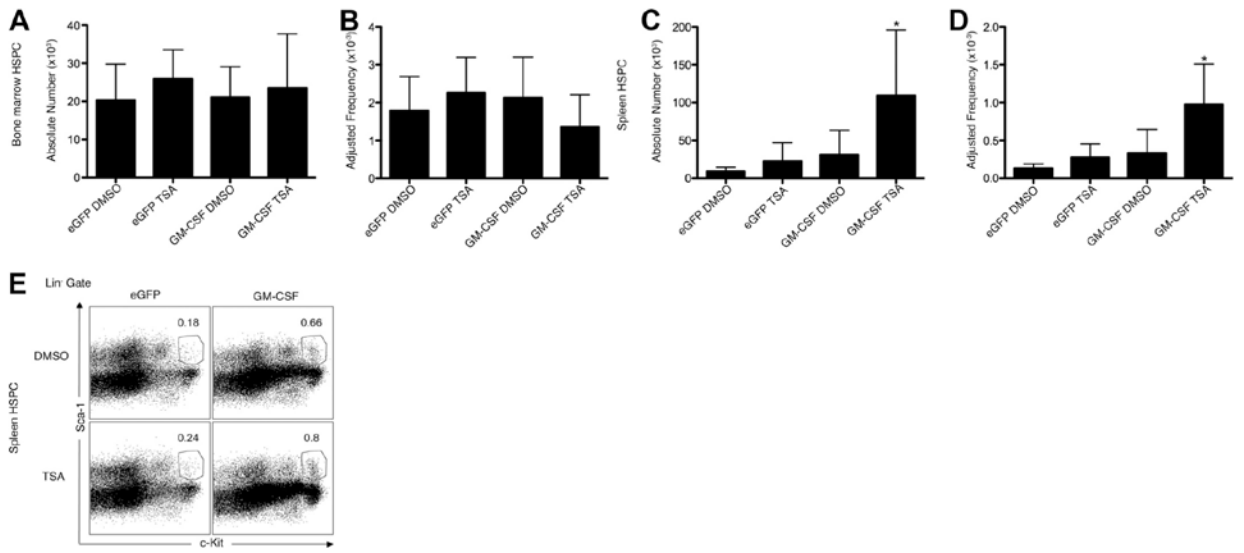

Figure 2.9 TSA increases the mobilization of peripheral HSPC following GM-CSF administration

B6 mice given GM-CSF or eGFP plasmid were treated with TSA or vehicle (DMSO). HSPC ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$) were identified in BM cell (A)-(B) and splenocyte populations (C)-(D) by flow cytometry. The absolute number of HSPC in the BM from one femur (A) and the spleen (C) was determined from the adjusted frequency of HSPC in the BM (B) and spleen (D). Data are plotted as means + 1SD of n=5-9 from 3 independent experiments. Significance values (*, $p<0.05$) were determined by unpaired Student's 't' test. (E) Representative flow cytometry plots of Lin^--gated cells demonstrating splenic HSPC.

Unlike BM cells (Figure 2.8A), the absolute number of splenocytes was not increased significantly in mice given TSA in combination with GM-CSF (Figure 2.10A). However, as observed in the BM (Figure 2.8B-D), TSA significantly increased the absolute number and frequency of splenic $CD11b^+Gr1^+$ cells in animals given GM-CSF (Figure 2.10B-D). $CD11b^+Gr1^+$ cells that expanded in the spleen were predominantly $Gr1^{lo}$. mDC expansion was not significantly increased by TSA in the spleens of mice given GM-CSF (data not shown), reflecting the need for IL-4 to allow expansion of mDC by TSA (Figure 2.7A). As in the BM, $B220^+$ cells were not increased significantly in the spleen (Figure 2.10B and 2.10C); however, the trend in increased absolute number reflected an increased absolute number of splenocytes in TSA-treated mice given GM-CSF (Figure 2.10A). These splenocytes yielded a significantly increased number of $Gr1^+$ cells (Figure 2.10E) that were functionally intact suppressors of

allogeneic T cell proliferation in MLR (Figure 2.10F). Thus, these data confirm *in vivo* our *in vitro* finding that TSA augments GM-CSF-mediated expansion of MDSC correlating with an increased number of splenic HSPC.

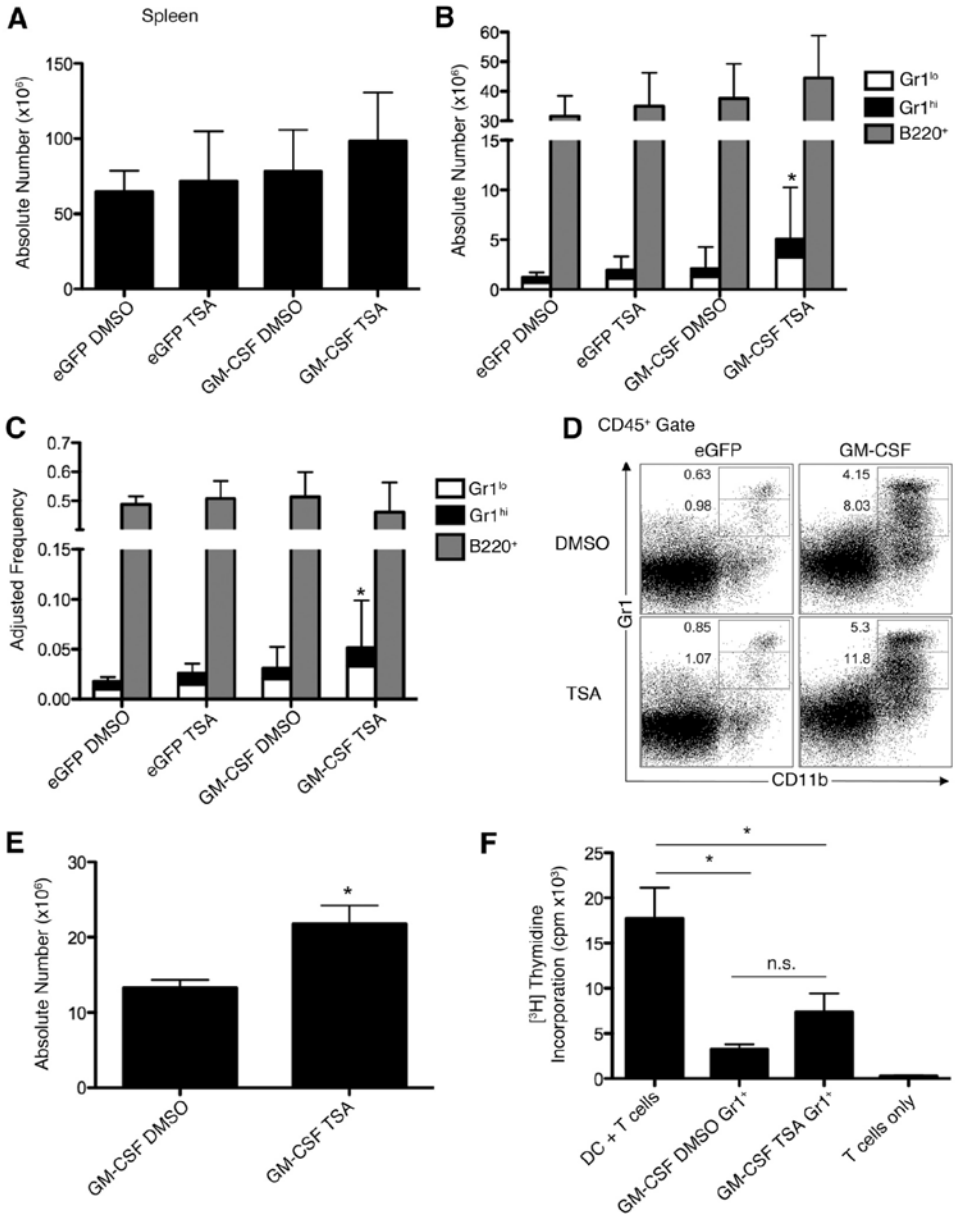

Figure 2.10 TSA enhances GM-CSF-mediated expansion of suppressive $CD11b^+Gr1^+$ cells in the spleen

(A) Splenocytes were enumerated using trypan blue exclusion from mice receiving hydrodynamic infusion of GM-CSF or eGFP plasmid and treated with TSA or vehicle (DMSO). The absolute number (B) and adjusted frequency (C) of $Gr1^{hi}$ and $Gr1^{lo}$ subsets of splenic $CD11b^+Gr1^+$ cells were determined by flow cytometry. $B220^+$ cells were included as a negative control. (D) Representative flow cytometry plots of $CD45^+$-gated cells ($5x10^4$ events) demonstrating $CD11b^+Gr1^{hi}$ and $CD11b^+Gr1^{lo}$ gates. (E) $Gr1^+$ cells were isolated by positive bead selection from $50x10^6$ pooled splenocytes of each treatment group and live cells enumerated by trypan blue exclusion. (F) Isolated $Gr1^+$ cells were tested as suppressors in MLR using B6 DC as stimulators and BALB/c $CD3^+$ T cells as responders. Positive controls consisted of DC + T cells, in the absence of added $Gr1^+$ cells. The data in (A)-(C) are plotted as means + 1SD for n=4 independent experiments, with 2-4 animals per group. Significance (*, $p<0.05$) was determined by unpaired Student's 't' test for total $CD11b^+Gr1^+$ cells and $B220^+$ cells. The data in (E)-(F) are means + 1SD of n=2 independent experiments, with 2-4 mice per group. Significance values (*, $p< 0.05$) were determined by unpaired Student's 't' test in (E) and paired Student's 't' test in (F).

2.5 DISCUSSION

Several recent studies have described the *ex vivo* expansion of early stem cell progenitors using HDACi (249-253). While others have shown (249, 251, 252) that TSA increases HSPC proliferation under conditions favoring stem cell renewal, our finding that TSA expands HSPC in GM-CSF ± IL-4-stimulated BM cell cultures reveals that this effect is maintained even under strong differentiation signals from GM-CSF. A similar effect has been seen with human $CD34^+$ cells stimulated with G-CSF in the presence of the HDACi valproic acid (250). To our knowledge, the present study presents the first evidence that *in vivo* administration of HDACi enhances peripheral HSPC, likely due to their increased mobilization from the BM since recent evidence demonstrates that HDACi may reduce HSPC adherence to BM stromal cells (254).

In addition, we found that addition of TSA to BM cell cultures stimulated with GM-CSF skews the differentiation of myeloid cells depending on the presence of IL-4. Exposure of BM cell cultures to TSA reduced the phenotypic differentiation of DC (Figure 2.1B), favoring a population of $CD11c^{-}CD11b^{+}$ cells. These observations are in agreement with previous reports that HDACi block DC differentiation (230, 233). The non-histone protein signal transducer and activator of transcription (STAT) 3 represents an intriguing target to further explain the mechanism of HDACi enhancement of MDSC expansion. STAT3 has a known acetylation site at lysine 685 that is required for its dimerization and transcriptional regulation (255), and exposure to HDACi promotes STAT3 activity by this mechanism (232). GM-CSF (245) and M-CSF (256) are among the soluble factors that induce MDSC expansion. Many of these factors activate STAT3, which is currently believed to be the most important transcription factor regulating MDSC expansion (6). Furthermore, STAT3 is indispensable for the maintenance of embryonic stem cell pluripotency and self-renewal (257). These findings make STAT3 a strong candidate for the molecular regulation of MDSC and potentially HSPC expansion in our model.

Importantly, our findings provide a novel platform for the expansion of MDSC *in vitro*. Typically, MDSC are expanded *in vivo* using either GM-CSF or inflammatory stimuli such as bacterial LPS. However, GM-CSF has been used to generate monocytic MDSC *in vitro* from BM cells, and these MDSC required iNOS for suppressive activity (25). Our data support these findings where GM-CSF-expanded monocytic MDSC required iNOS activity to suppress T cell proliferation, but they did not require arginase-1 activity (Figure 2.5E). In addition, MDSC isolated from BM cultures in our studies required HO-1 for their suppressive activity, similar to those isolated from endotoxin-exposed mice (77). In contrast, Highfill et al (64) used GM-CSF to expand monocytic $CD11b^{+}Ly6C^{+}$ MDSC, but in their system, the MDSC required arginase-1 for

suppressive activity. This discrepancy may result from our use of higher concentrations of GM-CSF (1000 U/ml vs. 250 U/ml), a longer culture period (7d vs. 4d) or different selection strategy (Gr1 vs. CD11b). TSA-expanded MDSC demonstrated a similar suppressive potency and mechanism of suppression as control MDSC, suggesting that the ability of HDACi to block complete myeloid differentiation (230, 233) leads to a build-up of immature myeloid cells and myeloid progenitors with intact suppressive function. In order to increase the potential to move this strategy to the clinic, further studies will be required to determine if this approach can also be used to expand human MDSC from peripheral blood mononuclear cells.

In vitro and *in vivo* methods to generate MDSC represent valuable tools to explore the potential use of these potent regulatory cells for therapeutic purposes. Two groups (64, 89) have generated MDSC *in vitro* that suppressed GVHD, and others have adoptively transferred MDSC to alleviate experimental inflammatory bowel disease (13) and promote skin allograft survival (84). The use of HDACi to enhance GM-CSF-mediated expansion of MDSC will allow for further interrogation of these cells to develop their therapeutic potential.

3.0 FLT3 LIGAND EXPANDS AND ACTIVATES MYELOID-DERIVED SUPPRESSOR CELLS IN A STAT3-INDEPENDENT MANNER

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3.1 ABSTRACT

The Flt3 (Fms-like tyrosine kinase 3)-Flt3 ligand (Flt3L) pathway is critically involved in the differentiation and homeostasis of myeloid cells, including dendritic cells (DC); however, its role in the expansion and function of myeloid-derived suppressor cells (MDSC) has not been determined. Herein, we describe the ability of Flt3L to expand and activate murine MDSC capable of suppressing allograft rejection upon adoptive transfer. While Flt3L expands and augments the stimulatory capacity of myeloid DC, MDSC expanded by Flt3L have increased suppressive activity. Although STAT3 is considered the central transcription factor for MDSC expansion and function, inhibition of STAT3 did not block, but augmented Flt3L-mediated MDSC expansion, without affecting their suppressive capacity. However, STAT3 inhibition reduced Flt3L-mediated DC expansion, signifying that STAT3 regulates the switch between normal DC differentiation and accumulation of suppressive myeloid cells. Together, these findings enhance understanding of the immunomodulatory properties of Flt3L.

3.2 INTRODUCTION

Myeloid-derived suppressor cells (MDSC) are recently-characterized innate immunoregulatory cells that expand under inflammatory conditions, such as cancer, sepsis, allograft rejection, and autoimmunity [reviewed (6, 258)]. Although mouse and human MDSC exhibit considerable heterogeneity, they share the ability to induce apoptosis or suppress T cell proliferation and secretion of cytokines (19, 258). In mice, MDSC are broadly identified as $CD11b^{+}Gr1^{+}$ cells, while cell morphology and differential surface expression of the Gr1 antigens Ly6G and Ly6C

distinguish granulocytic ($CD11b^+Ly6G^+$) and monocytic ($CD11b^+Ly6C^+$) subsets (6). Expansion and activation of MDSC occurs through the action of growth factors that promote myelopoiesis (90, 245) and pro-inflammatory cytokines (6, 90).

Fms-like tyrosine kinase 3 [Flt3; CD135; fetal liver kinase-2 (Flk2)] is a receptor tyrosine kinase expressed on hematopoietic stem cells and early precursors (259). The Flt3-Flt3 ligand (Flt3L) pathway is critically involved in dendritic cell (DC) homeostasis (234-236). Flt3L activates the transcription factor STAT3 (192), that is strongly implicated in MDSC expansion and function (6). However, the potential of Flt3L to support MDSC expansion/activation is undefined. Due to the potent ability of Flt3L to increase myeloid precursors and activate STAT3, we hypothesized that Flt3L-driven myelopoiesis would not only promote development of DC, but also suppressive MDSC.

Herein, we report that Flt3L expands and activates $Ly6G^+$ and $Ly6C^+$ MDSC. In contrast, DC expanded by Flt3L are more stimulatory than steady-state DC. Although DC expansion by Flt3L is dependent on STAT3, surprisingly, inhibition of STAT3 enhances Flt3L-induced mobilization of MDSC, without affecting their suppressive function. Adoptive transfer of Flt3L-mobilized MDSC, but not steady-state $CD11b^+Gr1^+$ cells, prolongs fully MHC-mismatched cardiac allograft survival.

3.3 MATERIALS AND METHODS

Animals and drug administration. 8-12 week old male BALB/c ($H2K^d$) or C57BL/6 (B6; $H2K^b$) mice were given r human Flt3L (Chinese hamster ovary cell-derived; 10 μg/d i.p., Amgen) for 10 d. The STAT3 inhibitor S31-201 (5 mg/kg; Selleck Chemicals) was administered i.p. as described (260). All studies were performed under an institutional animal care and use committee-approved protocol.

Flow cytometry. Cell surface and intracellular marker expression was analyzed as described (202, 238).

MLR and suppression assay. MDSC were isolated from splenocytes by positive selection with FITC anti-Ly6C, PE anti-Ly6G, or PE anti-Gr1 using anti-FITC or anti-PE microbeads (Miltenyi Biotec), as described (238, 261). DC were isolated by CD11c immunomagnetic bead selection and γ-irradiated (20 Gy). T cell proliferation using DC as stimulators was assessed in MLR at 72 h by [^{3}H] TdR incorporation.

Vascularized heart transplantation. Heterotopic intra-abdominal mouse heart transplantation was performed and graft survival monitored as described (261).

Statistics. Data are presented as means ± 1 standard deviation. Significant differences between means and survival curves were determined using a two-tailed, Student's 't' test and log-rank test, respectively.

3.4 RESULTS AND DISCUSSION

We first examined myeloid populations expanded by Flt3L. Total splenocyte number was increased (Figure 3.1A), and in agreement with previous studies (234, 236), Flt3L increased the frequency and absolute number of conventional DC (Figure 3.1B-D). Splenic $CD11b^{+}Gr1^{+}$ cells were also increased in frequency and absolute number by Flt3L; however, macrophage ($CD11b^{int}F4/80^{hi}$) frequency was unchanged (Figure 3.1B and 3.1C). Of the lymphoid populations examined, Flt3L only increased the frequency of $Foxp3^{+}$ regulatory T cells (Treg); however, the absolute number of all T cell populations was augmented (Figure 3.2A-C). This increase in natural thymic-derived Treg is thought to be due to DC-mediated expansion of Treg (262), and similar results have been seen in human subjects following Flt3L administration (263). Since Flt3L has been given to human subjects safely (263, 264), it will be necessary to determine if Flt3L expands human MDSC in the peripheral blood.

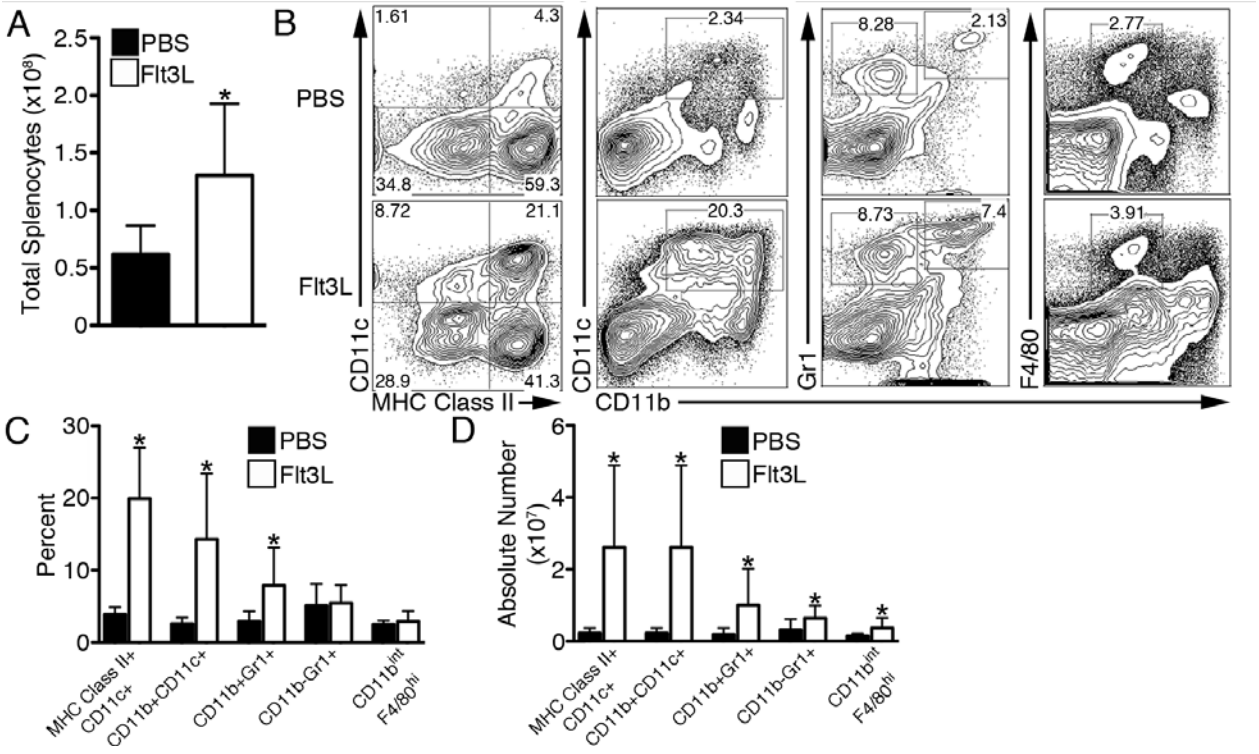

Figure 3.1 Flt3L expands myeloid DC and CD11b$^+$Gr1$^+$ cells

(A) Total viable splenocytes from Flt3L-treated mice were enumerated using trypan blue exclusion. (B)-(C) The frequency of splenic myeloid DC (CD11c$^+$MHC class II$^+$; CD11b$^+$CD11c$^+$), CD11b$^+$Gr1$^+$ cells, and macrophages (CD11b^{int}F4/80^{hi}) within CD45$^+$-gated cells was determined and (D) absolute number quantified. Data are representative of n≥2 experiments with n≥3 mice per group. * $p<0.05$.

We next sought to further characterize surface antigen expression on CD11b$^+$Gr1$^+$ cells expanded by Flt3L. Flt3L induced expansion of both CD11b$^+$Ly6C$^{int/hi}$ and CD11b$^+$Ly6G$^+$ cells (Figure 3.3A-C). CD11b$^+$Ly6C^{hi} cells expressed an intermediate level of F4/80 and were CD115 (M-CSF receptor)$^+$, consistent with surface antigen expression described for MDSC (Figure 3.3A) (6). CD11b$^+$Ly6C^{int} and CD11b$^+$Ly6G$^+$ cells were F4/80$^-$ and expressed only low levels of CD115 (Figure 3.3A and 3.3B). Previously, Solheim et al (265) described an increase in splenic CD11b$^+$Gr1$^+$ cells following adenoviral delivery of Flt3L to tumor-bearing mice; however, the suppressive function of these cells was not assessed. We now show that both Ly6C$^+$ monocytic and Ly6G$^+$ granulocytic MDSC from Flt3L-treated mice are suppressive in MLR (Figure 3.3D).

Moreover, both Flt3L-expanded Ly6C$^+$ and Ly6G$^+$ MDSC were significantly more potent suppressors than their counterparts from steady-state control mice (Figure 3.3D). By contrast, CD11c$^+$ DC isolated from Flt3L-treated mice demonstrated increased allogeneic T cell stimulatory capacity (Figure 3.3E). Thus, these data reveal that Flt3L has reciprocal capacities to expand functionally distinct populations of stimulatory DC and suppressive MDSC.

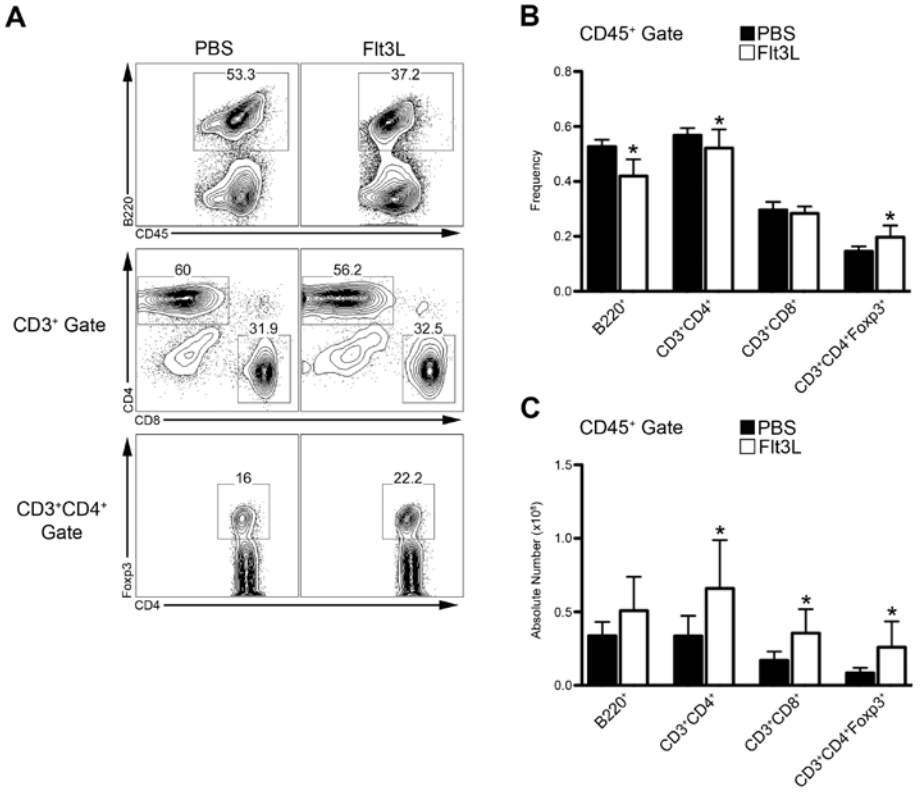

Figure 3.2 Flt3L reduces the frequency of splenic B cells and pan CD4$^+$ T cells, while Foxp3$^+$ Treg frequency is increased

(A) CD45$^+$-gated BALB/c splenocytes were analyzed for the frequency of lymphoid cell subsets, including B cells (B220$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$), CD8$^+$ T cells (CD3$^+$CD8$^+$), and Treg (CD3$^+$CD4$^+$Foxp3$^+$). (B) The frequency of lymphoid subsets was determined across multiple experiments and (C) absolute numbers quantified. Data are representative of n≥6 mice. * $p<0.05$ by two-tailed Student's 't' test.

STAT3 is considered the key regulator of MDSC expansion and suppressive function (6, 49), and Flt3L is a potent activator of STAT3 (192). Therefore, we next ascertained whether STAT3 is required for Flt3L-mediated MDSC expansion. Inhibition of STAT3 *in vivo* during

Flt3L administration reduced the frequency of myeloid DC (Figure 3.4A and 3.4B), consistent with earlier reports using conditional STAT3 knockout mice (192). By contrast, expansion of $CD11b^{+}Gr1^{+}$ cells by Flt3L was augmented by STAT3 inhibition (Figure 3.4A and 3.4B). Absolute numbers of $CD11c^{+}$ and $Gr1^{+}$ cells isolated from spleens of these mice demonstrated a similar pattern with STAT3 inhibition (Figure 3.4C-E). Flt3L causes an accumulation of common myeloid progenitors in conditional STAT3 knockout mice (192). Our data suggest that this increase in common myeloid progenitors resulting from blocked DC differentiation may serve as a source of immunosuppressive MDSC. Significantly, STAT3 inhibition at the time of MDSC mobilization by Flt3L did not block activation of their suppressive ability (Figure 3.4F). Further study will be required to elucidate the signaling pathway by which Flt3L expands MDSC. There is evidence that the Flt3 pathway is capable of activating STAT5 and MAP kinases (266-268), which are implicated in MDSC expansion and survival (49, 59, 61). MDSC suppress T cell proliferation through several immunosuppressive enzymes, including arginase-1, inducible nitric oxide synthase, heme oxygenase-1 (HO-1), and IDO (6, 77, 269). Both steady-state control and Flt3L-mobilized $Gr1^{+}$ cells independently required HO-1 and IDO for suppression of T cell proliferation (Figure 3.4F).

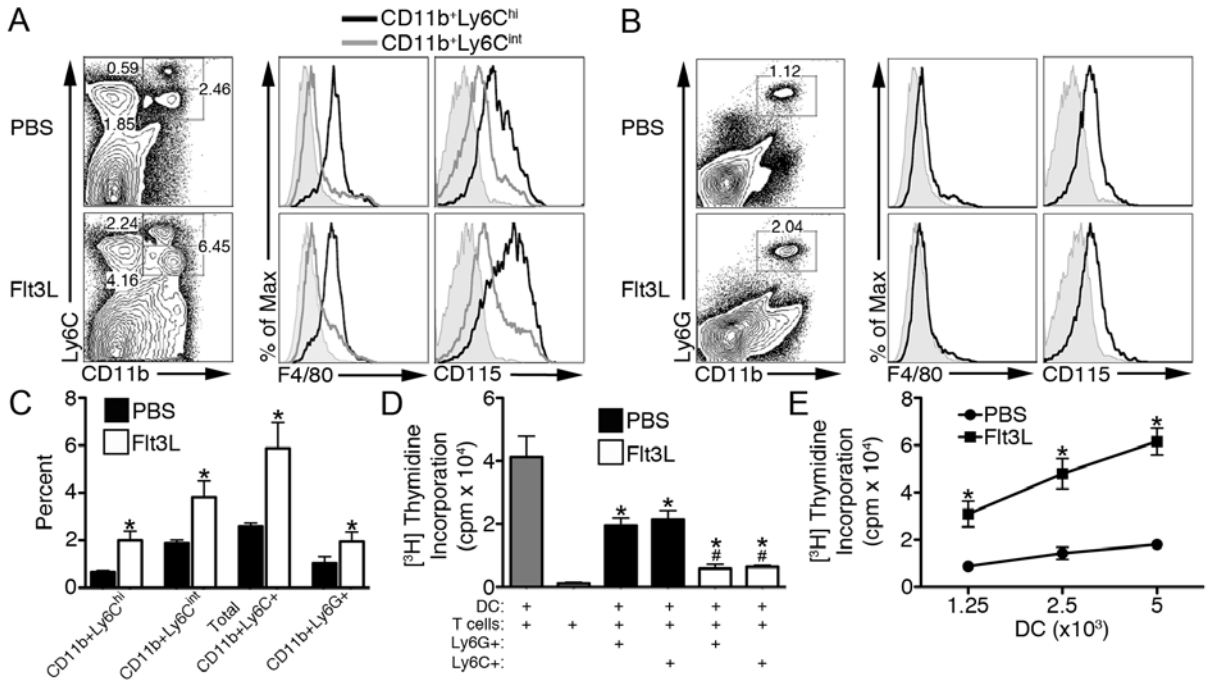

Figure 3.3 Flt3L expands myeloid DC with augmented T cell stimulatory capacity but suppressive CD11b$^+$Gr1$^+$ cells

F4/80 and CD115 expression was determined on (A) CD11b$^+$Ly6C$^{int/hi}$ and (B) CD11b$^+$Ly6G$^+$ splenocytes and (C) their frequency quantified. (D) BALB/c Ly6C$^+$ and Ly6G$^+$ splenocytes ($2x10^5$) were used as suppressors of B6 CD3$^+$ T cells ($2x10^5$) stimulated with Flt3L-mobilized BALB/c CD11c$^+$ DC ($2x10^4$). (E) BALB/c CD11c$^+$ DC were used to stimulate B6 CD3$^+$ T cells ($1x10^5$). Data are representative of ≥2 experiments with n≥3 mice per group. (A)-(C) and (E), * $p<0.05$. (D) *, # $p<0.05$ compared to DC + T cells and PBS administration, respectively.

Adoptively-transferred BM-derived MDSC inhibit graft-versus-host disease (64), and allogeneic skin transplant-activated MDSC transferred to skin graft recipients prolong survival (84). Furthermore, MDSC are required for the induction of organ transplant tolerance by costimulation blockade (23). In the present study, Gr1$^+$ cells isolated from splenocytes of Flt3L-treated mice, but not control mice, significantly prolonged fully MHC-mismatched cardiac allograft survival in the absence of additional immunosuppression (Figure 3.4G), thus demonstrating their *in vivo* suppressive function. Flt3L has been reported to have both pro- and anti-inflammatory effects in disease models (270-272). Thus, the varying impact of Flt3L on immune responses *in vivo* remains poorly understood, and the role of MDSC in these models has

not been explored. Our data show that Flt3L mediates STAT3-independent expansion of suppressive MDSC but STAT3-dependent expansion of stimulatory $CD11c^+$ DC. These findings have significant clinical relevance for the use of Flt3L as an immune modulating agent. Combination of Flt3L administration with STAT3 inhibition could promote effective immune regulation, given the expectation that STAT3 inhibition will counter Flt3L-driven DC generation but allow MDSC expansion and activation. Conversely, delivery of Flt3L with inhibitors of IDO or HO-1 would be expected to augment previously demonstrated immune adjuvant properties of Flt3L.

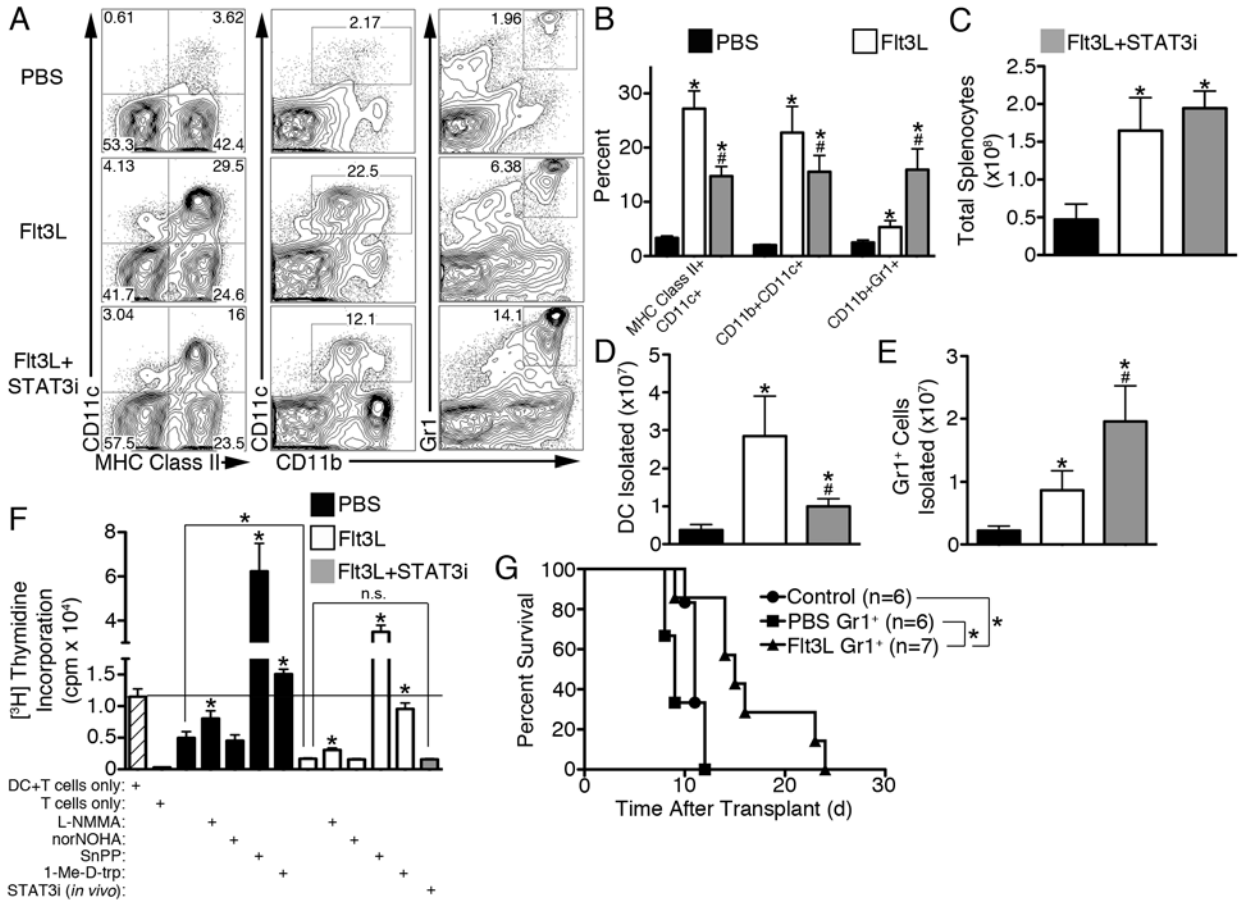

Figure 3.4 Flt3L-mobilized MDSC are expanded in a STAT3-independent manner and prolong cardiac allograft survival

(A) STAT3 inhibitor (STAT3i) was co-administered with Flt3L, and DC and MDSC frequency was determined and (B) quantified. (C) Total viable splenocytes, (D) DC, and (E) $Gr1^+$ cells were isolated and enumerated. (F) Splenic BALB/c $Gr1^+$ cells ($1x10^5$) were used as suppressors of BALB/c $CD3^+$ T cells ($1x10^5$) stimulated with B6 Flt3L-mobilized $CD11c^+$ DC ($1.25x10^4$). Inhibitors of nitric oxide synthase (N^G-Methyl-L-arginine; L-NMMA; 0.5 mM), arginase-1 (N^ω-Hydroxy-nor-L-arginine; norNOHA; 0.5 mM), HO-1 (tin protoporphyrin; SnPP; 0.15 mM), or IDO (1-methyl-D-tryptophan; 1-Me-D-trp; 0.2 mM) were added to co-culture where indicated. Horizontal line represents no suppression. (G) $5x10^6$ BALB/c $Gr1^+$ cells were administered to BALB/c recipients i.v., 1 d before B6 heart transplant, and allograft survival was monitored. Data are representative of n=2 independent experiments. (A)-(E) * and # $p<0.05$ compared to PBS and Flt3L groups, respectively. (F) * $p<0.05$ compared to PBS or Flt3L $Gr1^+$ cells in the absence of inhibitor unless otherwise indicated.

4.0 MURINE DENDRITIC CELL RAPAMYCIN-RESISTANT AND RICTOR-INDEPENDENT MTOR CONTROLS IL-10, B7-H1 AND REGULATORY T CELL INDUCTION

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4.1 ABSTRACT

Mammalian target of rapamycin (mTOR) is an important, yet poorly understood integrative kinase that regulates immune cell function. mTOR functions in two independent complexes,- mTOR complex (mTORC) 1 and 2. The immunosuppressant rapamycin (RAPA) inhibits mTORC1 but not mTORC2, and causes a paradoxical reduction in anti-inflammatory IL-10 and B7-H1 expression by dendritic cells (DC). Using catalytic mTOR inhibitors and DC lacking mTORC2, we show that restraint of STAT3-mediated IL-10 and B7-H1 expression during DC maturation involves a RAPA-insensitive and mTORC2-independent mTOR mechanism. Relatedly, catalytic mTOR inhibition promotes B7-H1- and IL-1β-dependent DC induction of regulatory T cells (T_{reg}). Thus, we define an immunoregulatory pathway where RAPA-sensitive mTORC1 in DC promotes effector T cell expansion and RAPA-insensitive mTORC1 restrains T_{reg} induction. These findings identify the first known RAPA-insensitive mTOR pathway that is not mediated solely by mTORC2 and have implications for the use of catalytic mTOR inhibitors in inflammatory disease settings.

4.2 INTRODUCTION

Dendritic cells (DC) are innate professional antigen (Ag)-presenting cells (APC) that initiate and regulate adaptive immunity (94, 95). DC control T cell reactivity by coordinating display of Ag to T cells in the context of major histocompatibility class (MHC) molecules with the delivery of co-stimulation and cytokines that dictate T cell differentiation and function. While co-stimulatory molecules support T cell responses, co-inhibitory molecules restrain T cell reactivity. Our understanding of the precise molecular mechanisms regulating expression of pro-inflammatory versus regulatory signals by DC remains unclear.

B7-homolog 1 [B7-H1, programmed death-1 ligand 1 (PD-L1); CD274] is a B7 family co-inhibitory molecule expressed on DC in a regulated manner that binds to programmed death-1 (PD-1; CD279) on activated T cells thereby reducing their proliferation and pro-inflammatory cytokine production (150, 274). The B7-H1/PD-1 pathway plays a crucial role in the maintenance of peripheral tolerance (275). B7-H1 stimulates T cell secretion of anti-inflammatory IL-10 (276) and promotes the induction, maintenance, and function of regulatory T cells (T_{reg}) from naïve T cells (277). Importantly, the precise upstream mechanisms regulating B7-H1 expression remain elusive, and the differential regulation of co-stimulatory versus co-inhibitory molecule expression is poorly understood, despite their central role in the activation and constraint of adaptive T cell responses by DC.

Mammalian target of rapamycin (mTOR) is a highly-conserved, serine/threonine kinase that controls APC and T cell function (172, 237). The mTOR kinase performs the catalytic function of two independent complexes, -mTOR complex (mTORC) 1 and mTORC2 (175, 176). mTORC1 consists of mTOR, raptor, mLST8 and PRAS40, while mTORC2 contains mTOR, rictor, mLST8, mSIN1, and PROTOR (177). Although rapamycin (RAPA) is a potent allosteric

inhibitor of mTORC1, it exerts little activity against RAPA-insensitive mTORC2 (175, 176). However, novel, highly-selective adenosine triphosphate (ATP)-competitive active site mTOR inhibitors that block both mTOR-containing complexes have revealed RAPA-resistant mTORC1 and mTORC2 signaling in non-immune cells (184, 185). mTORC1 inhibition suppresses conventional DC maturation and promotes their tolerogenicity (95, 172, 189). Conversely, RAPA has paradoxical, pro-inflammatory effects on DC, including increased secretion of IL-12p70 and IL-1β, with concomitant reduced secretion of IL-10 and expression of B7-H1 (195, 201-203, 278, 279). These effects on DC are mediated by augmentation of NF-κB activity and reduction in signal transducer and activator of transcription (STAT)3 activity (195, 278, 279).

RAPA-insensitive mTORC2 regulates the actin cytoskeleton in non-immune cells (175, 176), while insight is emerging into its function in T lymphocytes. Selective deletion of mTORC2 in T cells impairs their differentiation into Th1 and Th2 (204) or only Th2 subsets (205). In contrast to the well-defined role of mTORC1, little is known about the function of mTORC2 in APC or innate immunity. In this study, we sought to define the role of RAPA-resistant mTOR in molecular regulation of the ability of DC to promote T cell immunity.

We find that RAPA-resistant mTOR negatively regulates conventional DC STAT3-mediated IL-10 and B7-H1 expression. Deletion of the mTORC2 subunit rictor had the opposite effect, suggesting that residual RAPA-resistant mTORC1 activity or dual mTORC1 and 2 inhibition mediates this central anti-inflammatory pathway in DC. Enhanced STAT3 activation in DC exposed to ATP-competitive mTOR inhibitors correlated with a reduction in SOCS3. Functionally, mTORC1-inhibited DC were unable to stimulate proliferation of $Foxp3^-$ effector T cells (T_{eff}), while ATP-competitive mTOR inhibition additionally promoted the induction of $Foxp3^+$ T_{reg} in a B7-H1-dependent manner that also required IL-1β. These data reveal a novel,

RAPA-resistant anti-inflammatory pathway in DC that regulates IL-10 and B7-H1 and identifies divergent regulation of T_{eff} and T_{reg} responses by DC due to RAPA-sensitive and RAPA-resistant mTOR.

4.3 MATERIALS AND METHODS

Animals and drug administration. Male C57BL/6 (B6; $H2K^b$), BALB/c ($H2K^d$), B6.129S2-*Irf1*^{tm1Mak}/J (interferon regulatory factor-1; IRF-1 null), B6.129P2-*Il10*^{tm1Cgn}/J (IL-$10^{-/-}$), B6.129X1-*Ebi3*^{tm1Rsb}/J (Epstein-Barr Virus induced gene 3; Ebi$3^{-/-}$), and B6.129S1-*Il12b*^{tm1Jm}/J (IL-12/23p$40^{-/-}$) mice were purchased from The Jackson Laboratory. Male B6.129S2-*Il6*^{tm1Kopf}/J (IL-$6^{-/-}$) and IL-10-green fluorescent protein (IL-10-gfp) mice were kindly provided by Dr. A. Jake Demetris and Dr. David Rothstein, respectively (University of Pittsburgh). Femurs and tibiae from mice containing loxP-flanked FoxO1, FoxO3, and FoxO4 crossed to mice containing tamoxifen-inducible Cre recombinase under the ROSA26 promoter (FoxO1/3/$4^{fl/fl}$ x ROSA26-CreERT2) were used to generate BM-derived DC. B7-$H1^{-/-}$ mouse pairs on a B6 background were kindly provided by Dr. Lieping Chen (Johns Hopkins University) and bred at the University of Pittsburgh. Mice containing loxP-flanked rictor (rictor$^{fl/fl}$) and ROSA26-CreERT2 were maintained at the University of Pittsburgh. WYE-125132 (50 mg/kg (280) intraperitoneally; Selleck Chemicals) was dissolved in DMSO and administered in vehicle composed of 5% Tween 80 and 54% polyethylene glycol (Sigma-Aldrich; average M_n 300) at the time of LPS injection (*E. coli* 0111:B4; Sigma-Aldrich; 100 μg/kg intraperitoneally).

DC differentiation, purification, and stimulation. DC were generated from BM cells, as described (202, 238) using recombinant (r) mouse GM-CSF and r mouse IL-4 (both 1000 U/ml; R&D Systems). On d8 of culture, myeloid DC were selected from non-adherent cells by anti-CD11c immunomagnetic bead purification (Miltenyi Biotec). Torin1 (184) [kindly provided by Dr. Nathanael S. Gray (Dana-Farber Cancer Institute) or purchased from Tocris Bioscience], AZD8055 (281) (Selleck Chemicals), WYE-125132, or RAPA (LC Laboratories) were added to cultures on d2 at the concentration indicated and refreshed on d4 and d6. Where indicated, TLR4-specific LPS (*S. minnesota* R595; 100 ng/ml; Alexis Biochemicals) was used to stimulate DC cultures on d7 for 16-18h. In some experiments, 250 nM STAT3 inhibitor VII (EMD Chemicals) was added on d7, 2h before LPS stimulation.

Flow cytometric analyses. Cell surface and intracellular staining was performed as described (202, 238). Fluorochrome-conjugated mAbs were purchased from eBioscience, BD Bioscience, or Biolegend. P-STAT3 quantification by flow cytometry was performed as described (278). Data were acquired using an LSR II or LSR Fortessa flow cytometer (BD Bioscience) and analyzed using FlowJo 8.8.6 (Tree Star). Percent P-STAT3 positive cells were determined using Overton Subtraction (FlowJo) by comparing experimental samples with corresponding control samples stained with secondary antibody (Ab) only.

Mixed leukocyte reaction (MLR). γ-irradiated (20 Gy) DC (B6; $1x10^4$) were used as stimulators in 5d allogeneic MLR as described (190). Normal $CD4^+CD25^-$ BALB/c T cell responders ($1x10^5$) were isolated by negative selection and labeled with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's protocol (Invitrogen). In some experiments, neutralizing Ab was added to cultures at 10 μg/ml [αIL-1β (B122), αIL-10 (JES5-16E3; BD Bioscience), αB7-H1 (MIH5), or αPD-1 (RMP1-14; eBioscience)]. The absolute

number of cells in MLR was determined on d5 by trypan blue exclusion or flow cytometry using CountBright Absolute Counting Beads (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total cell numbers were multiplied by T_{reg} frequency to determine the absolute number of T_{reg} in MLR.

Cytokine quantification. Enzyme-linked immunosorbent assays were performed on cell-free DC supernatants according to the manufacturer's instructions to quantify IL-12p40 and IL-6 (Biolegend). IL-1β was quantified by cytometric bead array (BD Bioscience) according to the manufacturer's protocol.

Tamoxifen induction of Cre recombinase. 100 nM (Z)-4-hydroxytamoxifen (4OHT; Sigma-Aldrich) was added to BM cultures on d0 to delete FoxO transcription factors from FoxO1/3/4$^{fl/fl}$ x ROSA26-CreERT2 BM-derived DC. Tamoxifen (15 mg/ml; Sigma-Aldrich) was administered intraperitoneally in sunflower seed oil (Sigma-Aldrich) every 2d for 3 doses to rictor$^{fl/fl}$ x ROSA26-CreERT2 mice. BM cells were harvested from these mice 7d later and cultured to generate rictor$^{-/-}$ BM-derived DC.

Immunoblot. Immunoblotting was performed on DC lysates as described (202, 238) using primary Abs from Cell Signaling with the exception of GAPDH (Novus Biologicals) and SOCS5 (Thermo Scientific). Densitometry was performed using ImageJ (National Institutes of Health).

Human monocyte-derived DC (MoDC) generation. Peripheral blood mononuclear cells (PBMC) were isolated from normal leukopacks (Central Blood Bank, Pittsburgh, PA) by Ficoll-Hypaque density gradient centrifugation followed by CD14 immunomagnetic bead selection (Miltenyi Biotec, Auburn, CA). PBMC ($1.1x10^6$ cells/ml) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with pooled human AB serum (5% v/v, Gemini Bio-

Products, West Sacramento, CA), L-glutamine (2 mM, Cellgro Manassas, VA), penicillin/streptomycin (100 U/ml, Lonza, Walkersville, MD), rhGM-CSF (1000 U/ml, R&D Systems, Minneapolis, MN) and rhIL-4 (1000 U/ml, R&D Systems) for 6 d. One half of the medium was refreshed every 2 d. Cultures were stimulated with TLR4-specific LPS (100 ng/ml; *S. minnesota* R595, Alexis Biochemicals, San Diego, CA) on d4. RAPA (10 ng/ml; LC Laboratories, Woburn, WA) or Torin1 (Tocris Bioscience, Minneapolis, MN) were added on d2 and d4.

Human CD34$^+$ cell-derived DC. Human CD34$^+$ cell-derived DC were generated from immunomagnetic bead-selected (Miltenyi Biotec, Germany), cryopreserved CD34$^+$ cells from blood donors after informed consent, as described (282) with minor modifications. Briefly, 4x10^4 CD34$^+$ cells/ml were expanded in 6-well plates (Greiner, Germany) for 6 d with GM-CSF (50 ng/ml, Miltenyi Biotec), stem cell factor (25 ng/ml, Miltenyi Biotec) and TNFα (2.5 ng/ml, Miltenyi Biotec) in RPMI-1640 supplemented with L-glutamine, penicillin/streptomycin and 10% heat-inactivated FCS (PAA, Germany). On d6, cells were washed then cultured in fresh medium for an additional 7 d with GM-CSF (50 ng/ml) and IL-4 (50 ng/ml, eBioscience, Germany). Vehicle control or mTOR inhibitors were added on d2 at the indicated concentration and refreshed every 2 d. DC were stimulated with LPS (100 ng/ml; 0111:B4 strain, Sigma-Aldrich, Germany) for 24 h.

Statistical Analyses. Results are expressed as means + 1 standard deviation (SD). Unpaired, two-tailed Student's 't' test was used to determine the significance of differences between means (GraphPad Prism).

4.4 RESULTS

4.4.1 RAPA-resistant mTOR is a negative regulator of conventional DC B7-H1 expression

We first examined the surface phenotype of B6 BM-derived conventional DC propagated under conditions of either mTORC1 inhibition with RAPA or mTORC1 and 2 inhibition with the ATP-competitive mTOR inhibitor Torin1. As reported previously for RAPA (201), DC differentiated in Torin1 were small and homogenous compared to control DC (Figure 4.1A). Although mTOR inhibition did not affect $CD11c^+$ DC differentiation (Figure 4.1B), both RAPA and Torin1 reduced the DC yield from BM cell cultures (Figure 4.1C). In agreement with previous studies (195, 278), RAPA reduced the expression of CD80, CD86, B7-H1, and B7-DC on both unstimulated and LPS-stimulated DC (Figure 4.1D-E and Figure 4.2). While Torin1 also reduced CD80, CD86, and B7-DC expression, by contrast with RAPA, Torin1 selectively and dose-dependently enhanced B7-H1 expression on DC (Figure 4.1D-E). Expression of MHC molecules, CD40, CD54, CD80 and B7RP-1 was similarly regulated by RAPA and Torin1 (Figure 4.2). B7-H3 and B7-H4 were not detected (data not shown). Torin1 reduced CD86 expression similarly to RAPA on human monocyte-derived DC (Mo-DC) and $CD34^+$ cell-derived DC; however, B7-H1 expression was spared by Torin1 resulting in significantly higher expression than on RAPA-exposed DC (Figure 4.3).

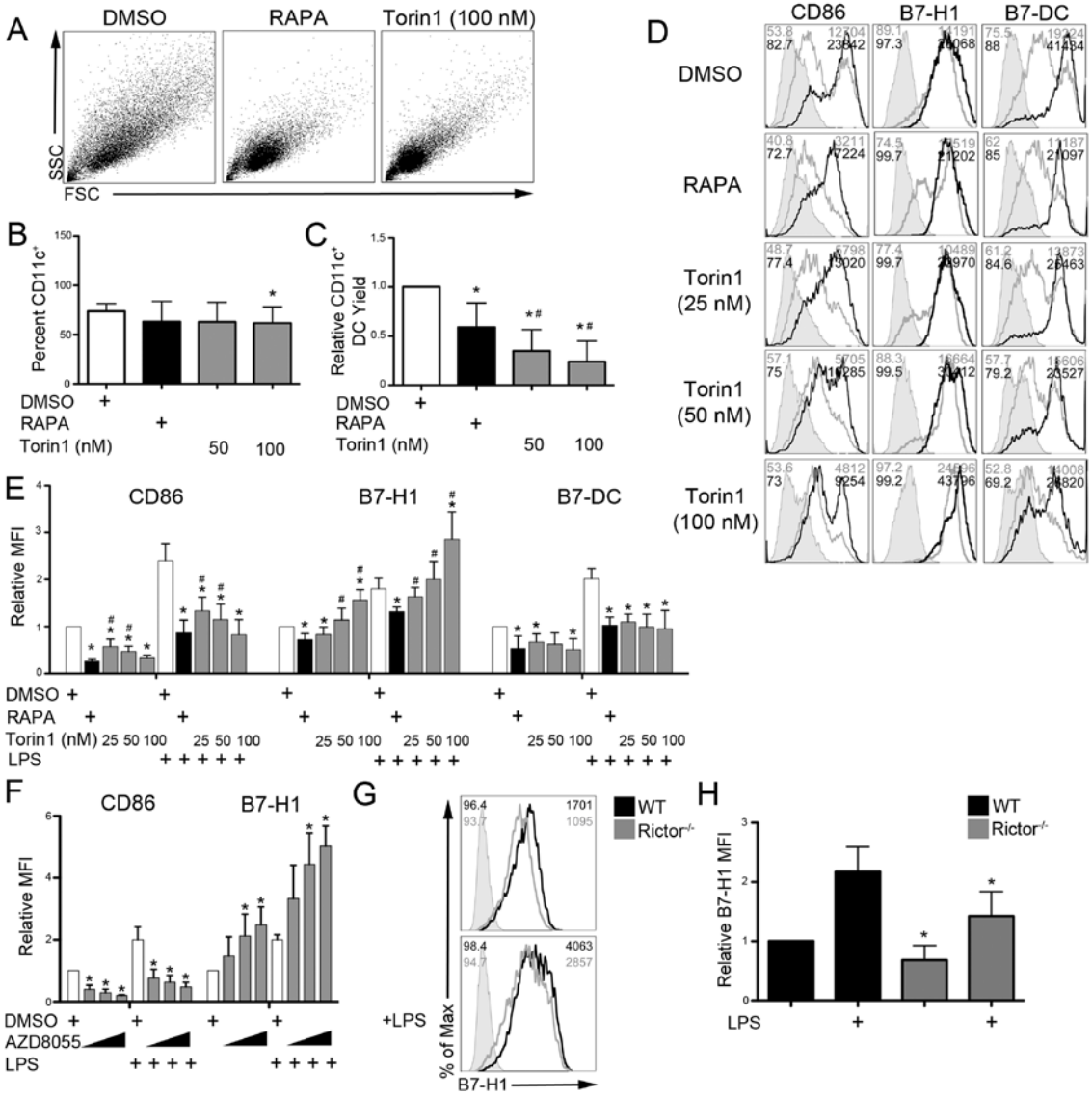

Figure 4.1 ATP-competitive dual mTORC1 and 2 inhibition dose-dependently and selectively upregulates DC B7-H1 expression

B6 mouse BM-derived conventional DC were differentiated in the presence of vehicle (DMSO) or the indicated mTOR inhibitor (10 ng/ml RAPA or various concentrations of Torin1 or AZD8055). (A) Forward scatter (FSC) vs. side scatter (SSC) flow cytometry plots of $CD11c^+$-purified DC. (B) The frequency of $CD11c^+$ DC in BM cell cultures was determined on d8. (C) mTOR inhibition reduced the yield of $CD11c^+$ DC isolated from BM cell cultures on d8. Viable cell numbers were determined by trypan blue exclusion. (D) CD11c-gated cells were analyzed for CD86, B7-H1 (PD-L1) and B7-DC (PD-L2) expression by flow cytometry in unstimulated cultures and cultures stimulated with LPS on d7 for 18h. Isotype controls are indicated by the shaded histogram; unstimulated (gray line) and LPS-stimulated cells (black line) are also shown. The percent of cells staining positive and the MFI are indicated in the upper left and right corners, respectively. (E) Quantification of CD86, B7-H1 and B7-DC expression (MFI) across multiple experiments. (F) CD11c-gated cells from BM cultures exposed to increasing concentrations of AZD8055 (400, 800, and 1200 nM) were assessed for CD86 and B7-H1 expression. (G) B7-H1 expression on wild-type (WT) or $rictor^{-/-}$ BM-derived DC. Percent positive cells and MFI are indicated in the upper left and right corners, respectively. (H) Quantification of (G) across multiple experiments. Bar graph values are normalized to WT or the DMSO treatment condition. $n \geq 3$ experiments for all data presented. * and # indicate $p<0.05$ when compared to control and RAPA, respectively.

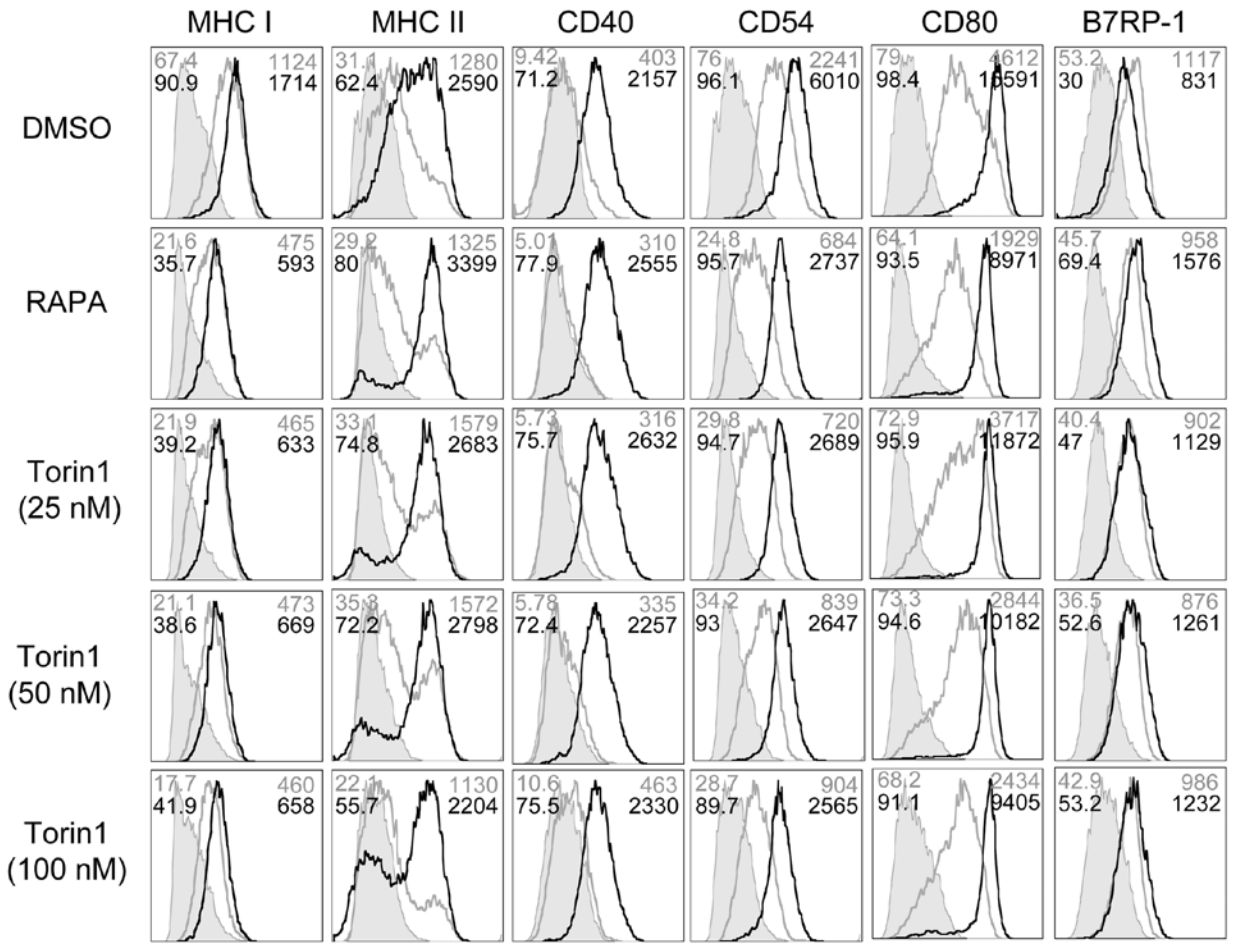

Figure 4.2 RAPA- and Torin1-exposed DC phenotype

RAPA or the indicated concentration of Torin1 was added to BM cell cultures from d2-d8, and the phenotype of CD11c-gated cells analyzed on d8 following overnight LPS stimulation. Isotype controls are indicated by the shaded histograms, and unstimulated (gray) and LPS-stimulated (black) conditions are indicated by solid lines. The percent of positive cells and MFI are indicated in the upper left and right corner, respectively. Data are representative of $n \geq 3$ experiments.

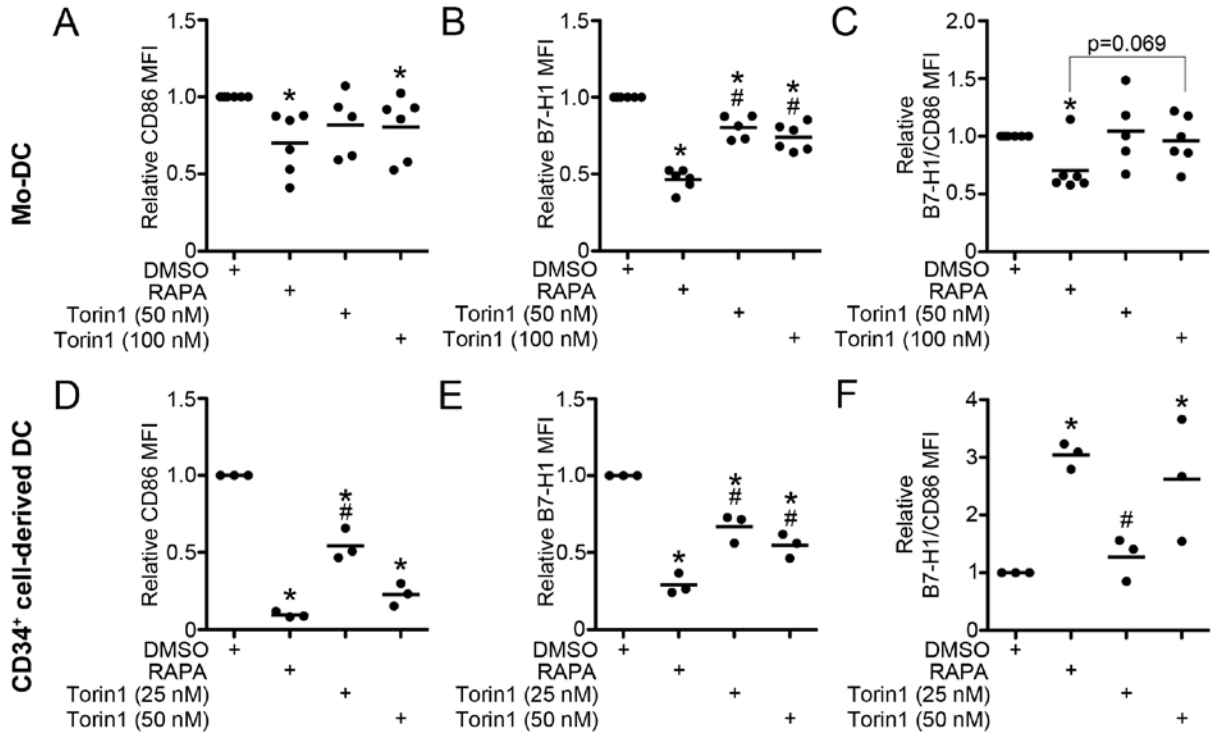

Figure 4.3 Torin1 spares B7-H1 while similarly reducing CD86 expression compared to RAPA on human DC

(A)-(B) Human Mo-DC were cultured in RAPA or Torin1 and matured overnight with LPS. (A) CD86 and (B) B7-H1 expression was normalized to DMSO. (C) The B7-H1 to CD86 expression ratio was calculated and normalized to DMSO. (D)-(E) $CD34^+$ cell-derived DC were stimulated with LPS overnight and (D) CD86 and (E) B7-H1 expression determined. (F) Normalized B7-H1 to CD86 expression was calculated. * and # indicate $p<0.05$ when compared to control and RAPA, respectively. Each data point represents one individual donor.

AZD8055, another ATP-competitive mTOR inhibitor, similarly increased B6 DC B7-H1 expression (Figure 4.1F and Figure 4.4A), thus confirming B7-H1 upregulation was due to on-target mTOR inhibition. Surprisingly, $rictor^{-/-}$ DC lacking mTORC2 activity displayed diminished B7-H1 expression following LPS stimulation (Figure 4.1G-H). DC differentiation was not affected, while the DC yield was reduced slightly in $rictor^{-/-}$ BM cultures (Figure 4.4B-C). Together, these data suggest that RAPA-resistant mTORC1 is a negative regulator of DC B7-H1 expression downstream of TLR4 while mTORC2 is a positive regulator of B7-H1 expression.

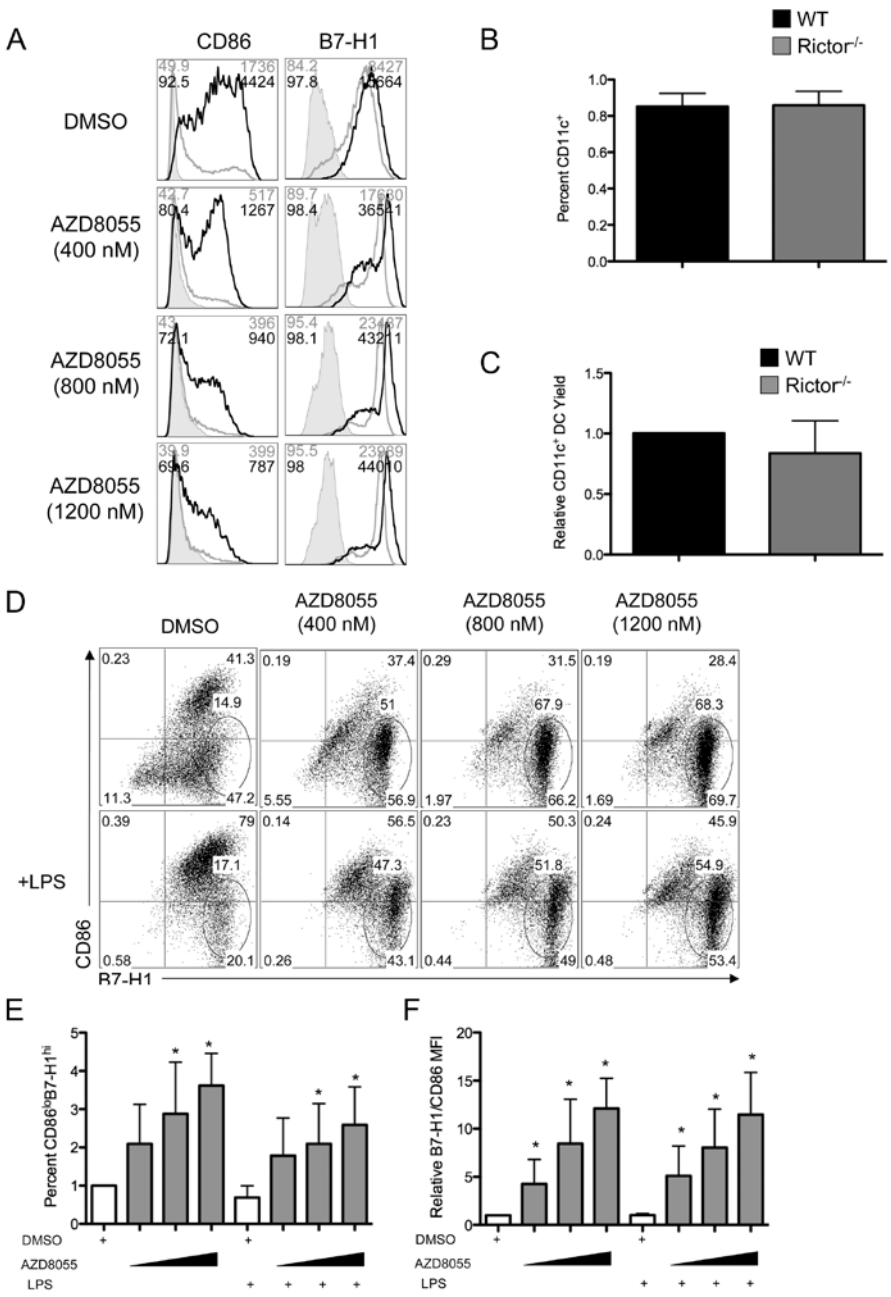

Figure 4.4 AZD8055 enhances the ratio of B7-H1 to CD86 expression and promotes CD86^{lo}B7-H1^{hi} DC

(A) CD86 and B7-H1 expression was analyzed on CD11c-gated cells following culture with AZD8055 at the indicated dose. Unstimulated DC (gray lines) or those stimulated with LPS overnight (black lines). The percent of positive cells and MFI are indicated in the upper left and right corners, respectively. Isotype controls are indicated by shaded histograms. (B) Percentage of CD11c^{+} DC in WT and rictor$^{-/-}$ BM cultures. (C) Absolute number of CD11c^{+} DC isolated from WT and rictor$^{-/-}$ BM cultures. (D) Co-expression of CD86 and B7-H1 was analyzed on CD11c-gated cells cultured in AZD8055. (E) CD86^{lo}B7-H1^{hi} DC gated in (D) were quantified relative to DMSO controls. (F) B7-H1 and CD86 expression was normalized to DMSO control DC expression and the ratio determined for each treatment group. AZD8055 concentrations ranged from 400 nM to 1200 nM. Data are from n ≥ 3 independent experiments. *, $p<0.05$ compared to DMSO or DMSO+LPS controls.

Further analysis revealed that enhanced B7-H1 expression on Torin1-conditioned DC occurred predominantly on immature $CD86^{lo}$ cells (Figure 4.5A-B). Similarly, AZD8055-conditioned DC were mainly $CD86^{lo}B7\text{-}H1^{hi}$ (Figure 4.4A and 4.4D-E). The ratio of co-inhibitory (B7-H1) to co-stimulatory (CD86) B7 family molecule expression was enhanced on Torin1- and AZD8055-conditioned DC to a greater extent than on RAPA-conditioned DC, with or without LPS stimulation (Figure 4.5C and Figure 4.4F). Human Mo-DC, but not $CD34^{+}$ cell-derived DC, differentiated in Torin1 exhibited a trend towards an elevated B7-H1 to CD86 expression ratio compared to those differentiated in RAPA (Figure 4.3C and 4.3F).

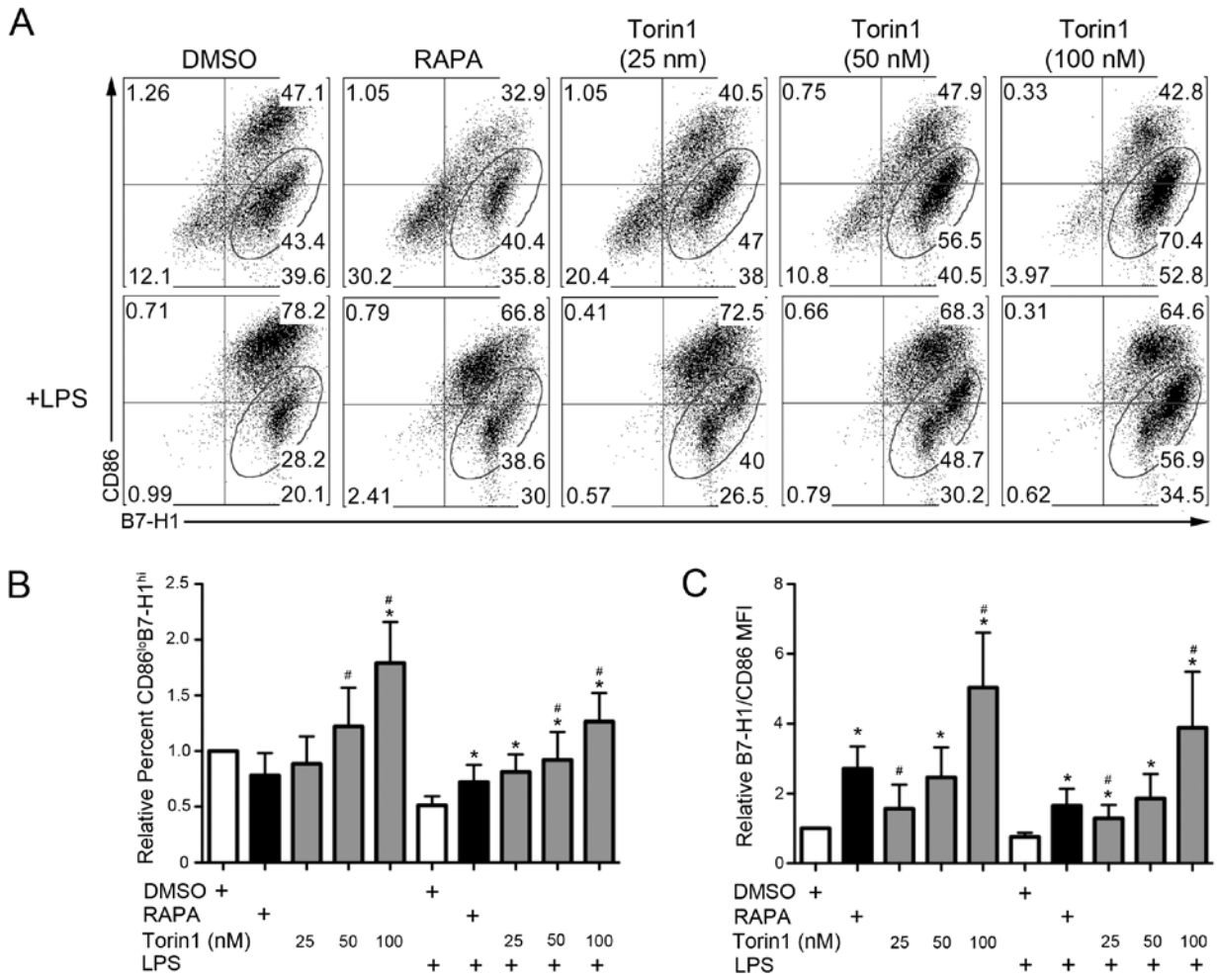

Figure 4.5 Torin1-conditioned DC are predominantly CD86^{lo}B7-H1^{hi}

(A) Analysis of the co-expression of CD86 and B7-H1 on CD11c-gated cells with percentage of CD86^{lo}B7-H1^{hi} DC indicated in the gate. (B) Quantification of the frequency of CD86^{lo}B7-H1^{hi} DC across multiple experiments. (C) Ratio of the normalized B7-H1 MFI divided by the normalized CD86 MFI for the indicated culture conditions. $\geq$5 experiments for all data presented. * and # indicate $p<0.05$ when compared to DMSO and RAPA, respectively.

4.4.2 STAT3 but not IRF-1 is required for upregulation of B7-H1 on DC by Torin1

The B7-H1 promoter contains two IRF-1 binding sites that are required for constitutive expression and IFNγ-induced upregulation of B7-H1 in cancer cell lines (283). We analyzed B7-H1 expression on wild-type and IRF-1 null Torin1-conditioned DC, but IRF-1 was not required for augmented DC B7-H1 expression (Figure 4.6A-B and Figure 4.7A-C).

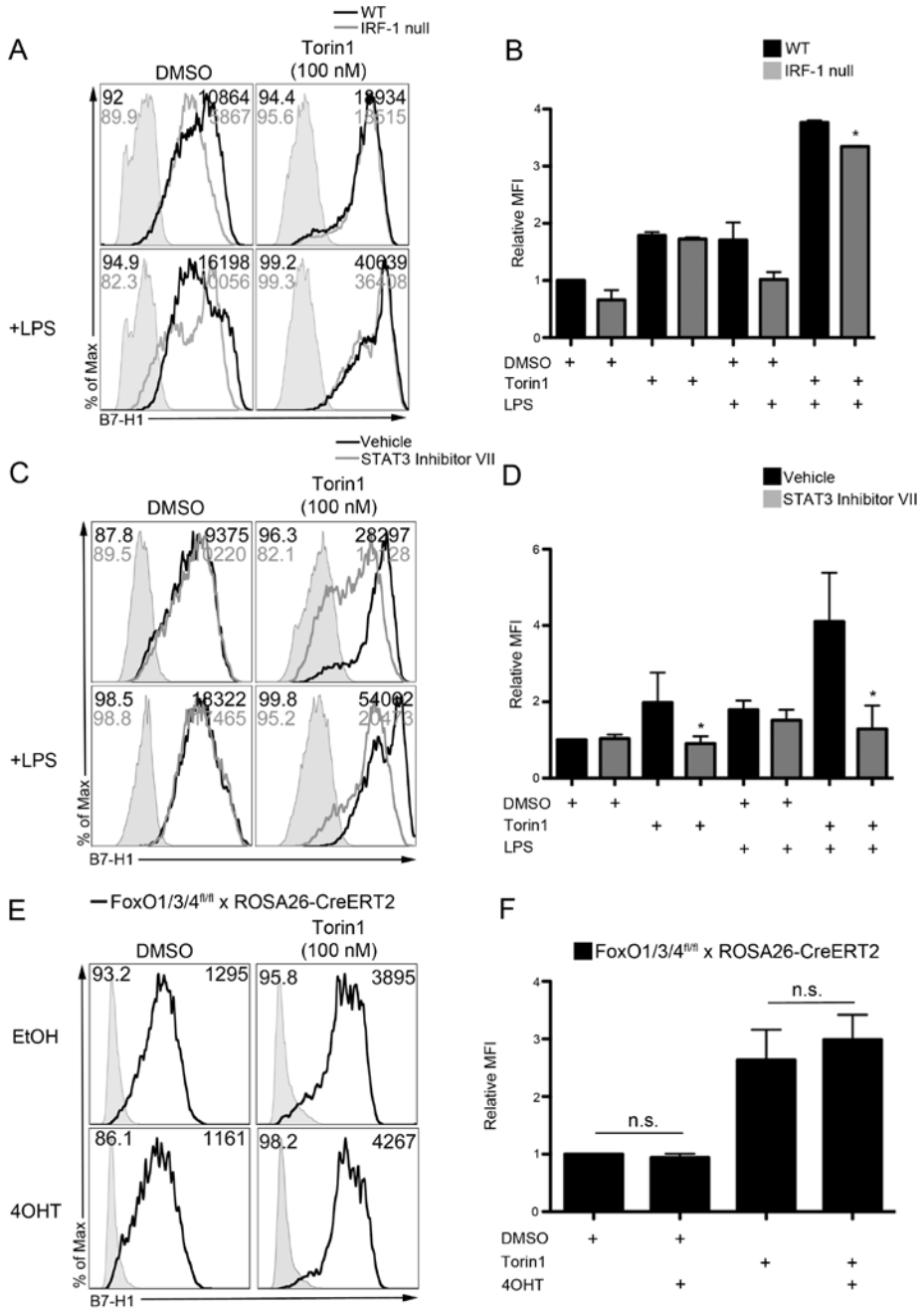

Figure 4.6 RAPA-resistant mTOR negatively regulates B7-H1 expression by reducing STAT3 activation independent of FoxO

(A) WT or IRF-1 null BM cell cultures were exposed to Torin1 (100 nM), stimulated with LPS (100 ng/ml) overnight on d7, and $CD11c^{+}$ DC interrogated for B7-H1 expression by flow cytometry on d8. (B) Quantification of B7-H1 MFI from (A) relative to DMSO control. * = $p<0.05$ compared to WT. (C) DMSO or Torin1-exposed DC were cultured as described in the methods. STAT3 inhibitor VII (250 nM) was added to cultures 2h before LPS stimulation for 18h and B7-H1 expression analyzed on CD11c-gated DC. (D) Quantification of B7-H1 MFI from (C) across multiple experiments normalized to DMSO control DC expression. * = $p<0.05$ compared with the corresponding group not receiving STAT3 inhibitor VII. (E) FoxO1/3/$4^{fl/fl}$ x ROSA26-CreERT2 BM cells were exposed to 4OHT, and B7-H1 expression determined on CD11c-gated DC on d8. (F) Quantification of B7-H1 MFI from (E) normalized to the DMSO+EtOH group. Data are from n=2-4 independent experiments.

STAT3 binds the B7-H1 promoter and is required for B7-H1 expression by lymphoma cell lines (284). Importantly, STAT3 has also been identified as a critical regulator of B7-H1 on human APC (285). STAT3 inhibition reversed enhanced B7-H1 expression (Figure 4.6C-D), eliminated concomitant $CD86^{lo}B7\text{-}H1^{hi}$ expression (Figure 4.7D-E) and reduced the B7-H1/CD86 ratio on Torin1-conditioned DC (Figure 4.7F). These data demonstrate that enhanced DC B7-H1 expression induced by Torin1 occurs through an IRF-1-independent and STAT3-dependent pathway.

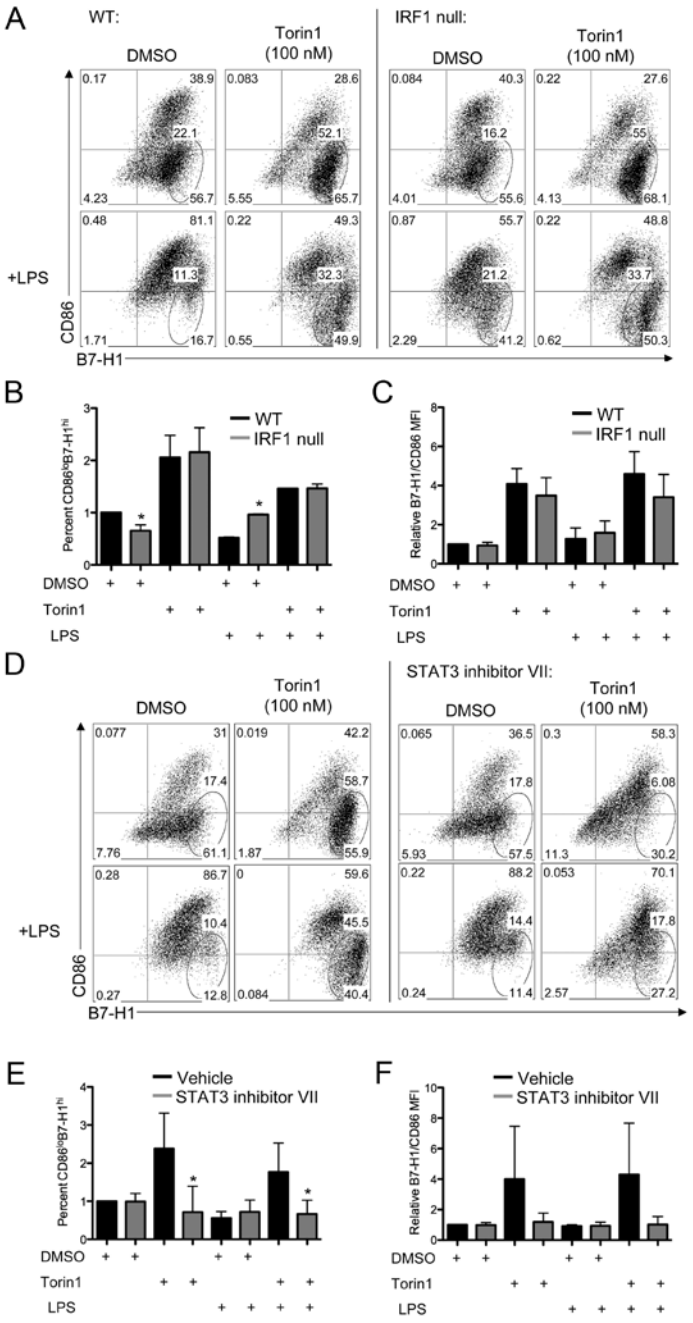

Figure 4.7 STAT3 but not IRF-1 is required for generation of CD86^{lo}B7-H1^{hi} DC by ATP-competitive mTOR inhibition

(A) WT or IRF-1 null BM cell cultures were exposed to Torin1 (100 nM) from d2-d8 as described in the methods. Cultures were stimulated with LPS (100 ng/ml) overnight on d7 and CD11c^{+} DC interrogated for CD86 and B7-H1 expression by flow cytometry. (B) Quantification of CD86^{lo}B7-H1^{hi} DC. (C) The ratio of B7-H1 to CD86 expression was determined on CD11c^{+} DC on d8. (D) DC were differentiated in the presence of Torin1 and exposed to STAT3 inhibitor VII on d7 for 2h before stimulation with LPS overnight. Representative dot plots of CD86 vs. B7-H1 expression are shown. (E) Quantification of CD86^{lo}B7-H1^{hi} gate in (D) normalized to DMSO controls. (F) Ratio of B7-H1 to CD86 MFI normalized to DMSO controls. Bar graphs demonstrate the mean + SD from n=2-4 independent experiments with * indicating $p<0.05$ when compared to WT DC (A-C) or samples not treated with STAT3 inhibitor (D)-(F).

O class forkhead-box (FoxO) transcription factors mediate immune homeostasis (286) and control the ability of DC to limit T cell expansion following viral infection (287). FoxO promotes STAT3 activity, and FoxO3 transcription correlates with elevated B7-H1 expression by tumor-associated DC (288). Torin1-conditioned DC were generated from FoxO1/3/4$^{-/-}$ BM cells by exposing FoxO1/3/4$^{fl/fl}$ x ROSA26-CreERT2 BM cells to 4OHT (289). Deletion of FoxO3a was confirmed by immunoblot (data not shown). FoxO was not required for B7-H1 upregulation by Torin1 (Figure 4.6E-F), thus suggesting a STAT3-dependent but FoxO-independent mechanism of RAPA-resistant mTOR negative regulation of B7-H1.

4.4.3 ATP-competitive mTOR inhibition enhances STAT3 signaling by reducing SOCS3 expression

Studies with RAPA have implicated mTORC1 as a positive regulator of STAT3 in DC (195, 278); however, reduced IL-10 secretion by RAPA-exposed DC leads to reduced autocrine STAT3 activation (279). DC were pre-incubated with RAPA or Torin1 for 2h before LPS stimulation (hereby referred to as 'short-term' mTOR inhibition). As reported (177), RAPA abolished mTORC1 signaling (P-S6K T389), but only Torin1 blocked mTORC2 signaling (P-Akt S473; Figure 4.8A). Both short-term RAPA and Torin1 inhibited STAT3 phosphorylation following LPS stimulation (Figure 4.8B-C). In contrast, DC differentiated in the presence of Torin1, but not RAPA, from d2-d8 (hereby referred to as 'RAPA- or Torin1-conditioned DC') demonstrated augmented STAT3 phosphorylation 3h after LPS stimulation (Figure 4.8D-F). Suppressor of cytokine signaling (SOCS) proteins regulate cytokine signaling by inhibiting Janus kinase (JAK)-STAT pathways (290). RAPA conditioning led to augmented SOCS1 and SOCS5, which are negative regulators of STAT1 (291) and STAT6 (292), respectively (Figure 4.8G).

Torin1 suppressed expression of the key STAT3 negative regulator SOCS3 (Figure 4.8E and 4.8G) (293). As such, we make the novel observation that extended mTORC1 and 2 inhibition by Torin1, but not mTORC1 inhibition by RAPA, downregulates SOCS3 and enhances STAT3 signaling.

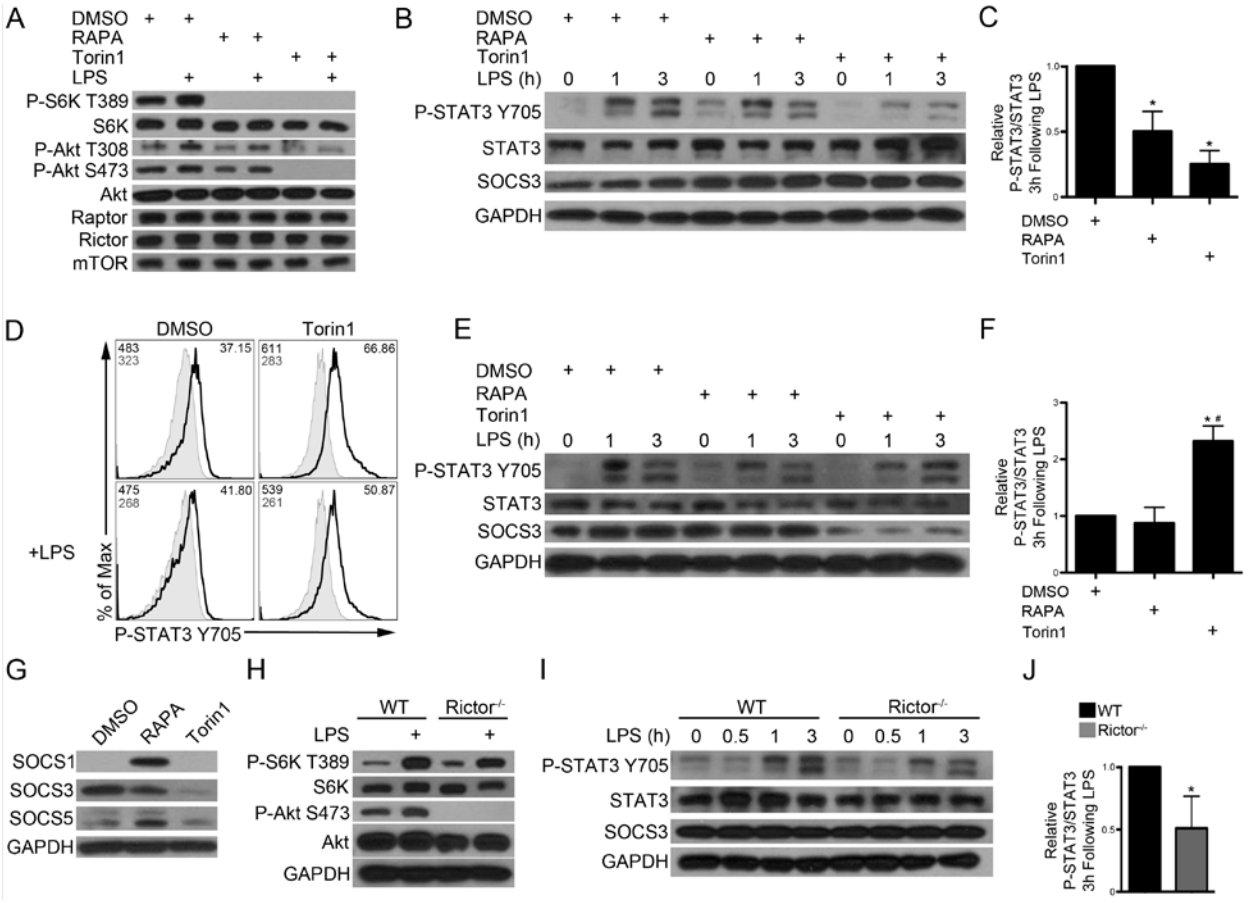

Figure 4.8 Extended exposure to ATP-competitive mTOR inhibition reduces SOCS3 expression, resulting in sustained STAT3 activation

$CD11c^+$ cells were isolated from 7d BM cell cultures and pre-treated with DMSO, RAPA (10 ng/ml) or Torin1 (100 nM) for 2h before LPS stimulation for 30 min (A) or 1-3h (B). Total cell lysates were immunoblotted for the indicated protein. (C) P-STAT3 signal was quantified relative to total STAT3 signal at 3h following LPS stimulation and normalized to control. (D) DC were differentiated in the presence of DMSO (vehicle) or Torin1 (100 nM) from d2-d8. DC were isolated by CD11c immunomagnetic purification, and P-STAT3 signal determined by flow cytometric analysis following LPS stimulation for 3h. Grey histogram depicts samples stained with secondary Ab only. MFI for P-STAT3 signal and secondary Ab only are indicated in the top left corner, and percent positive cells are indicated in the upper right corner. (E) RAPA- or Torin1-conditioned DC were purified as described in (D), stimulated with LPS for 0-3h, and total cell lysates immunoblotted for the indicated protein. (F) Quantification of P-STAT3/STAT3 signal 3h after LPS stimulation. (G) SOCS protein was assessed in RAPA- or Torin1-conditioned DC. WT or $rictor^{-/-}$ DC were stimulated with LPS for (H) 30 min or (I) 0-3h and probed as indicated. (J) P-STAT3/STAT3 signal was quantified at 3h post-LPS. Data are representative of n=2-3 independent experiments. * and # indicate $p<0.05$ when compared to DMSO or WT and RAPA, respectively.

Rictor$^{-/-}$ DC were unable to phosphorylate Akt S473; however, mTORC1 signaling was intact (Figure 4.8H). These cells exhibited diminished P-STAT3 following LPS stimulation, and there was no change in SOCS3 expression (Figure 4.8I-J). Importantly, these data establish that RAPA-resistant mTORC1 negatively regulates STAT3 while mTORC2 is a positive regulator of STAT3.

4.4.4 RAPA-resistant mTOR inhibition augments IL-10 production, but it is not required for B7-H1 upregulation

STAT3 phosphorylation occurs late (1-3h) after LPS stimulation of DC (Figure 4.8), suggesting autocrine signaling that induces phosphorylation. Autocrine IL-10 signaling is required for STAT3 phosphorylation following LPS stimulation (279). IL-10 production was increased markedly in B7-H1^{hi} Torin1-conditioned IL-10-gfp reporter DC (Figure 4.9A and 4.9C). The IL-10 MFI was increased in Torin1-conditioned DC compared to RAPA-conditioned and control DC (Figure 4.9B and 4.9D). Both RAPA- and Torin1-conditioned DC secreted increased IL-12/23p40 (Figure 4.9E). Rictor$^{-/-}$ DC secreted elevated IL-12p40 also (data not shown). IL-6 and IL-1β secretion by DC after LPS stimulation was reduced and increased respectively, by RAPA, but unaffected by Torin1 (Figure 4.9F-G).

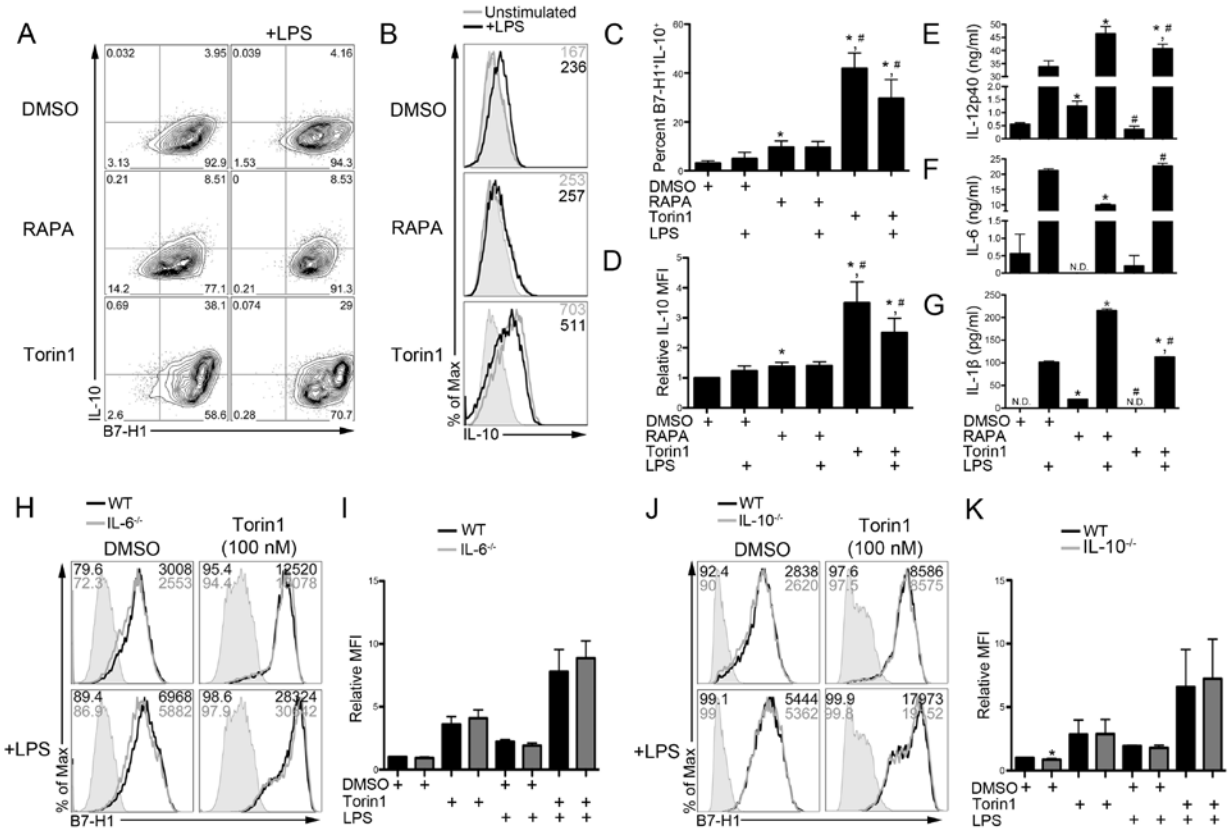

Figure 4.9 Torin1-exposed DC produce increased IL-10, but B7-H1 upregulation does not require autocrine IL-6 or IL-10

(A) BM from IL-10-gfp reporter mice was differentiated in RAPA or Torin1 from d2-d8. DC were stimulated on d7 where indicated, and interrogated for B7-H1 and IL-10 co-expression by flow cytometry. (B) IL-10-gfp histogram for CD11c-gated DC. MFI is indicated in the upper right corner. (C) The percentage of B7-H1^{+}IL-10^{+} DC from (A) was quantified across multiple experiments. (D) Quantification of IL-10 MFI across multiple experiments normalized to DMSO control DC. Cell-free supernatants were assessed for (E) IL-12/23p40, (F) IL-6 and (G) IL-1β; N.D. indicates not detected. *, # $p<0.05$ compared to DMSO or DMSO+LPS and RAPA or RAPA+LPS, respectively. DC were differentiated from IL-6$^{-/-}$ (H,I) or IL-10$^{-/-}$ (J,K) BM cells in the presence of Torin1. B7-H1 expression was analyzed on CD11c-gated DC by flow cytometry and quantified. * $p<0.05$ compared to WT or WT+LPS. Data are from n=2-3 independent experiments.

IL-6, IL-10, IL-23, and IL-27 all stimulate STAT3 phosphorylation (293-295). Neither autocrine IL-6 nor IL-10 was required for enhanced B7-H1 expression by Torin1 on control or LPS-stimulated DC (Figure 4.9H-K). Autocrine IL-23 and IL-27 were also not required, as determined using IL-12/23p40$^{-/-}$ and Ebi3$^{-/-}$ BM cell cultures, respectively (Figure 4.10A-D). Collectively, these results identify a RAPA-resistant mTOR pathway that co-regulates B7-H1 and IL-10 expression that does not depend on autocrine cytokine stimulation.

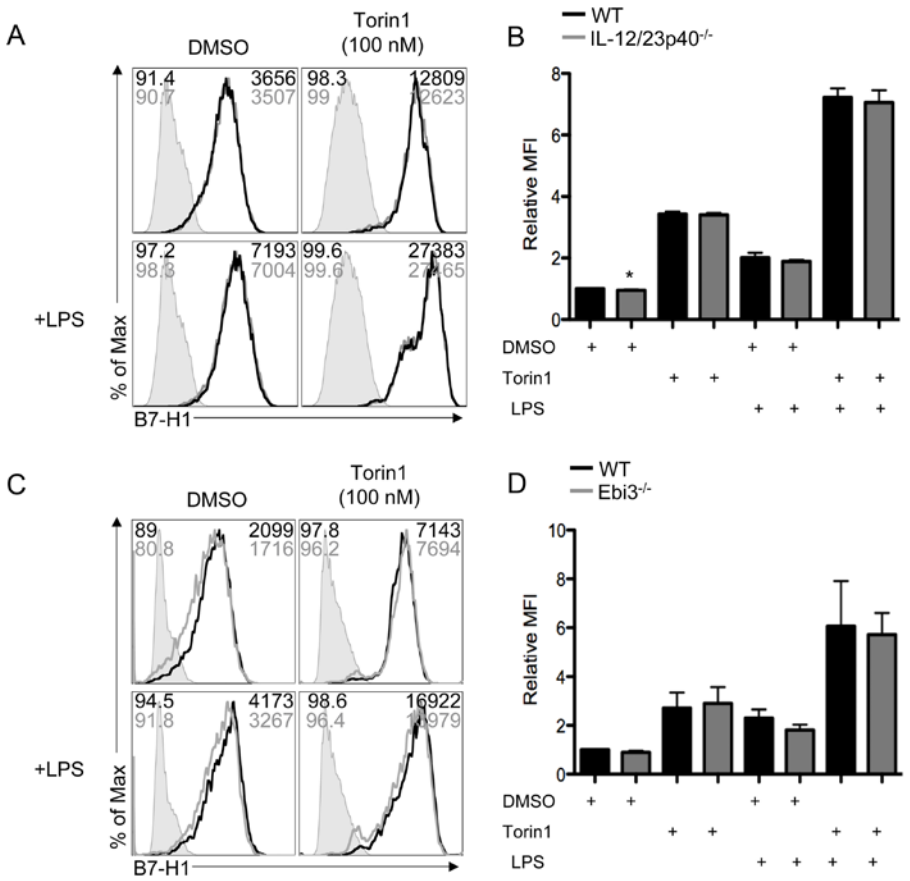

Figure 4.10 B7-H1 upregulation by Torin1 does not require autocrine IL-12/23 or IL-27

CD11c^{+} DC differentiated from (A) and (B) IL-12/23$^{-/-}$ (IL-12/23p40$^{-/-}$) or (C) and (D) IL-27$^{-/-}$ (Ebi3$^{-/-}$) BM cells differentiated in Torin1 from d2-d8 were analyzed for expression of B7-H1. *, $p<0.05$ when compared to WT DC. Data are from n=3 independent experiments.

4.4.5 DC mTORC1 promotes T_{eff} expansion while RAPA-resistant mTOR restrains T_{reg} induction via B7-H1

We next sought to determine how ATP-competitive mTOR inhibition modulates the T cell stimulatory function of DC. RAPA reduces the T cell stimulatory capacity of DC (188-190, 201, 202). Torin1 shared this ability, although its effect was less than that of RAPA (Figure 4.11A-B). Although B7-H1 has been shown to restrain T cell proliferation (150), B7-H1$^{-/-}$ RAPA-DC that express low CD80 and CD86 were less stimulatory than wild-type DC (Figure 4.11A-B). These data are in agreement with early reports describing a T cell proliferation-promoting effect of B7-H1 (276).

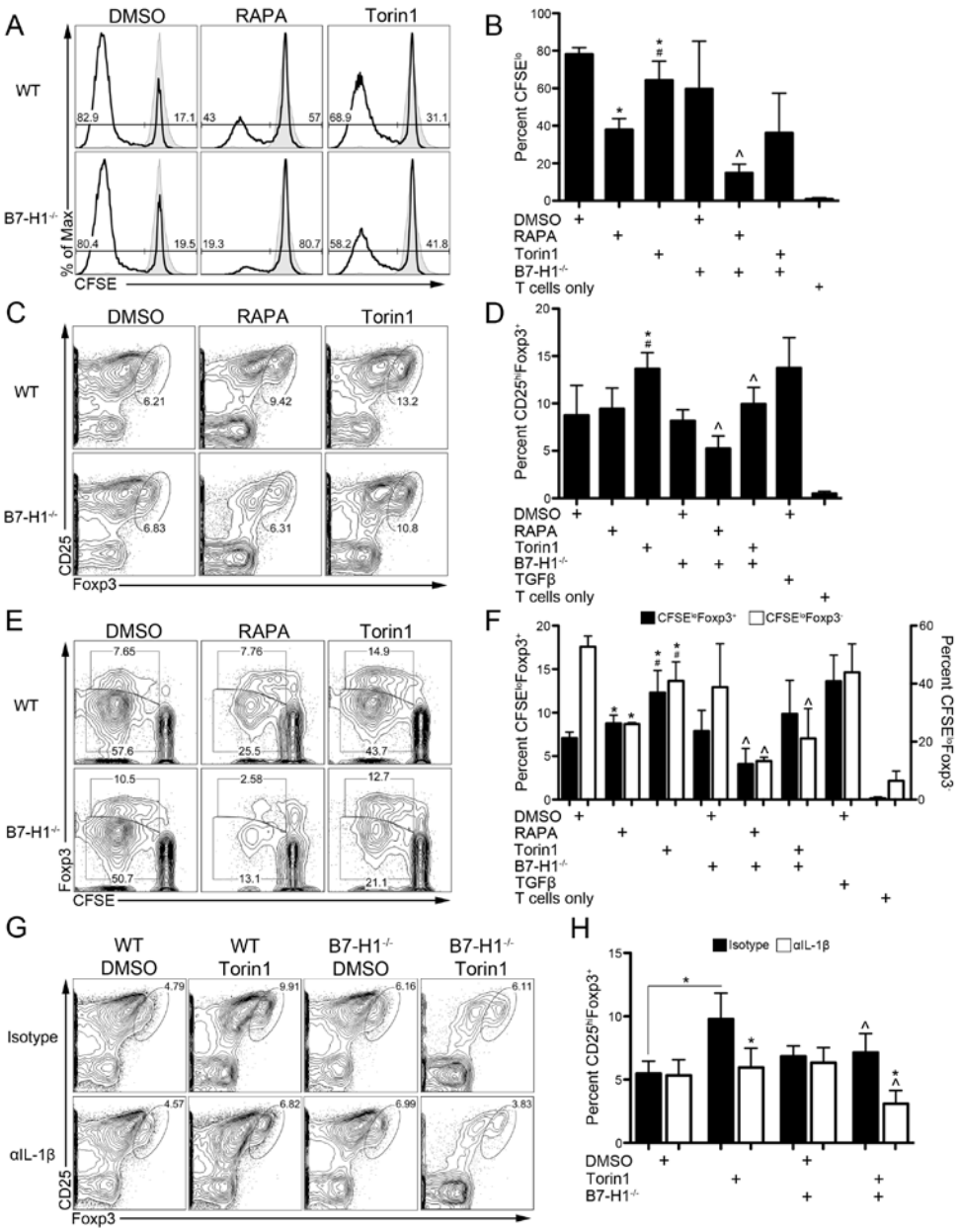

Figure 4.11 Enhanced induction of T_{reg} by Torin1-exposed DC is B7-H1- and IL-1β-dependent

(A) WT or B7-H1$^{-/-}$ DC propagated in the indicated mTOR inhibitor were washed extensively and used as stimulators of CD4^{+}CD25^{-} normal BALB/c T cells in a 5d CFSE-dilution MLR. (B) Percent of proliferating T cells (CFSE^{lo}) was calculated across multiple experiments. (C) Representative contour plots depicting the percentage of induced T_{reg} (CD25^{hi}Foxp3^{+}) in the MLR from (A) are shown. (D) The data from (C) were calculated across multiple experiments. (E) Representative contour plot of CFSE dilution of Foxp3^{+} and Foxp3^{-} T cells. (F) Quantification of multiple experiments from (E). *, #, and ^ $p<0.05$ compared to WT DMSO, WT RAPA, and the corresponding WT condition, respectively. (G) IL-1β was neutralized in MLR and T_{reg} induction determined as in (C) and (H) quantified across experiments. *, ^ $p<0.05$ compared to corresponding isotype control and WT conditions, respectively, unless otherwise indicated. All data are representative of n≥3 independent experiments. In some experiments, TGFβ (10 ng/ml) was added at the start of culture as a positive control and T cells without DC stimulators were included as a negative control.

B7-H1 is a key regulator of T_{reg} induction and function (277). Since Torin1-conditioned DC express elevated levels of B7-H1, we determined their ability to induce T_{reg} (Figure 4.11C-F). Torin1-conditioned DC induced a significantly greater frequency of T_{reg} compared to control and RAPA-conditioned DC (Figure 4.11C-D). Enhanced induction of T_{reg} by Torin1-conditioned DC was dependent on B7-H1 (Figure 4.11C-D) but independent of PD-1 (Figure 4.12A-B). Although Torin1-conditioned DC made increased IL-10 (Figure 4.9A-D), it was not required for their ability to induce T_{reg} (Figure 4.12A-B). Neither genetic deletion nor neutralization of B7-H1 reduced T_{reg} induction by control DC (Figure 4.11C-D and Figure 4.12A-B). The absolute number of T_{reg} induced by RAPA-conditioned DC was reduced dramatically compared to control (Figure 4.12C). Torin1-conditioned DC induced a greater number of T_{reg} compared to RAPA-conditioned DC, which was reduced in the absence of B7-H1 (Figure 4.12C).

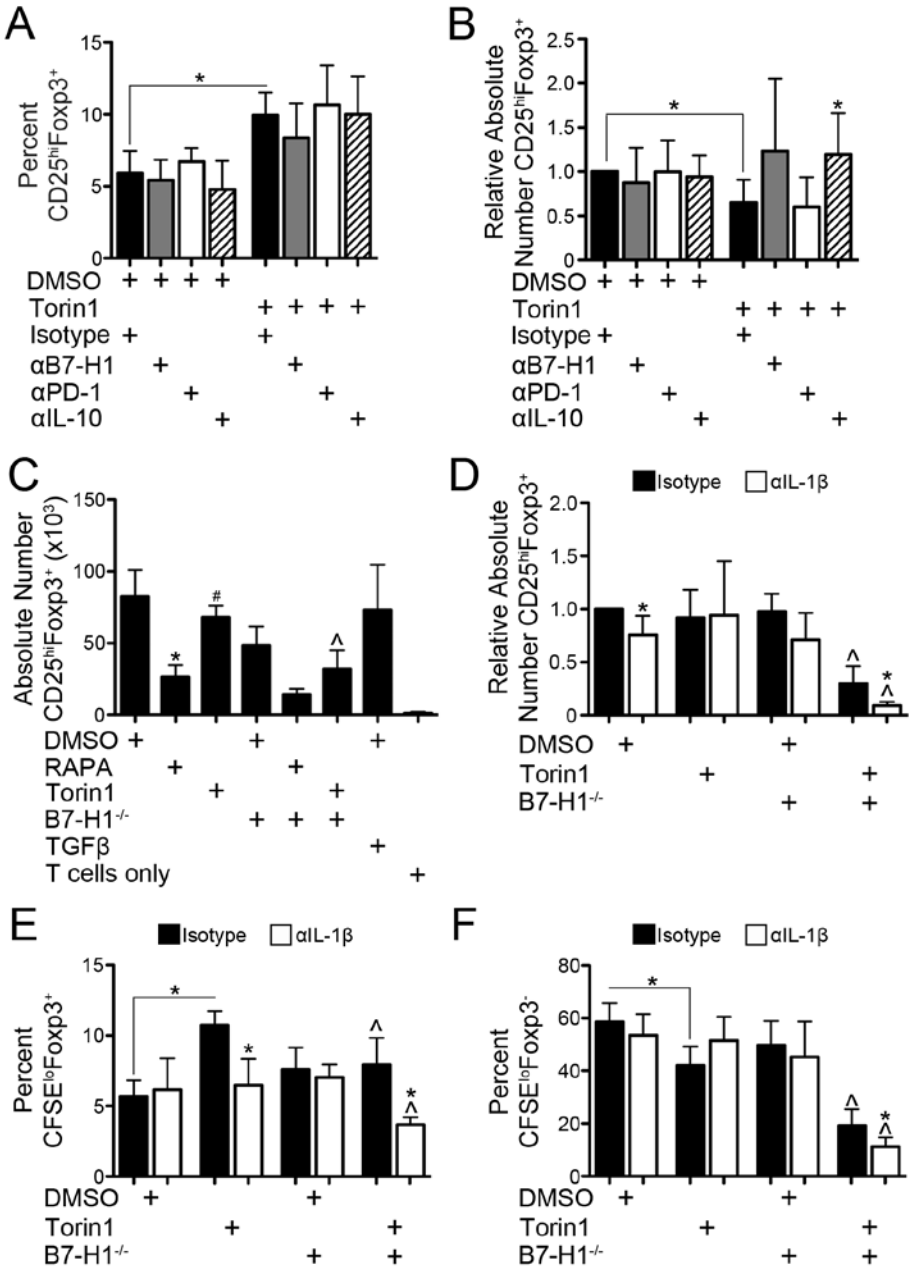

Figure 4.12 Enhanced T_{reg} induction by Torin1-DC is PD-1- and IL-10-independent

(A)-(D) WT or B7-H1$^{-/-}$ B6 DC generated in DMSO, RAPA or Torin1 were used as stimulators of CFSE-labeled CD4^{+}CD25^{-} BALB/c T cells in 5d MLR. (A) B7-H1, PD-1 or IL-10 were neutralized by addition of Ab at the start of MLR and the percent of T_{reg} determined. (B) Absolute numbers of T_{reg} in MLR from (A) were determined by flow cytometry and normalized to control. (C) Total viable cell numbers were determined on d5 by trypan blue exclusion, and the absolute number of induced T_{reg} was calculated. Exogenous TGFβ (10 ng/ml) was added to cultures as a positive control, where indicated. (D) IL-1β was neutralized in MLR. The absolute number of T_{reg} was determined by flow cytometry and normalized to control. (E,F) Quantification of (E) CFSE^{lo}Foxp3^{+} and (F) CFSE^{lo}Foxp3^{-} cells in MLR with IL-1β neutralization. (A-B and D-F) *, ^ $p<0.05$ compared to corresponding isotype control and WT condition, respectively. (C) *, #, and ^ $p<0.05$ compared to WT DMSO, WT RAPA, and the corresponding WT condition, respectively. Data are representative of n≥3 independent experiments.

CFSE dilution profiles of $Foxp3^+$ and $Foxp3^-$ T cells were used to determine the relative contribution of inhibition of effector T cell (T_{eff}; $Foxp3^-$) proliferation to induction of T_{reg} (Figure 4.11E-F). Consistent with our previous report (190), RAPA-conditioned DC stimulated T_{eff} poorly but stimulated $CFSE^{lo}Foxp3^+$ T cells similarly to control DC (Figure 4.11E-F). Torin1-conditioned DC also stimulated T_{eff} cells less than control DC; however, they approximately doubled the frequency of $CFSE^{lo}Foxp3^+$ T cells (Figure 4.11E-F). These data demonstrate that ATP-competitive mTOR inhibition in DC enhances their ability to induce T_{reg} in a B7-H1-dependent manner and augment $CFSE^{lo}Foxp3^+$ T cells, while RAPA-conditioned DC specifically reduce proliferation of T_{eff} without affecting $CFSE^{lo}Foxp3^+$ T cells.

There is evidence that IL-1β promotes T_{reg} induction (296), and mTORC1 regulates IL-1β production by DC (201, 203). Although Torin1 did not alter IL-1β secretion by DC (Figure 4.9G), neutralization of IL-1β suppressed T_{reg} induction by Torin1-conditioned DC (Figure 4.11G-H). T_{reg} induction and augmentation of $CFSE^{lo}Foxp3^+$ T cells were further reduced when IL-1β-inhibited Torin1-conditioned DC were B7-H1-deficient (Figure 4.11G-H and Figure 4.12D-F). These data suggest that B7-H1 and IL-1β act cooperatively to promote T_{reg} induction by Torin1-conditioned DC.

4.4.6 ATP-competitive mTOR inhibition elevates DC B7-H1 expression *in vivo* and augments their ability to induce T_{reg}

To determine if RAPA-resistant mTOR modulates DC B7-H1 expression in a more physiologic setting, we investigated *in vivo* modulation of DC by ATP-competitive mTOR inhibition. WYE-125132 is an ATP-competitive mTOR inhibitor with *in vivo* bioavailability (280). We first verified that WYE-125132 augmented DC B7-H1 expression while reducing CD86 expression *in*

vitro (Figure 4.13A-D). When given as a single dose at the time of LPS administration, WYE-125132 enhanced splenic DC B7-H1 expression while reducing CD86 expression (Figure 4.13E-G). Furthermore, the B7-H1 to CD86 expression ratio was increased (Figure 4.13H). These DC augmented T_{reg} induction *ex vivo* compared to control DC (Figure 4.13I-J). Together, these findings confirm that RAPA-resistant mTOR controls B7-H1 expression and modulates the ability of DC to induce T_{reg} *in vivo*.

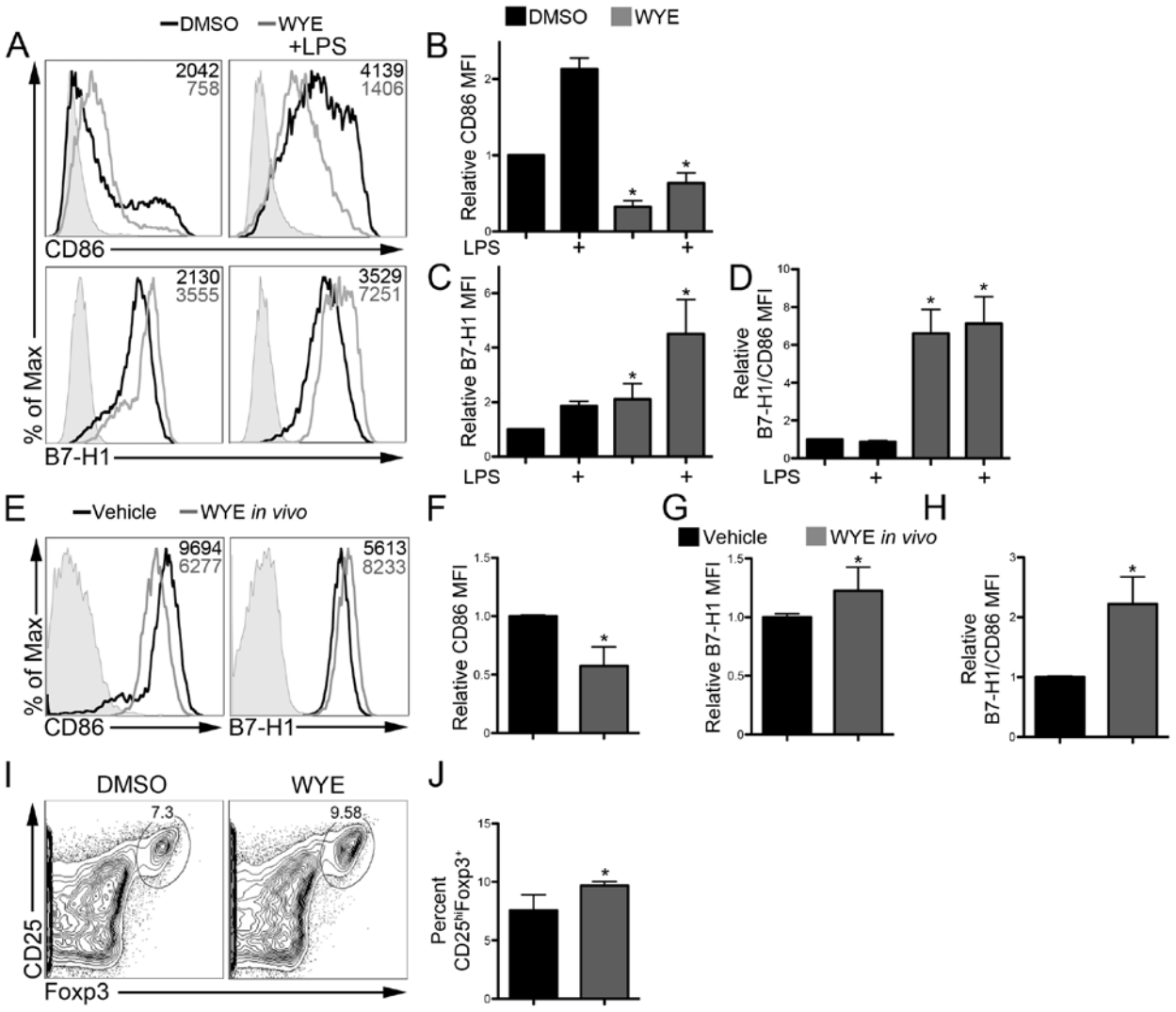

Figure 4.13 ATP-competitive mTOR inhibition *in vivo* augments DC B7-H1 expression and their ability to induce T_{reg}

(A)-(D) DC were differentiated in the presence of DMSO or WYE-125132 (WYE; 400 nM) from d2-d8 and stimulated with LPS. (A) Representative histograms and (B) quantification of CD86 and (C) B7-H1 expression. MFI is indicated in the upper right corner of each histogram. (D) B7-H1 to CD86 expression ratio. Values were normalized to unstimulated DMSO DC. (E)-(H) Mice were treated with WYE (50 mg/kg) and given LPS (100 µg/kg). Data are representative of n=3 independent experiments. (E) Splenic $CD11b^{+}CD11c^{+}$ DC were analyzed by flow cytometry for CD86 and B7-H1 expression 18h later with representative histograms shown. MFI values are shown in the upper right corner of each histogram. (F) CD86, (G) B7-H1 and (H) the B7-H1 to CD86 ratio were determined across several experiments and normalized to the vehicle control. (I) Splenic DC isolated in (E) were used to induce Treg *ex vivo* from $CD4^{+}CD25^{-}$ BALB/c T cells. (J) T_{reg} induction was quantified across multiple experiments. Data are representative of n=4 mice per treatment group. * $p<0.05$ compared to DMSO.

4.5 DISCUSSION

Herein we describe a novel RAPA-resistant and mTORC2-independent signaling pathway in conventional DC that controls B7-H1 and IL-10 expression downstream of TLR4. This novel signaling network coordinates the ability of DC to stimulate T_{eff} and T_{reg} responses. DC conditioned in ATP-competitive mTOR inhibitors dose-dependently and selectively upregulated B7-H1 expression. Elevated IL-10 production by Torin1-conditioned DC was found in a B7-H1^{hi} population. B7-H1 upregulation by Torin1 was STAT3-dependent, but did not require FoxO1/3/4 or autocrine IL-6, IL-10, IL-12/23, or IL-27 signaling. Augmented STAT3 phosphorylation correlated with a reduction in SOCS3 expression. Rictor$^{-/-}$ DC did not exhibit augmented STAT3 phosphorylation or B7-H1 expression. ATP-competitive mTOR inhibition resulted in DC that markedly enhanced B7-H1-dependent but PD-1 independent T_{reg} induction in the absence of exogenous TGFβ. IL-1β, but not IL-10, was required for enhanced T_{reg} induction by DC conditioned in Torin1 and acted additively with B7-H1. Together, these findings establish how distinct mTOR complex signaling coordinates to regulate DC stimulatory function at a molecular level. The proposed mechanisms by which RAPA-sensitive and RAPA-resistant mTOR controls DC STAT3 activation and B7-H1 expression are summarized in Figure 4.14.

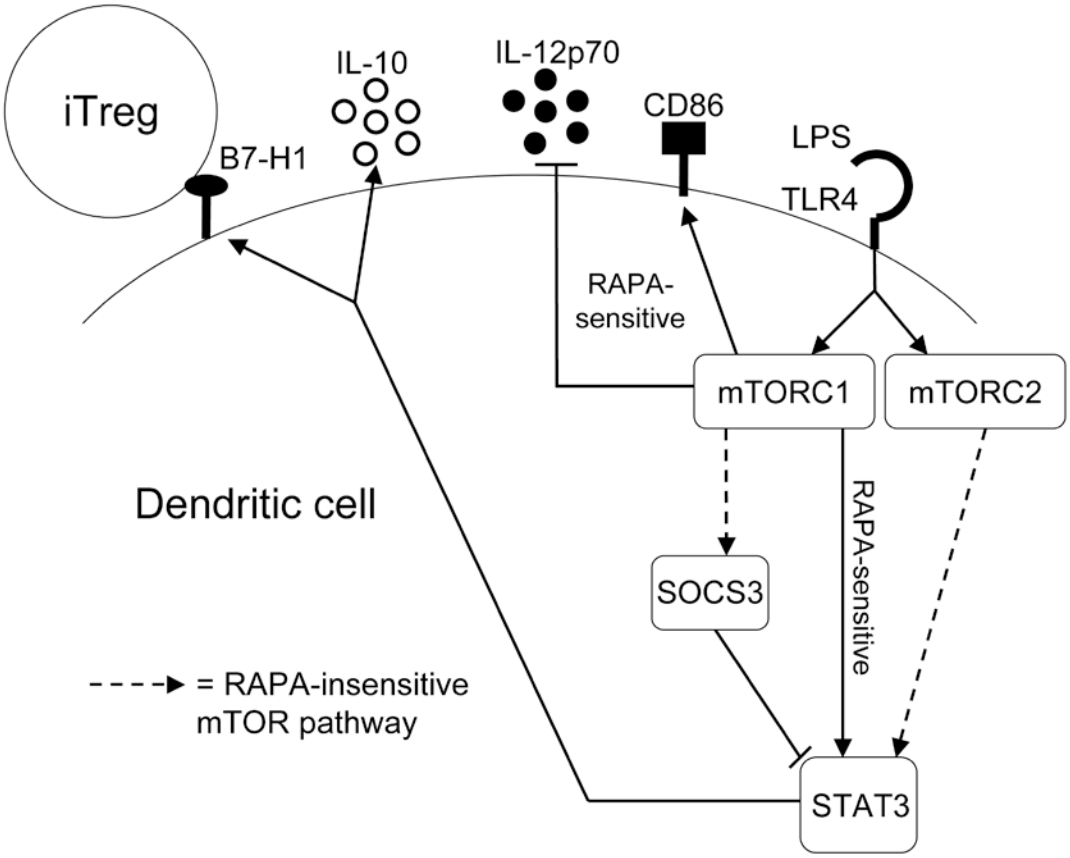

Figure 4.14 Proposed model of mTOR regulation of STAT3 activation and B7-H1 expression in DC

mTORC1 and mTORC2 both independently regulate STAT3 activation through RAPA-sensitive and RAPA-resistant pathways. RAPA-sensitive mTORC1 is a positive regulator of STAT3 activity; however, a parallel and RAPA-insensitive mTORC1 pathway exists where mTORC1 positively regulates SOCS3, a negative regulator of STAT3 activity. Inhibition of this pathway with ATP-competitive mTOR inhibitors results in reduced SOCS3 expression, increased STAT3 phosphorylation following LPS stimulation and increased expression of B7-H1 and IL-10. DC differentiated in the presence of ATP-competitive mTOR inhibitors promote Treg induction in a B7-H1-dependent, but PD-1-independent, manner. Additionally, RAPA-sensitive mTORC1 regulates CD86 and IL-12p70 expression. mTORC2 is a RAPA-insensitive positive regulator of STAT3 activity.

mTOR is a central regulator of T cell function (237) that is targeted for clinical immunosuppression, yet the impact of mTOR, particularly RAPA-resistant mTOR, on innate immune cells is poorly understood (172, 297). RAPA is a potent and selective mTORC1 inhibitor; however, prolonged exposure can inhibit mTORC2 by preventing its assembly in certain cell types (182). After verifying specific mTOR complex specificity of RAPA and ATP-competitive mTOR inhibitors, we used this strategy to dissect the function of RAPA-sensitive

and RAPA-resistant pathways in conventional DC. RAPA-sensitive mTOR (mTORC1) promoted co-stimulatory CD80 and CD86 expression and reduced IL-12 secretion. RAPA inhibits phosphorylation of inhibitory residues on glycogen synthase kinase-3 leading to NF-κB p65 activation and increased IL-12 secretion after TLR4 ligation (195, 202, 278, 279, 298). Our data support these findings where RAPA-sensitive mTORC1 suppresses IL-12 production. A recent study demonstrated a similar role for mTORC2 in DC following LPS activation where rictor knockdown enhanced pro-inflammatory cytokine and reduced IL-10 secretion. However, elevated IL-12 secretion in these cells did not require NF-κB, but was due to hyperactive FoxO1 (299). The present study identifies, for the first time, a RAPA-resistant mTORC1 pathway that is critical for controlling the immune regulatory properties of DC and further elaborates on signaling events downstream of TLR4.

ATP-competitive mTOR inhibition promoted DC IL-10 and B7-H1 expression and enhanced STAT3 activation. Enhanced STAT3 activation and reduced SOCS3 expression were only seen following prolonged ATP-competitive mTOR inhibition. These data demonstrate that the kinetics of mTOR inhibition (short-term exposure vs. long-term conditioning), in addition to the complexes being targeted, are critical to understanding the function of mTOR in DC. Initial publications describing ATP-competitive mTOR inhibitors revealed unexpectedly, that RAPA did not inhibit mTORC1 completely; however, ATP-competitive mTOR inhibitors fully inhibited residual, RAPA-resistant mTORC1 (184, 185). Further studies have since shown that RAPA-resistant mTOR provides broad transcriptional regulation that does not occur with inhibition of mTORC1 by RAPA (300). To our knowledge, the present report describes the first RAPA-resistant mTOR pathway that is not dependent on RAPA-sensitive mTORC1; however, the contribution of transcriptional level control by RAPA-resistant mTOR in the proposed

pathway is unknown. Contrastingly, RAPA produces the opposite effects, whereby B7-H1 and IL-10 are diminished. Raptor-deficient DC lacking RAPA-sensitive and RAPA-resistant mTORC1 activity display reduced IL-10 production (301). Extended exposure of rictor$^{-/-}$ DC to RAPA was not sufficient to upregulate B7-H1 (data not shown). Our findings can therefore be ascribed to a novel, RAPA-resistant mTORC1 pathway that is subordinate to RAPA-sensitive mTORC1, or to concomitant inhibition of RAPA-resistant mTORC1 and mTORC2.

Our data highlight the distinct function of mTOR-containing complexes in DC when compared with T cells. mTOR-deficient T cells show reduced STAT3 phosphorylation (302), similarly to DC exposed to short-term mTOR inhibition. However, extended exposure of DC to ATP-competitive mTOR inhibition augmented STAT3 activation. While rictor$^{-/-}$ DC demonstrate reduced STAT3 activation, rictor$^{-/-}$ T cells exhibit normal STAT3 activation (204, 205). Furthermore, resting rictor$^{-/-}$ T cells express reduced SOCS3 (205). Our data show that extended ATP-competitive mTOR inhibitor conditioning reduces DC SOCS3 expression, but rictor deletion has no effect on SOCS3 in DC. These apparent inconsistencies support how mTOR signaling occurs differentially in DC and T cells.

Evidence is accumulating for a role of mTOR in DC in shaping T cell responses. Our data demonstrate that, while RAPA-sensitive mTORC1 promotes co-stimulatory molecule expression and Foxp3^{-} T_{eff} proliferation, RAPA-resistant and rictor-independent mTOR reduces IL-10 secretion and restrains T_{reg} induction by downregulating co-inhibitory B7-H1. Interestingly, although DC B7-H1 expression was required for augmented T_{reg} induction by Torin1-conditioned DC, the only known receptor for B7-H1, PD-1, was not required. PD-1-independent B7-H1 activity has been reported (303), and our data also suggest that unidentified B7-H1 receptors may exist. IL-1β was required for T_{reg} induction and functioned additively with

B7-H1 on Torin1-conditioned DC. Neutralization of IL-1β (data not shown) and genetic deletion of B7-H1 also reduced T_{reg} induction by RAPA-conditioned DC but not control DC, which suggests that other effects of mTORC1 inhibition are required that function collectively with IL-1β and B7-H1 to promote T_{reg} induction.

ATP-competitive inhibition to target RAPA-resistant mTOR may be useful for the treatment of inflammatory disorders. mTORC2 signaling is required for Th1 and Th2 (204) or only Th2 (205) differentiation, and deletion of mTOR in T cells causes diversion to $Foxp3^+$ T_{reg} following stimulation (302). Our data supports the development of ATP-competitive mTOR inhibitors for clinical use, especially since similar findings regarding B7-H1 expression were obtained in human DC cultures and following administration of an ATP-competitive mTOR inhibitor to mice. Further development of ATP-competitive mTOR inhibitors will be required since some have brief half-lives (281), and their *in vivo* pharmacology is poorly understood. Given the paradoxical enhancement of IL-12p70 (195, 202, 298) and IL-1β (201, 203) production by RAPA-conditioned DC and the reported pulmonary inflammation that can be associated with RAPA (304), augmentation of DC-derived IL-10 secretion and lack of increased IL-1β production by ATP-competitive mTOR inhibitors may mitigate untoward side effects of mTORC1 inhibition. Together, these studies demonstrate a novel, immune regulatory pathway mediated by RAPA-resistant mTOR downstream of TLR4 that is relevant for clinical applications of mTOR inhibition.

5.0 SUMMARY AND FUTURE DIRECTIONS

Myeloid lineage cells, including MDSC and DC, are innate immune cells that regulate adaptive immunity and are increasingly recognized as targets to modify immune responses. The data presented identify novel pharmacologic and biologic agents able to modify the expansion and function of these cells. HDACi and Flt3L both promote the expansion of MDSC, and Flt3L activates their suppressive function. The HDACi SAHA is a clinically-approved anti-neoplastic agent, and numerous immunomodulatory effects of HDACi have been described, including enhancement of Treg expansion and function (305) and inhibition of DC stimulatory functions (229-231, 306, 307). Following encouraging results in mouse models of GVHD (231, 242), HDACi are undergoing evaluation in clinical trials for therapy of acute and chronic GVHD. The role that MDSC play in the immune modulatory properties of HDACi in inflammatory disease settings is currently unknown. Evaluation of MDSC expansion by HDACi in appropriate, clinically-relevant transplantation models would be of benefit to determine if these cells contribute to immune modulation. Additionally, it will be necessary to investigate the ability of MDSC generated in HDACi *in vitro* to prolong long-term allograft survival following adoptive transfer to determine if this method of augmenting MDSC production *ex vivo* will have benefit for cellular therapy applications. Furthermore, the ability of HDACi, particularly FDA-approved SAHA, to expand MDSC in human PBMC cultures will be critically important to move these findings into the clinic.

Flt3L also strongly promoted MDSC expansion; however, the mechanism remains unclear. STAT3 was not required for MDSC expansion by Flt3L when inhibited with a chemical agent *in vivo*, but future studies will be required to verify this by genetic ablation of STAT3 within the hematopoietic or myeloid lineage using a Cre-Lox system. This system will also be useful to determine if STAT3 is required for MDSC expansion by HDACi. There is evidence that HDACi promote STAT3 acetylation and activation, and STAT3 prevents DC maturation and reduces T cell stimulatory function (232, 255, 308). The varying role of STAT3 in MDSC expansion in these studies highlights the shortcomings of our current understanding of factors regulating MDSC expansion since it appears to be model-dependent. Nevertheless, the finding that STAT3 inhibition during Flt3L administration diminishes immunostimulatory DC expansion while augmenting MDSC expansion suggests that targeting this pathway should be evaluated in experimental organ transplantation. Also, given the safety and efficacy of Flt3L in expanding DC in human volunteers (263, 264), it will be important to validate MDSC expansion by Flt3L in humans.

The dearth of knowledge of the influence of clinical immunosuppressants on MDSC expansion and function currently hinders targeting these cells to alleviate anti-allograft immune responses. mTOR is a central regulator of myeloid cell differentiation and immune function (172, 191), thus it will be interesting to determine if mTOR inhibition promotes MDSC expansion due to inhibition of DC differentiation. Inhibition of mTOR with RAPA or ATP-competitive mTOR inhibitors did not reduce the percentage of $CD11c^+$ DC in BM cell cultures stimulated with GM-CSF + IL-4, but conditions favoring MDSC expansion (i.e. excluding IL-4 in culture) were not investigated. Further studies will be required to determine if mTOR inhibition favors or is permissive to MDSC expansion in transplantation and chronic inflammatory disease.

Aside from MDSC immunobiology, mTOR inhibition regulates DC expansion, maturation and T cell stimulatory function (172). Historically, studies have interrogated mTOR function in DC using RAPA; however, until the recent discovery of RAPA-insensitive mTORC1 and mTORC2 (175, 176, 184, 185), the immunologic function of RAPA-insensitive mTOR was largely unknown. It has since been shown that mTORC1, but not mTORC2, is required for Langerhans cell homeostasis (309) and that mTORC2 negatively regulates TLR4-mediated pro-inflammatory cytokine secretion by DC (299). The data presented herein demonstrate a rictor-independent but RAPA-resistant function of mTOR that regulates anti-inflammatory STAT3, IL-10 and B7-H1. The signals that preferentially stimulate mTORC1 or mTORC2 in DC are not known, as both are activated by LPS stimulation. These data highlight the need for further investigation of RAPA-sensitive versus RAPA-resistant mTOR immune regulation to determine if targeting these pathways will be beneficial for subverting alloimmune responses. Indeed, progress is being made to improve the pharmacologic properties of ATP-competitive mTOR inhibitors *in vivo* since their half-lives currently limit their efficacy.

Together, the data presented within this dissertation highlight the opportunities moving forward to exploit myeloid cell immunobiology at the levels of differentiation, expansion or function of terminally differentiated myeloid cells to skew immune reactivity. Further studies will be required to translate these findings to *in vivo* disease models to advance the potential of HDACi, Flt3L and ATP-competitive mTOR inhibitors as therapeutic agents. It is anticipated that strategies harnessing manipulation of myeloid cells will become an important new approach to manipulate immune responses for the promotion of transplant acceptance.

APPENDIX A

PUBLICATIONS, ORAL PRESENTATIONS, AWARDS AND FELLOWSHIPS

A.1 PUBLICATIONS

Turnquist HR, Cardinal J, Macedo C, **Rosborough BR**, Sumpter TL, Geller DA, Metes D, Thomson AW. mTOR and GSK-3 shape the CD4+ T cell stimulatory and differentiation capacity of myeloid DC following exposure to LPS. *Blood*. 2010;115(23):4758-4769. PMID: 20335217

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Nace GW, Huang H, Klune JR, Eid RE, **Rosborough BR**, Korff S, Li S, Shapiro RA, Stolz DB, Sodhi CP, Hackam DJ, Geller DA, Billiar TR, Tsung A. Cellular specific role of toll-like receptor 4 in hepatic ischemia-reperfusion injury. *Hepatology*. 2013;58(1):374-387. PMID: 23460269

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Huang H, Chen H, Yan W, **Rosborough BR**, Nace GW, Ding Q, Evankovich J, Beer-Stolz D, Billiar TR, Esmon CT, Tsung A. Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. Under revision at *J Immunol*.

Rosborough BR, Raïch-Regué D, Turnquist HR, Thomson AW. Regulatory myeloid cells in transplantation (Overview). Under revision at *Transplantation*.

A.2 PUBLISHED ABSTRACTS

Rosborough BR, Turnquist HR, Thomson AW. HDAC inhibition enhances myeloid dendritic cell proliferation and induces reversible impairment of their T cell allostimulatory activity. *Am J Transplantation* 2009; 9(Suppl. 2): 476 (Abstract).

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A.3 ORAL PRESENTATIONS

Rosborough BR, Castellaneta A, Natarajan S, Thomson AW, Turnquist HR. Histone deacetylase inhibition facilitates GM-CSF-mediated expansion of myeloid-derived suppressor cells in vitro and in vivo. Block symposium, Session: Phagocytes. Immunology 2011 (American Association of Immunologists).

Rosborough BR, Matta BM, Turnquist HR, Thomson AW. mTORC2 negatively regulates DC PD-L1 and IL-10 through SOCS3 and STAT3. Block symposium, Session: Leukocyte activation and effector function in innate immunity, leukocyte activation in innate immunity. Immunology 2012 (American Association of Immunologists).

Rosborough BR, Matta BM, Turnquist HR, Thomson AW. Concomitant inhibition of mTORC1 and mTORC2 in dendritic cells reduces SOCS3 expression resulting in enhanced immunoregulatory PD-L1 expression and IL-10 secretion. Concurrent oral session, Session: Experimental immunobiology. 24^{th} International Congress of The Transplantation Society 2012.

Rosborough BR, Raïch-Regué D, Matta BM, Lee K, Boothby M, Turnquist HR, Thomson AW. Rapamycin-resistant mTORC1 regulates dendritic cell B7-H1 expression that acts in synergy with IL-1β to promote regulatory T cell induction. Concurrent session 61: Innate signaling in alloimmunity. American Transplant Congress 2013.

A.4 AWARDS

American Association of Immunologists Trainee Abstract Award, 2011

American Association of Immunologists Trainee Abstract Award, 2012

American Association of Immunologists Trainee Abstract Award, 2013 – Awarded but declined

American Transplant Congress Young Investigator Award, 2013

A.5 FELLOWSHIPS

Predoctoral Fellowship; Interdisciplinary Training in Transplantation Biology; NIH/NIAID T32AI074490; September 2010-June 2011

American Heart Association Great Rivers Affiliate Predoctoral Fellowship (11PRE7070020). Rapamycin-resistant mTOR regulation of dendritic cell function and heart allograft rejection. July 2011-June 2013

APPENDIX B

ABBREVIATIONS

B.1 ABBREVIATIONS BEGINNING WITH A NUMBER OR A-M

1-Me-D-trp, 1-methyl-D-tryptophan; 4OHT, (Z)-4-hydroxytamoxifen; Ab, antibody; Ag, antigen; APC, antigen-presenting cell; ATP, adenosine triphosphate; B6, C57BL/6; B7-H1, B7-homolog 1; Bcl-xL, B cell lyphoma-extra large; BM, bone marrow; C/EBP, CCAAT-enhancer-binding protein; CAV, chronic allograft vasculopathy; CCL, chemokine C-C motif ligand; CCR, chemokine receptor; cDC, conventional dendritic cell; CDP, common dendritic cell progenitor; CFSE, carboxyfluorescein succinimidyl ester; CMP, common myeloid progenitor; COX, cyclooxygenase; CSF1R, colony stimulating factor 1 receptor; CTLA-4, cytotoxic T lymphocyte antigen-4; d, day; DC, dendritic cell; DEPTOR, DEP domain-containing mTOR-interacting protein; DMSO, dimethyl sulfoxide; DST, donor splenocyte transfusion; Ebi3, Epstein-Barr Virus induced gene 3; eGFP, enhanced green fluorescent protein; Erk, extracellular-signal-related kinase; FKBP12, FK506-binding protein 1A, 12 kDa; Flt3; Fms-like tyrosine kinase 3; Flt3L, Fms-like tyrosine kinase 3 ligand; FoxO, O class forkhead box; FoxP, P class forkhead box; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte-colony stimulating factor; gfp, green fluorescent protein; GM-CSF, granulocyte macrophage-colony

stimulating factor; GVHD, graft-versus-host disease; h, hour; HDACi, histone deacetylase inhibitor/inhibition; HO-1 heme oxygenase-1; HSC, hematopoietic stem cell; Hsp, heat shock protein; HSPC, hematopoietic stem and progenitor cells; IC_{50}, half maximal inhibitory concentration; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; IRF1, interferon regulatory factor 1; JAK, Janus kinase; LC, Langerhans cell; Lin, lineage; LN, lymph node; L-NMMA, N^G-Methyl-L-arginine; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAMP, microbe-associated molecular pattern; M-CSF, macrophage-colony stimulating factor; mDC, myeloid DC; MDP, monocyte and dendritic cell progenitor; MDSC, myeloid-derived suppressor cell; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; mLST8, mammalian lethal with Sec13 protein 8; MoDC/Mo-DC, monocyte-derived dendritic cell; MP, myeloid progenitor; mTOR, mammalian/mechanistic Target of Rapamycin; mTORC, mTOR complex; MyD88, myeloid differentiation primary response gene 88

B.2 ABBREVIATIONS BEGINNING WITH N-Z

NADPH, nicotinamide adenine dinucleotide phosphate; NK, natural killer; NLR, NOD-like receptor; NO, nitric oxide; NOD, nucleotide oligomerization domain; norNOHA, N^{ω}-hydroxy-nor-L-arginine; PBMC, peripheral blood mononuclear cell; PD-1, programmed death-1; pDC, plasmacytoid dendritic cell; PD-L1, programmed death-1 ligand 1; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PRAS40, proline-rich substrate of Akt of 40 kDa; pre-DC, precursor dendritic cell; PROTOR, protein associated with rictor; PRR, pattern recognition receptor; PTEN, phosphatase and tensin homolog; r, recombinant; RAPA,

rapamycin; raptor, regulatory associated protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; RIG, retinoic acid-inducible gene; RLR, RIG-I-like receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid; SCF, stem cell factor; SD, standard deviation; SLO, secondary lymphoid organ; SnPP, tin protoporphyrin; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TCR, T cell receptor; Teff, effector T cell; TGF, transforming growth factor; Th, T helper; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg/T_{reg}, regulatory T cell; TSA, trichostatin A; VEGF, vascular endothelial growth factor; WT, wild-type

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