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The Role of Tumor Suppressors, SHIP and Rb, in Immune Suppressive Cells

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The Role of Tumor Suppressors, SHIP and Rb, in Immune Suppressive Cells

by

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DEDICATION

I dedicate this work…

To my parents… I have been blessed with wonderful and supportive parents, Maria E. Chanlatte Ruiz and Ramon Collazo Gonzalez and their equally wonderful and supportive partners Luis M. Chanlatte and Rosaura Rios Padilla, respectively. Thank you for always believing in me and trusting the choices I have made. I love and appreciate you so much.

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TABLE OF CONTENTS

LIST OF FIGURES

The Role of Tumor Suppressors, SHIP and Rb, in Immune Suppressive Cells

Michelle M. Collazo Ruiz

ABSTRACT

Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) have been extensively studied in the past 30-40 years. Their potent suppressive capacity shown in several pathological and clinical settings, such as cancer and transplantation, has made it evident that better understanding their development and function is critical.

Specifically, Tregs play a pivotal role in preventing autoimmunity, graft-versus-host disease (GvHD), and organ graft rejection. We previously demonstrated that germline or induced SH2 domain–containing inositol 5-phosphatase (SHIP) deficiency in the host abrogates GvHD. Here we show that SHIP-deficiency promotes an increase of FoxP3^{+} cells in both the CD4^{+}CD25^{+} and the CD4^{+}CD25^{-} T cell compartments with increased expression of Treg-associated markers. Importantly, SHIP-deficiency does not compromise Treg function. Interestingly, like conventional Tregs, SHIP$^{-/-}$ CD4^{+}CD25^{-} T cells are unresponsive to allogeneic stimulators and suppress allogeneic responses by T cells *in vitro*, and can mediate reduced lethal GvHD *in vivo*. Thus, SHIP limits the immunoregulatory capacity of CD4^{+} T cell, particularly in allogeneic settings.

SHIP-deficiency expands the number of immunoregulatory cells in both the T lymphoid and myeloid lineages. Here, we examined if these increases are interrelated. Specifically, we found that myeloid specific SHIP-deficiency leads to expansion of both MDSC and Treg numbers. Conversely, T lineage specific ablation of SHIP leads to

expansion of Treg numbers, but not expansion of MDSC, indicating an intrinsic role for SHIP in limiting Treg numbers. Interestingly, MDSC lack SHIP expression suggesting that another SHIP-deficient myeloid cell promotes MDSC and Treg expansion. Also, increased levels of G-CSF, a myelopoietic growth factor, in $SHIP^{-/-}$ mice may extrinsically promote MDSC expansion since we found that G-CSF is required for the expansion of splenic MDSC in mice with induced SHIP-deficiency.

MDSC consist of two distinct subsets, granulocytic-MDSC (G-MDSC), and monocytic-MDSC (M-MDSC) that differ in morphology, phenotype, suppressive capacity and differentiation potential. Importantly, M-MDSC can further differentiate into dendritic cells, macrophages and preferentially into G-MDSC, in the presence of tumor-derived factors (TDF). The retinoblastoma gene (Rb1), a tumor suppressor gene and central regulator of the cell cycle and differentiation, has been shown to influence monocytic and neutrophilic lineage commitment and to limit myeloproliferative disease. Here, we examined the role of Rb1 in the biology of MDSC subsets in tumor-bearing mice. Firstly, M-MDSC expressed high levels of Rb1 which remained relatively stable in culture with GM-CSF. Conversely, freshly isolated G-MDSC initially expressed undetectable levels of Rb1 that increased over time in culture, which correlated with increased histone acetylation at the Rb1 promoter. This increased Rb1 expression and histone acetylation was accelerated by histone deacetylase inhibitors (HDACi) treatment, suggesting Rb1 expression may be controlled by histone modification. Furthermore, when treated with HDACi, M-MDSC did not differentiate into G-MDSC in culture, even with TDF present. Finally, induced Rb1 deficiency *in vivo* promoted an expansion of splenic $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ cells, similar to G-MDSC in tumor-bearing mice. Although further studies are required, these results strongly suggest that Rb1, like SHIP, plays a role in MDSC accumulation, particularly G-MDSC in cancer.

Chapter 1. Background

Regulation of Immune Responses

The immune system, consisting of two arms, the adaptive and the innate; is a complex system of biological structures and processes that protects an organism against disease. Although it is seemly "dormant" while healthy and "wakes up" only during sickness, the immune system actually works constantly to maintain our health. Disease occurs when the immune system is challenged in such a way that its steady state activity is compromised or it is no longer sufficient to overcome the challenge, be it a bacterial or viral infection, cancer or overwhelming stress. When functioning properly, the immune system elicits an effective immune response strong enough to eliminate the challenge while not being deleterious to the host. Then the immune system "turns off" or suppresses the mounted response and returns to its steady state of maintenance and surveillance. Equally critical, cells of a healthy immune system can distinguish between foreign or altered self from self, dangerous from innocuous; and can appropriately dispose of the foreign, altered-self and dangerous[1].

Clinically, understanding this intricate balance attained and maintained by the immune system is very important, particularly when this balance is disrupted. For example, when the distinction between foreign and self is not made, the immune system can turn itself against for example, normal self-constituents in autoimmune disease, harmless environmental substances in allergy[2], an allogeneic fetus in pregnancy[3], or commensal bacteria in certain inflammatory bowel diseases[4]. In transplantation

procedures, establishing stable immunological tolerance to a solid organ or bone marrow (BM) transplant (BMT) is essential for successful engraftment[5]. Conversely, in cancer, reversing immune unresponsiveness to autologous tumor cells is the main goal in immunotherapy[6].

Several concepts have been established to elucidate how the immune system acts in physiological and pathological settings. The concept of Immunological Tolerance explains how the immune system protects from autoimmunity. Furthermore, it provides great insight into how the immune system can be manipulated to tolerate allergens, to accept an allogeneic graft and to effectively eradicate cancer[7-9]. Immune Surveillance and Immunoediting are concepts that further describe the role of the immune system in tumor progression[10] and thus form the foundation, along with Immunological Tolerance, from which different cancer immmunotherapies are developed.

Immunological Tolerance. Immunological tolerance is said to occur when the immune system recognizes an antigen but does not attack. Specifically, 'self tolerance' or 'natural tolerance' occurs when the immune system avoids mounting a response to self-antigens. 'Induced tolerance' occurs when the immune system attains tolerance to external antigens. There are three types of immunological tolerance: central tolerance, peripheral tolerance and acquired tolerance[7].

Central tolerance begins with the development of T and B lymphocytes in primary lymphoid tissues, the thymus and BM, respectively. Here, specialized antigen presenting cells (APCs) present maturing lymphocytes with a variety of self-antigens in complex with major histocompatibility complex (MHC) molecules. Simply, those that recognize these self-antigens are deleted or eliminated, preventing them from being released into the periphery and developing into fully immune competent self-reactive

cells[11]. This selection process occurs primarily during fetal life, but persists throughout life as immature lymphocytes are produced[12, 13].

More specifically, in the thymus, T cells go through a selection process that includes both positive and negative selection, based on the affinity of their receptor (TCR) to the presented self-antigen. TCRs must have sufficient affinity for self-MHC molecules in order for the T cell to be positively selected and thus allowed to mature and released into the periphery. T cells with insufficient affinity are forced to undergo apoptosis, or cell death. T cells whose TCR has very high affinity to self-MHC molecules are negatively selected, also undergoing apoptosis. Throughout this process, some T cells are instead recruited to further differentiate into Tregs[14]. B cell tolerance occurs in the BM where B cells either undergo several rounds of receptor editing or clonal deletion, which only occurs when receptor editing has been unsuccessful. Similar to T cell tolerance, self-reactive B cells are eliminated according to their ability to recognize autoantigens[15].

Once released into the periphery, the mature T and B cells then undergo peripheral tolerance. For instance, self-reactive T cells that were not eliminated during selection in the thymus are suppressed by Tregs in the periphery[16]. Additionally, without appropriate co-stimulatory signals or with co-inhibitory signals, lymphocytes that encounter antigen presented by peripheral APCs become hyporesponsive or anergic[17]. When an antigen is at a concentration that is too low to activate the encountering T cell, the insufficient stimulation instead leads to cell death. In immune privileged sites, such as the testes, potentially self-reactive lymphocytes are not activated. Finally, anatomical barriers, like the blood-brain barrier, can separate self-reactive lymphocytes from the antigen[18, 19].

The immune system can also adapt and become non-responsive to external antigens that would otherwise induce a cell-mediated or humoral immune response, a process called acquired or induced tolerance. A natural example of this is during pregnancy, where the maternal immune system must acquire tolerance to the fetus and the placenta[20]. Acquired or induced tolerance can also be readily achieved by administering intravenously, sublingually or orally; very large doses of antigen, or doses less than that required for effective immune stimulation[21, 22]. Promoting immune suppression, by using immunosuppressive drugs or by the preferential expansion of immunosuppressive cells, for example, can also facilitate tolerance induction. This is of clinical importance, for example in organ or BMT, where the allogeneic organ or BM must be accepted by the host. If the host is not induced properly to acquire graft tolerance, the graft will be rejected[23, 24]. In addition, inflammatory bowel diseases, such as Crohn's disease, ulcerative colitis and irritable bowel syndrome, are partly, if not primarily, due to the failure of orally induced tolerance[25].

In change, acquired tolerance, which also occurs in cancer, is a significant barrier in the effectiveness of cancer immunotherapy. Specifically, tolerance induced against tumor antigens has been clearly shown to thwart effective anti-tumor immune responses[26]. Furthermore, this tumor-specific T cell anergy is induced early in the course of tumor progression[27]. Interestingly, *in vivo* activation of APCs using antibodies against CD40 preserved the responsiveness of tumor-specific T cells in tumor-bearing host and promoted the regression of established tumors in mice[17].

Immune Surveillance and Immunoediting. To prevent cancer, the immune system employs three main functions. First, it clears or suppresses viral infections and in so doing, protects against virus-induced tumors. The immune system also removes

pathogens and resolves inflammation promptly thereby preventing a tumor-promoting inflammatory environment from being established. Lastly, it can eliminate tumor cells in various tissues to prevent them from establishing malignancy[28]. This process, referred to as cancer immune surveillance, is primarily driven by tumor-specific antigens (TSAs) expressed on tumor cells that are recognized by immune effector cells, which in turn mediate the destruction of these tumor cells[10]. Despite this, cancer can still develop, thus evading immune surveillance. Several studies have provided evidence and insight into why cancer develops in spite of an intact functioning immune system that is constantly employing immune surveillance. In summary, what these studies have shown is that while the immune system eliminates transformed cells, it is also applying a selective pressure where tumor cell variants with less immunogenicity, enabled by their genetic instability, can prevail[29]. This notion forms the basis behind the concept cancer immunoediting, a mechanism that justifies how the immune system can both prevent and promote tumor formation and progression.

Cancer immunoediting is a dynamic process that occurs in three sequential phases: elimination, equilibrium and escape; the flow of which can be influenced by several external factors such as environmental stress, aging and immunotherapeutic intervention. Elimination is essentially a modernized version of cancer immune surveillance. As explained above, during this phase, the innate and adaptive immune systems work together to detect and destroy transformed cells that have overridden intrinsic tumor suppressor mechanisms, before they can form a clinically apparent tumor[28]. Although the precise interplay and sequence have not been clearly defined, studies have clearly shown that immune molecules such as perforin, interferon-α/β (IFN-α/β), IFN-γ, TRAIL, NKG2D[30], and interleukin-12 (IL-12); and immune cells such as natural killer (NK) cells, T cells, macrophages (MΦ) and dendritic cells (DCs), among

others, participate in this phase. If successful, the process may end with the elimination phase, leaving the host cancer free[28].

Due to the genetic instability of transformed cells, a variant with less immunogenicity may emerge and remain untouched, denoting the beginning of the equilibrium phase. During this phase, the tumor remains functionally "dormant", its outgrowth kept in check by mechanisms employed specifically by the adaptive immune system, known to require such components as T cells[31, 32], IL-12, and IFN-γ[33]. Although "dormant", during this stage the transformed cells are undergoing an immune selective process mediated by the constantly interacting immune system that "edits" the tumor's immunogenicity, hence the term immunoediting. The immunoediting process may end here with the outgrowth of clinically detectable cancers restrained for a lifetime[34].

The constant immunoediting of genetically unstable tumor cells may eventually allow for the selection of tumor cell variants that are capable of entering the escape phase. In order to do so, the tumor cell variant may acquire one or more of the following qualities: (1) become undetectable by adaptive immunity by losing expression of TSAs or by developing defective antigen-processing or presentation machinery; (2) acquire resistance to cytotoxicity induced by immune effector functions mediated, for example, by inducing anti-apoptotic mechanisms and hyper-activation of pro-oncogenic transcription factors[35]; (3) induce an immunosuppressive microenvironment within the tumor by producing immunosuppressive cytokines, such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) and indoleamine 2,3-dioxygenase (IDO), among others, and by recruiting regulatory immune cells, such as Tregs and myeloid-derived suppressor cells (MDSC) that in turn also mediate immune suppression[36]. Although there are several other immune cells capable of suppressing immune responses, this dissertation will focus on Tregs and MDSC.

Immune Cells that Suppress Immune Responses

Regulatory T cells (Tregs). With their initial discovery in the 1970's, Tregs have been extensively studied in several immune contexts prevailing as critically important in active immune regulation. The discovery of Tregs, their development, mechanisms of suppressive function, their involvement in pathological conditions and clinical implications are discussed below.

Discovery of Tregs. Gershon and Kondo, in 1970, observed that T cells can both enhance and dampen an immune response, and that this dampening was mediated by a T cell population distinct from helper T cells[37], coined the name suppressor T cells. Several studies that followed described various types of suppressor T cells according to antigen specificity, secretion of suppressive factors, phenotype and suppressive mechanism[38]. Though, by the late 1980's, several factors came together causing researcher to avoid describing "suppressor T cells" in the context of immunological suppression or inhibition. These factors were, but not limited to, the scrutiny of the mouse MHC gene complex[39], failure in finding markers that reliably distinguished suppressor T cells from other T cells, ambiguity in the molecular basis of suppression, difficulty of preparing antigen-specific suppressor T cell clones to perform more detailed studies, and lack of clinical evidence for suppressor T cells as the primary cause of any immunological disease[40]. Most of these studies were focused on analyzing tolerance or suppression experimentally induced towards a particular exogenous antigen. Though not successful in clearly defining the suppressor T cell population whose sole purpose

was to down-regulate immune responses, these experiments did lead to the discovery and characterization of various immunosuppressive cytokines, such as IL-10[41] and TGFβ [42]. Immune suppression mediated by T cells was then being attributed to T cells that could be induced to secrete these immunosuppressive cytokines. This gave rise to the accepted existence of Tr1 cells that secrete IL10[43], and Th3 cells that secrete TGF-β[44], both of which were cells propagated via antigenic stimulation of naïve T cells. These cells are now known as induced Tregs (iTregs)[45].

In 1969, Nishizuka and Sakakura took a different approach to investigating T cell suppression and instead examined how autoimmune diseases can occur and be inhibited. This route clearly elucidated that the immune system did indeed harbor a specialized thymocyte population capable of suppressing autoimmunity[46]. They found that neonatal thymectomy of normal mice performed between the second and fourth day after birth resulted in manifestations of autoimmunity, specifically destruction of the ovaries and inflammatory tissue damage in other organs[47]. Similar studies performed on adult rats produced comparable results, including autoimmune thyroiditis[48] and type1 diabetes[49]. Furthermore, the autoimmune disease could be transferred by adoptively transferring $CD4^+$ T cells from a mouse suffering of an autoimmune disease to a T cell deficient mouse. The transferred $CD4^+$ T cells functioned as helper T cells and effector T cells that mediate immune destruction[50]. Importantly, transfer of syngeneic $CD4^+$ T cells and $CD4^+CD8^-$ mature thymocytes to mice than underwent a thymectomy prevented the onset of autoimmune diseases[51]. These and other experiments clearly showed that the normal thymus continuously produces a $CD4^+$ T cell population, now known as natural Tregs (nTregs) that protects the host from autoimmunity. In addition, it was clear that in the periphery of normal mice, there co-exist two types of $CD4^+$ T cells, one that can mediate autoimmunity and the other that can dominantly suppress them[52].

The studies that followed were in search of reliably distinguishing between these two cell populations and clearly identifying the $CD4^+$ T cell population responsible for preventing autoimmunity, particularly by the expression of surface markers. Initially, it was shown that this population existed within the $CD5^{high}$, $CD45RB^{low}$ $CD4^+$ T cell population[52, 53]. In 1995, Sakaguchi *et al* further discerned that cells expressing the CD25 molecule (high-affinity IL-2 receptor a-chain) within the $CD5^{high}$, $CD45RB^{low}$ $CD4^+$ T cell population more specifically represented the subset of T cells with suppressive capacity, which comprise about 5-10% of peripheral $CD4^+$ T cells[54]. These cells are now well-known as Tregs. CD25 signaling is in fact required for Treg function. IL-2, CD25 and CD122 (the IL-2R β-chain) -deficient mice spontaneously developed severe autoimmunity and have considerably reduced Treg numbers[55].

Finally, several years later, FoxP3, an X-linked transcription factor belonging to the fork-head family, was identified as the gene responsible for causing severe autoimmune and inflammatory disease in Scurfy mice[56] and in IPEX patients[57, 58]. Researchers then turned to examining FoxP3 and its possible relationship to Tregs, which was confirmed in 2003 when FoxP3 was described as a major regulator of Treg development and function[59]. In fact, retroviral transduction of FoxP3 confers normal $CD25^-CD4^+$ T cells with Treg-like phenotype and function, such as suppressive capacity and hypoproliferation, reduced IL-2 production and increased expression of CD25 and other Treg associated molecules, such as glucocorticoid-induced tumor necrosis factor receptor–related protein (GITR) and cytotoxic lymphocyte antigen 4 (CTLA-4)[59, 60]. It was now obvious that Tregs are vital regulators of immune responses and self-tolerance.

***Development of Tregs*.** There are two types of $CD4^+$ Tregs, “natural” Tregs (nTregs) and induced Tregs (iTregs), which differ primarily in where they develop.

nTregs develop in the thymus and undergo positive and negative selection, while iTregs develop in the periphery from conventional $CD4^+$ T cells that have been stimulated with antigen under various conditions. Together, they must achieve a fine balance between maintaining peripheral tolerance by suppressing autoimmunity, while controlling responses against foreign pathogens[45].

nTregs arise from progenitor cells in the BM and undergo their lineage commitment and maturation in the thymus, from which they migrate from into the periphery as early as after 3 days of life[61]. Like other T cells, Tregs are selected by peptides presented by thymic APCs, such as DCs, medullary thymic epithelial cells (TEC) or cortical TEC in the thymus[14]. Although not fully understood, it is known that their development is influenced by affinity between TCR and antigen, the location and context within the thymus were antigen is encountered, and cytokines and co-stimulatory molecules[45].

The signal strength received from the APCs that present self peptides is given by the degree of affinity or avidity between the TCR and MHC:peptide complexes, which in turn dictates the T cell selection process, as mentioned earlier[14]. nTregs seem to differ in the TCR affinity/signal strength required for positive selection in the thymus compared to conventional T cells[62]. Additionally, Tregs may require a specific composition of self peptide and amount of signal to promote their development since they do not develop in mice expressing a single TCR[63]. Recently, the TCR of hundreds of conventional T cells and nTregs were sequenced. This data suggests that nTregs actually express a polyclonal TCR repertoire comparable to conventional T cells, in contrast to what was alleged before, that nTregs preferably recognize self-antigen[64, 65]. This allows for an alternative theory. nTregs may have a lower activation threshold that conventional T cells. In other words, Tregs may have a differential ability to propagate a signal that

mediates a functional outcome versus conventional T cells[45]. Accordingly, human Tregs have been shown to be responsive to TCR stimulation at 10- to 100- fold lower antigen concentrations than that required to activate conventional T cells[66]. Furthermore, it has been proposed that T cells are committed to the Treg lineage before they encounter self peptide[67] and that even a weak TCR:self peptide-MHC interaction is sufficient to promote survival during thymic selection[68, 69].

Where the antigen is encountered, the niche within the thymus seems to also influence the thymocyte selection process. When quantitatively changing the level of thymic expression of a specific T cell epitope, there was increasing deletion of conventional T cells with increasing expression of the thymic epitope while no change in the absolute number of nTregs[69]. Control of peptide repertoire expressed in the thymus, which is controlled primarily by the autoimmune regulator gene (*Aire*) also affects T cell development[70, 71]. Some studies have suggested that *Aire*-expressing stromal cells may enhance FoxP3 expression in $CD4^+$ thymocytes and thus may play a role in nTreg thymic development[72]. Though, other studies show that in the absence of *Aire,* nTreg frequency and function stay the same with a slight alteration in TCR specificity[73]. Thus, the critical importance of *Aire* in Treg development and function is controversial and remains to be resolved.

Signals mediated by co-stimulatory molecules and cytokines are not only important for nTreg thymic development but also for peripheral maintenance. When deficient in co-stimulatory molecules, CD28[74], CD80/86 (B7)[75], CD40[76] and IL-2Rβ[77], mice exhibited reduced numbers of nTregs with defective suppressive ability. The cytokines, IL-2 and to a lesser extent TGFβ, are also of critical importance[78, 79]. Both in the thymus and in the periphery, Tregs express high levels of CD25 but do not themselves produce IL-2, thus being dependent on paracrine IL-2 for survival and

growth[78]. The existence of a CD25^{high} precursor population that is poised to express FoxP3 upon IL-2 or IL-15 stimulation and thus becoming nTregs, has been suggested[80]. Although nTreg development seemed unaltered in TGFβ receptor dominant negative mice, sustained TGFβ was recently shown to be required for maintenance of FoxP3 expression and suppressive capacity of peripheral nTregs both *in vitro* and *in vivo*[79].

As mentioned before, IL-10 induced type 1 Tregs (Tr1)[43], and TGFβ induced T helper 3 cells (Th3)[81] are the two main subsets of iTregs. Not only do they differ from nTregs in their point of origin, but also in how antigen exposure and specific factors expressed in different settings dictate their differentiation and contribution to the overall immune response[82, 83]. While differentiation of Tr1 and Th3 cells are promoted by different cytokines, IL-10 and TGFβ, respectively, they exert similar suppressive ability by secreting the same cytokine that drives their own existence. Depending on the experimental conditions, they have been shown to also secrete other cytokines. nTregs and iTregs have overlapping suppressive mechanisms and share a similar phenotype with activated T cells, such as CD25, CTLA-4, GITR, CD26L and CD45RB^{lo} expression[84, 85]. FoxP3 expression is seen in Th3 cells upon activation and TGFβ stimulation, though not seen in Tr1 cells[86]. Cytokines IL-4 and IL-13, which signal through the IL-4Rα chain, have also been shown to induce FoxP3 expression in naïve T cells[87].

While nTreg development is dictated primarily by self antigen in the thymus, iTreg development is promoted by exogenous antigen that sub-optimally engages the TCR in the periphery[88], usually in an inflammatory setting with anti-inflammatory cytokines. Though, it still has not been excluded that the TCR repertoire of iTregs may include TCRs with high affinity for self-antigen. Additionally, different to nTregs, iTregs do not require CD28 co-stimulation for development and suppressive function both *in*

vitro and *in vivo*[89, 90]. In fact, co-stimulation may hinder iTreg development *in vitro*[91]. Other factors that contribute to iTreg generation includes route of exposure to antigen, tissue specific factors and APCs. For example, exposure to antigen intranasal or orally preferentially promotes iTreg generation[92]. APCs such as gut-associated DCs[93], and tumor-associated DCs [94], MΦ associated with the lamina propria[95], monocyte-derived DCs, like plasmacytoid DCs (pDCs)[96] are all particularly efficient at generating iTregs. Finally, Tregs can induce their own generation, a process called "infectious tolerance[97]. For example, T cells from tolerized mice retain their tolerant state when transferred to another mouse, a process later shown to be maintained by Tregs who promoted iTreg generation from the suppressed T cells[98]. When placed in a co-culture with nTregs, naïve $CD4^+$ T cells were converted to iTreg with suppressive capacity dependent on IL-10[99] and TGFβ[100]. Though, it must be noted that both subsets of iTregs can be generated in mice completely lacking nTregs, suggesting that they are indeed developmentally distinct[98, 101].

Recently, a third population of iTregs has been described, termed iTr35. Treatment with IL-35 alone induced the conversion of naïve human or murine T cells to iTr35, a process that was augmented by low dose IL-10. Like Tr1 and Th3 cells, iTr35 cells mediate suppression by secreting the cytokine that promotes its generation, IL-35. These cells do not express nor require FoxP3 and exhibit stable suppressive capacity in several *in vivo* models. Compared to control T cells, IL-35 treated T cells did not exhibit an increased expression of CD25 nor CTLA-4, surface molecule previously described as mediators of nTreg suppression[102]. This demonstrates that these cells are indeed a completely different suppressive T cell population.

It is also important to note that recent studies have started to clearly elucidate another developmental property of Tregs, namely that in the periphery, they exhibit

significant plasticity. For example, a reciprocal relationship has been described between Th17 cells and Tregs[103]. As mentioned, TGFβ promotes iTreg development from naïve $CD4^+$ T cells, but in the presence of IL-6 and IL-21, this process is inhibited and instead this cytokine combination promotes the development of Th17 cells[104, 105]. In fact, in the presence of IL-6 and high levels of TGFβ, activated Tregs undergo conversion into Th17 cells[106, 107]. On the other hand, when retinoic acid (RA) is present, Th17 differentiation is inhibited and Treg induction is promoted, most likely due to increased TGFβ signaling and inhibited IL-6 signaling[108]. Finally, other studies have also explored the ability of Tregs to convert to other Th cells, such as Th1 and Th2 cells. Although contradictory results have been reported, Tregs have been shown to produce high levels of INFγ, a Th1 associated cytokine, when treated with large amounts of exogenous IL-2 and under Th1-polarizing conditions *in vitro*[109, 110]. No evidence has been reported of Tregs converting to Th2 cells, and thus this remains unknown.

Suppressive Mechanisms of Tregs. Tregs were discovered because of their ability to actively mediate dominant immune suppression and peripheral tolerance in pathological and physiological conditions. Tregs are capable of inhibiting several stages of target cell activity, namely, proliferation, differentiation and effector function, such as cytolytic activity, cytokine secretion and antibody production[111]. They do so by employing several mechanisms which can be divided into three categories: cell-cell contact, soluble suppressive factors, and competition[112]. Importantly, one mechanism is not more important that another and their use has been described in combination with each other, which seems to be context or disease dependent.

Mechanisms employed in cell-cell contact-dependent suppression mediated by Tregs are perhaps the most controversial since reproducibility of experimental results is

inconsistent in different settings. In addition, *in vitro* data does not directly translate to the *in vivo* setting. The suppressive ability of Tregs on naïve T cells is lost when co-cultured using an *in vitro* transwell system, which is consistent with the finding that membrane-bound TFG-β contributes to suppression[113]. Other cell surface molecules expressed by Tregs, such as cytolysis molecules Fas[114] and Granzyme B[115], LAG3[116], cytotoxic T-lymphocyte antigen 4 (CTLA-4)[117] and glucocorticoid-induced tumor necrosis factor receptor family–related gene (GITR)[118] have been implicated in their suppressive ability. Specifically, LAG3 interacts with MHC class II molecules on APC rendering them less capable of subsequently activating T cells[119]. By engaging CD80/CD86 ligands on target cells, CTLA-4 transmits 'outside-in' suppressive signals to activated T cells and to DCs[120]. In DCs, this signal activates indoleamine 2,3-dioxygenase, an immunoregulatory tryptophan catabolizing enzyme[121, 122]. Lastly, Tregs are also capable of regulating cyclic adenosine monophosphate levels in target cells which has been shown to inhibit proliferation, differentiation and cytokine production[123]. Evidence suggests that Tregs may do so by directly delivering cAMP to the target cell via gap junctions[124] or by indirectly increasing the production of adenosine by surface bound ectonucleotidases CD73 and CD39[125]. Adenosine then binds to the adenosine A2A receptor and causes an intracellular increase of cAMP[126]. How increased cAMP levels leads to suppression is not fully understood. A recent study has demonstrated that Treg-mediated increase in cAMP intracellular levels facilitates the nuclear accumulation of inducible cAMP early repressor (ICER) and suppression of nuclear factor of activated T cell c1 (NFATc1) and IL-2 production[127].

Soluble suppressive factors employed by Tregs include mediators such as IL-10, TGFβ, IL-35 and adenosine. Because of their ability to promote iTreg development, IL-10 and TGFβ are indisputably important suppressive mediators. Though, their

contribution to nTreg function is still debatable since Treg function has been largely accepted to be contact-dependent[128]. *In vitro* data has shown that use of neutralizing antibodies or of T cells unresponsive to IL-10 and TGFβ does not affect Treg suppressive capacity, suggesting that these cytokines are not important for Treg function[129]. Though, this is not the case *in vivo.* IL-10 or TGFβ alone or in combination with each other has been shown to be essential for the suppression mediated by Tregs in several pathological conditions, including allergy[130, 131] and autoimmunity[132], diabetes[133], irritable bowel disease[134], and cancer[135, 136]. Furthermore, several studies have found that IL-10 or TGFβ production by the Tregs themselves is not required for the observed Treg induced suppression[137, 138].

The primary biological effect of IL-10 is seen on APCs, namely DCs and MΦ by affecting antigen presentation, differentiation and maturation. Specifically, MHC class II expression and costimulatory molecule upregulation is inhibited by IL-10. These effects in turn prevent APCs from producing Th1- and Th2-associated cytokines. IL-10 also inhibits MΦ and DCs from producing proinflammatory cytokines, such as IL-1, IL-6 and IL-12, chemokines of both the CC and CXC type and matrix metalloproteases[139]. Finally, IL-10 also affects naïve $CD4^+$ T cells directly by inhibiting CD28 signaling[140].

TGFβ suppresses immune responses by regulating cells of both the adaptive and innate immune system and does so by either inhibiting the function of inflammatory cells or as mentioned, by generating iTregs[141]. TGFβ, which can be both membrane bound or secreted, was first described to suppress T cell and B-cell proliferation[142, 143]. It does so by inhibiting the production of IL-2, a cytokine necessary for the survival and activation of T cell, NK cell and other types of immune cells[142, 144]. Additionally, it suppresses $CD4^+$ T cell effector function and thus differentiation into Th1 and Th2 effector cells[145]. TGFβ can also regulate $CD8^+$ T cell proliferation and effector functions, such as expression of

IFNγ and perforin and granule exocytosis[146]. Finally, in NK cells, TGFβ negatively regulates INFγ production thereby indirectly controlling the induction of Th1 differentiation from $CD4^+$ T cells[147].

IL-35, formed by the pairing of the IL-27 β-chain and IL-12α, was recently found to contribute significantly to Treg suppression. Unlike IL-10 and TGFβ, IL-35 is required both *in vitro* and *in vivo*. IL-35 expression is increased in Tregs and seems to be a downstream target of FoxP3[148]. In fact, ectopic expression of IL-35 in T cells confers suppressive ability[149]. Though, it is still unclear which cell types are responsive and whether IL-35 is specifically expressed only by Tregs. As mentioned earlier, IL35 also promotes the generation of a third and novel iTreg subset, iTr35.

The last category of suppressive mechanisms employed by Tregs is competition or alteration of growth factors, such as cytokines and costimulatory molecules on APCs. Tregs express high levels of CD25, the high affinity receptor for IL-2, but do not themselves produce IL-2. Thus, Tregs have an advantage to consume IL-2 over naïve T cells, which express CD25 only after activation[111]. By consuming IL-2, Tregs are able to promote cytokine deprivation-induced apoptosis in the surrounding target cells[150] and exploit it for the induction of IL-10 production[151]. This was shown to be dependent on Bcl-2 interacting mediator of cell death (BIM). Importantly, preactivated IL-2 deficient T cells, which similar to Tregs, expressed high levels of CD25 but do not produce IL-2, were able to suppress proliferation and induce apoptosis in wild-type (WT) responder cells, though not as efficiently as Tregs. Additionally, IL-2-deficient responder cells still underwent apoptosis when cocultured with WT Tregs. Thus, Tregs may be consuming growth cytokines other than IL-2 since they do express components of other receptors specific for IL-4, IL-7 and IL-15[150].

Tregs also express CTLA-4 which specifically interacts with the costimulatory ligands CD80/CD86 on APC. Thus, they may be able to compete with conventional T cells for these costimulatory molecules and/or modulate APC costimulatory function. Once binding to CD80/CD86, CLTA-4 indeed sends an intracellular inhibitory signal to DCs thereby dampening their ability to strongly activate T cells[120]. T cell activation and proliferation also requires a reducing microenvironment and a supply of cysteine which is used to synthesize glutathione (GSH), an antioxidant essential for DNA synthesis. DCs are the main shapers of this redoxing microenvironment and producers of cysteine[152]. A recent study showed that Tregs mediate suppression by interfering with the extracellular redox potential, a process that is CTLA-4 dependent, antigen dependent but nonspecific, and cell-cell contact dependent. Specifically, they showed that Tregs modulate DC and effector T cell metabolism of GSH and competitively consume extracellular cysteine, thereby depriving effector T cells of it[153].

As it can be appreciated, Tregs have several suppressive mechanisms at their disposal. The importance of each mechanism and their differential use individually or in combination depends on the microenvironment and the immune pathology being suppressed.

Tregs in Pathological Conditions and Clinical Implications. Much of the experiments yielding the most clinically relevant and informative data on Tregs have been performed *in vivo* where immune responses are either deregulated, such as in tissue-specific autoimmunity; or overly regulated as seen in cancer and as desired in transplantation procedures. The examples, GvHD, inflammatory bowel disease (IBD) and cancer, discussed here after, are of most relevance to the studies presented further in this dissertation.

Patients that suffer from a blood cancer, anemia or a severe immunodeficiency syndrome are candidates for a BMT to replace their damaged or destroyed BM with healthy BM. Even if there is a good match between the donor and the host, seen usually among close family members, the host still runs the risk of succumbing to GvHD. This is the most common complication of allogeneic BMT and can be fatal[154]. GvHD is instigated primarily by conventional donor T cells found in the BM graft that perceive the host as "foreign" and launch an attack against host organs. Specifically, host APCs present major and/or minor histocompatibility antigens to host-reactive donor T cells, which in turn become activated and expanded. These activated donor T cells then infiltrate and destroy target tissues, primarily the gut, liver and skin[155]. When depleting BMT of donor T cells, the host is effectively protected from GvHD but also loses the beneficial effects mediated by donor T cell, such as graft-versus-hematopoiesis or -leukemia/-lymphoma effects, the last being the main therapeutic purpose in allogeneic BMT[156]. Furthermore, the BMT preparative regimen may also cause damage to the host thymic epithelium, compromising its ability to negatively select autoreactive T cells. These autoreactive T cells may also then participate in allogeneic GvHD, particularly in the autoimmune manifestations of chronic GvHD[23].

Because of their functional properties, anergy, suppression and dominant regulation of self tolerance, many have examined Tregs and their affect on host-reactive effector T cells after allogeneic BMT in mouse models[157]. One such study showed donor Tregs alone do not induce GvHD when transplanted in a completely MHC mismatched host[158]. When adoptively transferred at a high ratio (1:1) with conventional donor T cells, they provide protection from GvHD, which was partly mediated by Treg derived IL-10[159] and the capacity of Tregs to home effectively to lymph nodes (LNs)[160]. Similar to how host APCs activate donor T cells that mediate GvHD, host APCs and their presentation

of alloantigen also promote the induction of protection against GvHD by donor Tregs[161]. Importantly, these donor Tregs did not restrain conventional T cells from mediating graft-versus-leukemia-effect[158].

After being described as a lineage-defining transcription factor specific to nTregs, FoxP3 has been used as a reliable marker for Treg identification. As mentioned before, conventional T cells can be induced to express FoxP3 and acquire *in vitro* suppressive capability by TCR stimulation and TGFβ exposure[162]. This makes the *in vitro* generation of potentially therapeutic iTregs very feasible, though their *in vivo* efficacy is debatable[163]. Using FoxP3-reporter mice, Koenecke *et al* elegantly showed that while suppressive *in vitro*, allospecific iTregs were not able to prevent recipient animals from succumbing to GvHD, contrary to that seen with polyclonal nTregs. They found that when re-isolated from the pro-inflammatory setting characteristic to GvHD, this transferred iTreg population, but not nTregs, had rapidly lost their expression of FoxP3 and suppressive activity[164]. This, though negative results, points to the importance and requirement of a stable and sufficiently strong FoxP3 expression to maintain the Treg phenotype. It has been shown that FoxP3 expression in nTregs is controlled by epigenetic modifications, where nTregs exhibit complete demethylation, while conventional T cells and iTregs exhibit full methylation at conserved CpG-rich noncoding regions of the FoxP3 locus[165]. Thus, further studies that combine the use of *in vitro* FoxP3 induction and chromatin modifications that result in sustained FoxP3 expression are required to conceivably generate Treg products that are therapeutic in GvHD as well as in other diseases.

IBD, which includes Crohn's disease and ulcerative colitis, is characterized by chronic inflammation aberrantly orchestrated against the normal bacterial flora of the gastrointestinal tract. In order to understand the cause and process behind IBD, several

different murine models have been utilized[45]. For example, the T cell-induced colitis model is a well established system allowing researchers to analyze the role Tregs play in controlling IBD. Specifically, when transferred into an immune deficient mouse, a small number of $CD4^{+}CD45RB^{hi}$ T cells differentiate into Th1 cells and mediate colitis in about 4-6 weeks. This process can be prevented by co-transferring Tregs, and even reversed by transferring Tregs after the onset of colitis[166]. Most studies have made clear that IL-10 is a key cytokine used by Tregs in protecting from IBD. Further, although IL-10 derived from other cell types alleviates the symptoms to a degree, it is the Treg-derived IL-10 that effectively suppresses local inflammation[137]. This coincides with the finding that IL-10-producing Tregs are particularly enriched in the colonic lamina propria and secondary lymphoid organs in IBD[167]. Similarly in humans, intestinal $CD4^{+}$ T cells from Crohn's disease patients are defective in producing IL-10[168]. In addition to IL-10, other cytokines such as TGFβ[169], and more recently IL-35[149], have also been implicated in Treg mediated protection of IBD. Additionally, expression of specific chemokine receptors, such as CCR7[170], and of L-selectin (CD62L)[171] was found to be critical for Treg activity at inflammation sites characteristic to IBD.

As mentioned earlier, once cancer successfully progresses through to the escape phase of the immune editing theory, an immunosuppressive environment is established, in which Tregs contribute greatly to. Tregs have been shown to actively suppress anti-tumor specific T cells[172, 173] from mounting a successful immune response against the growing tumor. In addition, Tregs inhibit NK cells[174], B cells[175] and other immune cells. Tregs are found in increased numbers in the tumor microenvironment of patients with melanoma[176], lymphoma[177], breast[178], gastric[179] and lung cancer[180], among others; which has been correlated, in some cases, with poor prognosis[181]. Using mouse tumor models, several investigators have shown that the depletion of Tregs, for example

by use of anti-CD25 antibody, is permissive to an anti-tumor response[182, 183]. Furthermore, local depletion of Tregs specifically inside the tumor promoted a shift in the cytokine milieu, elimination of well-established aggressive tumors and long-term antitumor memory[184]. The mechanisms that have been described to be employed by Tregs in cancer are those that have already been mentioned. In short, use of suppressive cytokines such as TGFβ[135] and IL-10[136], modulation and suppression of APC function[185], and granzyme B-dependent cytolysis[115] have all been described to be used by Tregs to control the tumor-specific immune response. Interestingly, use of cytolysis by Tregs has only been described in the tumor microenvironment, representing a potentially disease-specific mechanism[45].

It must be noted that controversy exists as to whether the presence of tumor-infiltrating Tregs is an indicator of prognosis in human cancer. Some reports have shown that increased Treg numbers correlates with poor prognosis[181, 186] while others have shown the opposite[187-189]. An obstacle to discerning what is in fact true is the detection method used in these studies. Specifically, the expression of FoxP3 was used to determine Treg numbers. This is not an accurate representation of human Tregs since conventional human T cells have been shown to transiently express FoxP3 upon activation[190].

Also, the applicability and feasibility of targeting Tregs in cancer has not been clear-cut and straightforward. Specifically, while depletion of Tregs has been shown to promote a better anti-tumor immune response, very few reports have shown that this ultimately resulted in tumor regression[191]. Furthermore, when depleting Tregs, their numbers have been shown to quickly rise again from T cells converting to Tregs[192]. In addition, the approach used to deplete Tregs also ends up depleting anti-tumor T effector cells[193]. Moreover, before considering Treg depletion as a treatment for human

cancer, the distinction between solid cancers and cancers of hematopoietic origin should be made. Given that Tregs inhibit lymphocyte function[175], Tregs may retard the progression of some hematopoietic cancers, particularly those involving B and T cell lineages. Thus, when designing immunotherapeutic approaches that involve Treg depletion, improvement of anti-tumor T cell responses must be weighed against the direct suppression of cancer cells mediated by Tregs[194].

Myeloid Derived Suppressor Cells (MDSC). The discovery of MDSC in the 1980's instigated a field of study that has continued to become more complex and very exciting. Because of their heterogeneity and their varying presence in several pathological conditions, deciphering the biology of MDSC is instrumental for understanding and treating several diseases. The discovery, development and heterogeneity of MDSC, as well as their suppressive mechanisms, involvement in pathological conditions and clinical implications are discussed below.

Discovery of MDSC. The first description of MDSC could be dated back to the early 1980's in studies examining the effects of cancer on the immune system. One particular study showed that mice transplanted with mammary carcinoma exhibited abnormal hematopoiesis resulting in an expansion of a population of cells devoid of T and B-cell surface antigens in the spleen (SPL) and BM. These "null" cells seemed to further differentiate to neutrophilic granulocytes with an undefined function. This study suggested that the tumor influenced the primary lymphoid organs resulting in granulocytopoiesis and marked changes in lymphocyte populations[195]. Young *et al* later made similar observations in mice bearing a metastatic variant of Lewis Lung carcinoma LLC-C3 that stimulated an increase in the frequency of monocytes in the peripheral

blood, SPL and BM. They took it one step further by functionally characterizing these cells, at least those isolated from the BM, as being suppressive to T-lymphocyte blastogenesis. In addition, their experiments suggested that there was a colony stimulating factor secreted by the LLC-C3 that promoted the generation of these suppressor cells with immature monocytes-macrophage characteristics[196]. A few years later, it was found that treatment of tumor-bearing mice with a monoclonal antibody against granulocytes inhibited the growth of cancer[197] and even promoted tumor rejection mediated by $CD8^+$ T cells[198]. Although not fully understood, several other studies supported the observation that tumor progression correlated with increased immune suppression. Furthermore, this suppression was mediated by a tumor-derived factor that promoted an increase in a suppressive immature myeloid population[199], which was sometimes referred to as natural suppressors, suppressor MΦ or macrophage precursors, among many other names. Fu *et al*, who also observed the correlation between immune suppressive MΦ and tumor progression, provided compelling data suggesting that GM-CSF specifically, released by the tumor, mediated immune suppression by promoting the expansion of these MΦ[200]. Shortly after, the same lab identified two mechanisms employed by these MΦ to suppress immune responses in tumor-bearing mice, PGE2 production and cell-cell contact, which was MHC-independent[201]. During this same time, similar cells were also described to control immune responses associated with infectious diseases like *Trypanosoma*[202] and *Salmonell*a[203]. Eventually, Bronte *et al* described a suppressive population co-expressing the cluster of differentiation molecule 11b (CD11b), also known as integrin alpha M, and the granulocyte receptor-1 (Gr1) that accumulates following a potent primary immune response to a highly effective immunization strategy[204]. The co-expression of Gr1 and CD11b, a seemingly immature myeloid phenotype, and

suppressive capacity soon became the most reliable markers used to study MDSC, a name coined several years later in 2007 to eliminate confusion[205]. It must be noted that this definition remains very broad and thus includes a variety of cells that fit this criteria yet differ in anatomical compartmentalization, expression of other surface markers, differentiation state, suppressive mechanism, and pathological condition with its corresponding microenvironmental stimuli which promote their accumulation. Thus another characteristic of these cells is their extensive heterogeneity. Although the identity of this population has been most extensively studied in cancer, it is greatly appreciated that MDSC also play a role in other inflammatory diseases such as chronic infection, autoimmunity and trauma, among other pathological conditions[206].

Development of MDSC. MDSC are comprised of a variety of myeloid precursor cells at different stages of differentiation that may and do give rise to granulocytes, monocytes, MΦ, and dendritic cells. Thus, in order to understand the development of MDSC in pathological conditions, the normal development of myeloid cells should be reviewed. It is widely accepted that in the BM, hematopoietic stem cells give rise to a common myeloid progenitor/precursor which further differentiates into a macrophage/dendritic cell precursor (MDP). MDPs represent an intermediate differentiation stage from which both monocytes and DCs arise and then go into systemic circulation where they can enter the tissues and further differentiate into DCs and MΦ. MDPs may also generate immature and mature polymorphonuclear cells, thus possibly being part of a broader granulocyte-monocyte progenitor population.

This is a very simplified schematic that is still not fully elucidated and that is further complicated by the fact that these cell types can be further divided into either distinct subsets or functional states depending on several factors. After leaving the BM,

the localization, microenvironment and interaction with other cells greatly affect the terminal differentiation, function and activation fate of these newly arriving cells. For example, MDPs can give rise to “resident” or “inflammatory” monocytes, plasmacytoid or classical DCs, and “classically” or “alternatively” activated MΦ, which are all different phenotypically and functionally[206]. Inflammation and cancer create further complexity due to the secretion of a vast array of cytokines, chemokines, and growth factors such as prostaglandins, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), MMP-9, CCL2, CXCL5/12, IL-1β, IL-6, , TGFβ , IL-10, IL-12, and IL-13[207]. These secreted factors then activate several signaling pathways that ultimately deregulate the main transcription factors involved in myelopoiesis, resulting in the observed accumulation of MDSC. It has been hypothesized that the accumulation of MDSC requires at least two signals. One signal is mediated by growth factors and cytokines that promote their development and expansion. The other signal mediated by pro-inflammatory molecules that dictate their activation, which translates into their suppressive ability[208]. Although not limited to these, some of the extensively studied signaling pathways that become activated during MDSC development involve members of signal transducer and activator of transcription (STATs) family, NF-κB, cyclooxygenase 2 (Cox-2) and prostaglandin E2 (PGE2). These are discussed below. Important to the studies presented in the following chapters, SHIP signaling also plays a role in the development of MDSC and is discussed separately in the subsection titled “SHIP in Myeloid Cells”.

Many of the above mentioned cytokines signal through the JAK (Janus tyrosine kinase)/STAT pathway. Particularly, STAT3, which is controlled by JAK2, has been shown to be hyperactivate in the presence of tumor-derived factors and in turn be critical

for the expansion[209], suppressive capacity of MDSC, and to their contribution to angiogenesis[210]. STAT3 signaling in myeloid cells prevents cells apoptosis and differentiation, and promotes cell proliferation by controlling the expression of Bcl-xL, c-myc, cyclin D1 and survivin[211]. MDSC suppressive activity is abolished *in vitro* by STAT3 inhibition. Targeting STAT3 signaling *in vivo* using the multi-targeting tyrosine kinase inhibitor, sunitinib, inhibited the expansion of MDSC in tumor-bearing mice[212].

There are many pathways downstream of STAT3 that may also regulate MDSC expansion and suppression. For example, the calcium-binding pro-inflammatory proteins S100A9 and S100A8 are upregulated upon STAT3 activation in hematopoietic progenitor cells (HPC). Further, HPCs with overexpressed S100A9 were impaired in their ability to differentiate into DC and instead differentiate into MDSC *in vitro*. Similarly, overexpression of S100A9 in mice exhibited defective DC and macrophage differentiation and accumulation of MDSC in tumor-free mice. Consistently, in tumor-bearing mice rendered S100A9 deficient, MDSC splenic expansion was blocked[213]. MDSC have also been shown to secrete high levels of S100A8 and S100A9 which accumulate systemically thereby promoting their own accumulation. Signaling triggered by S100A9 and S100A8 activates the NF-κB pathway in MDSC. It is proposed that S100A9, dimerized with S100A8, promotes NADPH oxidase (Nox2) formation, which in turn produces ROS in myeloid cells, a known inhibitor of myeloid cell differentiation[214]. In addition, it was found that activated STAT3 upregulated Nox2 levels directly by promoting the transcription of $p47^{phox}$ and $gp91^{phox}$, its subunits[215].

STAT3 has also been shown to regulate the expression and DNA binding activity of the transcription factor CCAAT-enhancer-binding protein beta (C/EBPβ) whose expression is induced by tumor secreted cytokines like GM-CSF, G-CSF and IL-6[216]. C/EBPβ, which is vital in emergency granulopoiesis, was found to be critical for the

differentiation of myeloid precursors to functional MDSC. Additionally, adoptive transfer of tumor-specific $CD8^+$ T cells mounted an effective response against the established tumor only in mice with C/EBPβ-deficiency in the myeloid compartment[217].

Several other signaling pathways involving STAT3 activity have been identified critical to MDSC accumulation and function. In short, STAT3 controls the expression of acute-phase proteins, which in turn play a role in MDSC mobilization and survival. Heat-shock protein 72 (Hsp72) induces MDSC function by activating STAT3 in an IL-6, TLR2, and myeloid differentiation primary response gene 88 (MyD88)-dependent manner. Finally, STAT3-induced down-regulation of PKCβII, which is required for DC differentiation from myeloid progenitor cells, may in turn promote the preferred differentiation and accumulation of MDSC in tumor-bearing mice[218].

Other STATs also play a role in MDSC accumulation and function. STAT1, which is activated by INFγ or IL-1β, regulates iNOS (inducible nitric oxide synthase) and arginase-1 (Arg-1) activity. Because of this, STAT1-deficient MDSC are not suppressive. Further, blocking secretion of INFγ in either T cells or MDSC also abrogated MDSC mediated suppression[219]. STAT5 also plays a role in MDSC accumulation particularly in the presence of GM-CSF, which is abundant in the tumor microenvironment. MDSC accumulation was prevented by sunitinib specifically in the SPL of tumor-bearing mice. But, within tumors, MDSC were resistant to sunitinib due to the abundance of GM-CSF, which activates STAT5 while inhibiting STAT3[220]. Finally, similar to STAT3, activation of STAT6, which results from IL-4 or IL-13 engagement of the receptor CD124, also dictates MDSC suppressive function. CD124 activation was shown to upregulate Arg-1 activity[221, 222] and TGFβ[223] production in MDSC. In addition, MDSC expansion after traumatic stress was dependent on STAT6 signaling[224].

As mentioned briefly above, NF-κB signaling influences MDSC mediated suppression and expansion. In myeloid cells, NK-κB activation occurs downstream of signaling initiated at the toll-like receptor 4 (TLR4), among other TLR family members, and transmitted through MyD88. Consistently, MDSC accumulate in infections, trauma and sepsis[225]. Particularly, in the presence of IFNγ, LPS, a bacterial endotoxin that engages TLR4, promoted the expansion of splenic MDSC *in vivo* and impeded DC differentiation from BM cells *in vitro*[226]. Another study showed that dust mite-induced airway eosinophilia and T-helper 2 (Th2) cytokine production was hindered by high dose LPS in mice. Furthermore, the development of lung-resident MDSC was promoted by this LPS treatment in a TLR4 and MyD88-dependent manner. These MDSC suppressed Th2 mediated allergic airway inflammation[227]. Consistently, in a model using tumor exosomes, MDSC from Mydd88$^{-/-}$ mice, different to WT counterparts, did not suppress T cell activity or cytokine release, and did not accumulate in the lung[228].

IL-1β, which also signals through the NF-κB pathway, activates MDSC and thus plays a role in MDSC development. For example, overexpression of IL-1β specifically in the stomach of transgenic mice caused spontaneous gastric inflammation and led to cancer. This is accompanied with the accumulation of MDSC in the stomach, all of which was inhibited by an IL-1β receptor antagonist[229]. Importantly, studies examining the role of NF-κB have shown that NF-κB signaling mainly dictates MDSC activation and acquisition of suppressive capacity more so than promoting MDSC accumulation.

The activity of prostaglandin E2 (PGE2), its receptor E-prostanoid 4, Cox-2, and Arg-1 are all interrelated and have been implicated in MDSC development. During PGE2 synthesis, Cox-2, expressed by many cell types, particularly tumor cells and MDSC, catalyzes the conversion of arachidonic acid to prostaglandin G2 that is further modified to PGE2 by PGE synthase. PGE2, a well known tumor-derived factor, then

engages its receptor, E-prostanoid 4, expressed on MDSC, and induces the expression and activity of Arg-1[230]. Furthermore, the use of Cox-2 inhibitors was shown to block the expression of Arg-1, prevent the accumulation of MDSC and elicit a lymphocyte-mediated antitumor response in a 3LL Lewis Lung carcinoma mouse model[231]. In a study using tumor exosomes that induced the accumulation of MDSC, the use of antibodies against PGE2 and TGFβ attenuated MDSC-mediated promotion of tumor progression by preventing MDSC induction[232]. These and many other studies have clearly shown that Cox-2 and PGE2 comprise a critical signaling pathway dictating both MDSC differentiation and function.

Heterogeneity of MDSC. In accordance with the variety of factors produced by tumors and infectious agents that promote the development of these cells; MDSC, as defined by suppressive capacity and co-expression of Gr1 and CD11b, are extensively heterogeneous morphologically, phenotypically and functionally. The confusion brought on by this heterogeneity has led researchers to further dissect MDSC into subsets according to their morphology, phenotype and suppressive capacity.

Initially, it was proposed that a more potent suppressive subset could be distinguished among MDSC by analyzing the expression of specific surface markers, specifically the M-CSF receptor (CD115), the IL-4 receptor (IL-4R) α chain (CD124), CD40, CD80 and CD49d. One such study using a mouse colon carcinoma model showed that $CD115^+$ MDSC were significantly more potent at suppressing antigen-specific T cell stimulation and inducing Treg development compared to $CD115^-$ MDSC[233]. Using a colon carcinoma mouse model, Gallina *et al* showed that expression of CD124 was required for MDSC-mediated suppression of alloreactive CTL generation *in vitro*. Furthermore, they showed that adoptively transferred tumor-specific $CD8^+$ T cells were

able to prevent tumor growth in myeloid-specific CD124 knockout tumor-bearing mice, while not so in WT tumor-bearing mice[219]. Another study using a mouse ovarian carcinoma, described the requirement of CD80 expression on MDSC to suppress antigen-specific immunity mediated by $CD4^+CD25^+$ Tregs and CD152 signaling[234]. The studies performed by Pan *et al* suggested that the expression of CD40, an immune stimulatory receptor, on $CD115^+$ MDSC was essential for their capacity to directly induce T cell tolerance and tumor-specific Treg expansion[235]. Finally, CD49d expression discovered using gene-expression analysis, subdivided MDSC into two distinct populations. $CD49d^+$ MDSC were mainly monocytic, while $CD49d^-$ MDSC were granulocytic. The $CD49d^+$ MDSC subset was more potent at suppressing T cell proliferation in an NO-dependent manner compared to the $CD49d^-$ subset[236]. Although these markers are indeed expressed by MDSC, further studies on other tumor models showed that these markers were actually more tumor model specific than universal markers defining the most immunosuppressive population among MDSC. Importantly, it is clear that MDSC consists of both monocytic cells, M-MDSC and granulocytic cells, G-MDSC.

Because MDSC express varying levels of Gr1, some researchers have suggested that the immune suppression capabilities of MDSC could be determined by Gr1 expression level. Specifically, when isolated from tumor-bearing mice, $CD11b^+Gr1^{int}$ MDSC, comprised mainly of M-MDSC and myeloid precursors, were potently suppressive of $CD8^+$ T cell effector functions. On the other hand, $CD11b^+$ $Gr1^{high}$ MDSC comprised mainly of G-MDSC, exhibited moderate suppression only in particular tumor models which required them to be present in high numbers. In addition, the $CD11b^+Gr1^{int}$ MDSC could further differentiate into CD11c and F4/80 expressing cells while the $CD11b^+Gr1^{high}$ cells could not[237, 238].

The antibody most commonly used to examine Gr1 expression, RB6-8C5, recognizes Ly6G, which is present on neutrophils, and Ly6C, which is present on neutrophils, dendritic cells and subpopulations of lymphocytes and monocytes[239]. Using antibodies that distinguish between these surface proteins allowed for the clear distinction of two subsets within MDSC in tumor-bearing mice[238], $CD11b^{+}$ $Ly6G^{+}Ly6C^{lo}$ and $CD11b^{+}$ $Ly6G^{-}Ly6C^{high}$. After morphological analysis, it was clear that the $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ MDSC were granulocytic, corresponding to G-MDSC or the $Gr1^{high}$ cells. The $CD11b^{+}Ly6G^{-}Ly6C^{high}$ were monocytic corresponding to M-MDSC or the $Gr1^{int}$ cells described above[240, 241]. These two major subsets differ also in the nature of their immune suppression, which is discussed in more detail in the next section. Briefly, G-MDSC suppress in an antigen-specific manner, which is mediated by their expression of Arg-1, high levels of ROS production and direct cell-cell contact with T cells. M-MDSC effectively suppress in a nonspecific manner, independent of cell-cell contact, by upregulating the expression of both iNOS and Arg-1, and by producing various suppressive cytokines[240, 242]. When examined on a per cell basis, some studies suggest that M-MDSC are more potent suppressors than G-MDSC[243]. Though, in most tumor mouse models, G-MDSC are significantly more abundant than M-MDSC in peripheral lymphoid organs[241]. Importantly, within the tumor, the ratio of G-MDSC to M-MDSC is much lower than that in the periphery, thus possibly shaping a distinct immunosuppressive environment within the tumor site. In addition, these two subsets differ in their proliferative potential, where M-MDSC are highly proliferative compared to G-MDSC, which are not as proliferative[244].

It must be noted that MDSC heterogeneity, as described by analyzing morphology, suppressive mechanism, proliferation and compartmental accumulation, is not restricted to these two major subsets. Intermediate groups of cells at different

stages of differentiation that possess varying phenotypes most likely exist. Other MDSC subsets not mentioned here have been described and more subsets will most probably be identified in the future.

Suppressive Mechanisms of MDSC. Several immunosuppressive mechanisms employed by MDSC have been described. It must be kept in mind that these mechanisms are very likely influenced by the specific microenvironment in which MDSC develop, by tumor characteristics and by the activation level of the lymphocytes being suppressed[245]. In summary, MDSC suppressive activity has been associated with L-arginine metabolism, production of reactive oxygen species (ROS) including peroxynitrites, induction of Tregs, sequestration of cysteine and downregulation of L-selectin on T cells[207, 246, 247]. These mechanisms are discussed below in more detail. Importantly, many of these mechanisms have not only been described in cancer but also in chronic inflammation, experimental autoimmune encephalomyelitis and infection[207]. In addition to suppressing T cell activity, MDSC also inhibit the innate immune response by affecting MΦ, NK cells and NKT cells activity[246].

L-arginine is a substrate for two enzymes highly expressed by MDSC: iNOS, which produces NO, and Arg-1, which uses L-arginine to produce urea and L-ornithine. Both M-MDSC and G-MDSC express high levels of Arg-1 while M-MDSC also express iNOS. The increased expression of these enzymes leads to enhanced L-arginine metabolism. MDSC can either import excess L-arginine from their environment through their CAT-2B transporter, as seen in murine MSDS, or release Arg-1 into circulation, as seen with human MDSC, specifically from renal cell carcinoma patients[248]. Regardless, either method depletes the amount of L-arginine available for T cells, which require L-arginine for protein synthesis. L-arginine deprivation inhibits T cell proliferation by

decreasing the expression of the CD3ζ chain[249], and the expression of cell cycle regulators cyclin D3 and cyclin-dependent kinase (CDK) 4[250]. Furthermore, NO has been shown to downregulate the activation of JAK3 and STAT5 required for IL-2 mediated T cell proliferation[251]. NO also inhibits the expression of MHC class II on a variety of cells[207, 252].

Increased ROS production, which is characteristic of G-MDSC and their increased Arg-1 activity, has been extensively described as a major mediator of MDSC suppression in cancer patients and tumor-bearing mice. In fact, *in vitro* inhibition of ROS production blocks the suppressive capacity of MDSC isolated from cancer patients and mice[211, 212]. ROS production, which requires STAT3 and NADPH activation, is induced by several known tumor-derived factors such as TGFβ, IL-10 and GM-CSF[253]. In addition, engagement of integrins expressed on MDSC upon interacting with T cells, has been shown to enhance ROS production by MDSC[254]. ROS has been shown to affect T cell activity in several ways. One study showed that increased ROS, particularly hydrogen peroxide, produced by MDSC inhibited cytokine production by T cells *in vitro*[255]. Another study showed that ROS production was responsible for MDSC-mediated suppression of antigen specific $CD8^+$ T cell responses[256].

Peroxynitrite ($ONOO^-$), a powerful oxidant produced when superoxide (O_2^-) reacts with nitric oxide (NO), causes the nitration and nitrosylation of amino acids cystine, methionine, tryptophan and tryosine[257]. Peroxynitrite found in high levels has been associated with tumor progression in several cancers[257-259] and with T cell unresponsiveness. For example, unresponsive $CD8^+$ T with high levels of nitrotyrosine were found within human prostate adenocarcinomas. The use of Arg-1 and iNOS inhibitors decreased tyrosine nitration in T cells and restored their anti-tumor activity[260]. Recently, it was more clearly elucidated that peroxynitrite produced by MDSC when in

direct contact with T cells, nitrated the TCR and CD8 molecules on the T cell. This altered the T cell's ability to bind to antigen/MHC complexes, and thus become activated upon antigen recognition. This phenomenon is antigen-specific, since the T cells were still responsive to nonspecific stimuli[261].

MDSC can also promote the development of Tregs, which requires the presence of IFNγ and IL-10, and the activation of tumor-specific T cells[233]. Contradictory studies have been presented regarding the requirement of TGFβ production by MDSC in promoting Treg development, suggesting that different MDSC subsets use diverse mechanisms to induce Tregs[223, 233]. Furthermore, other studies have argued against the idea that MDSC promote Treg development[240], thus requiring further studies to demonstrate this definitively. Regardless, MDSC and Tregs seem to be involved in a common immunoregulatory network.

Similar to L-arginine, cysteine is required by all cells for protein synthesis. Specifically, T cells require cysteine for activation and subsequent proliferation and differentiation. Though, they are not capable of importing cystine, the oxidized form of cysteine, or of producing cysteine themselves. Thus, T cells rely on exogenous sources of cysteine, which they import through the ASC neutral amino acid transporter[246]. Exogenous cysteine is supplied by APCs, such as DCs and MΦ, particularly during antigen presentation[262]. Recent studies have clearly demonstrated that MDSC, which also need cysteine, deplete their environment of cystine, disrupt the extracellular production of cysteine by thioredoxin, and do not export surplus cysteine as APCs do. Because MDSC are present during antigen presentation, they deplete the local microenvironment of cysteine and thereby inhibit T cell activation and proliferation[263].

Finally, MDSC prevent naïve T cell from homing to sites where they would otherwise undergo activation by mediating the down-regulation of L-selectin (CD62L)

surface expression on T cells. L-selectin facilitates leukocyte extravasation from the blood to LNs and inflammatory sites such as tumor microenvironments, where they encounter antigen and become activated[246]. Naïve T cells normally have an L-selectin^{high} phenotype. Conversely, in tumor-bearing mice and cancer patients, T cells have lower levels of L-selectin expression, which is inversely correlated to MDSC level and tumor burden. In fact, when co-cultured with MDSC, T cells acquire an L-selectin^{low} phenotype. MDSC directly down-regulate L-selectin expression because they constitutively express ADAM17, a disintegrin and metalloproteinase domain 17 that mediates the proteolytic cleavage and shedding of the L-selectin ectodomain by T cells[264].

MDSC also affect cells of the innate immune system, such as MΦ, NK cells, and NKT cells. MΦ can be activated "classically" and differentiate into so-called M1 MΦ, which promote an anti-tumor response; or "alternatively" and differentiate into so-called M2 MΦ, which enhance tumor development. When in contact with MDSC, which produce IL-10, MΦ acquire the M2 phenotype and decrease their production of IL-12[222, 265]. M2 MΦ also release IL-10, exert selective immunosuppressive activity, and inhibit T-cell proliferation. In addition, Ilkovitch *et al* demonstrated that in the liver, MDSC accumulate and interact with Kupffer cells, which are liver-residing specialized MΦ. This interaction upregulates the expression of PD-L1, a negative T cell costimulatory molecule, on Kupffer cells, which contributes to immunosuppression in tumor-bearing mice[266].

The effect of MDSC on NK cells varies. One study showed that MDSC inhibited NK cell cytotoxicity and perforin production, which required cell-cell contact[267]. In contrast, another study showed that a specific subset of MDSC expressing RAE-1, the ligand for the activating receptor NKG2D, promoted NK cell activity, particularly INFγ

production. Furthermore, these activated NK cells could eliminate MDSC when they were co-cultured *in vitro* or adoptively transferred *in vivo*[268].

The relationship between NKT cells and MDSC also varies depending on the NKT cell type in question. Type 1 or invariant NKT cells inhibited MDSC suppressive activity in a CD1d- and CD40- dependent manner[269]. In change, type II NKT cells produce IL-13[270] which has been shown to promote M2 macrophage and MDSC accumulation[222].

MDSC in Pathological Conditions and Clinical Implications. MDSC have been most extensively studied, particularly in cancer. As mentioned before, MDSC also play a role in other inflammatory diseases such as chronic infection, autoimmunity and trauma, among other pathological conditions[206]. Similar to Tregs, the more that is understood about the development and function of MDSC, the more effective it will be to target them, which is critical in cancer, or to exploit their suppressive ability as desired in transplantation procedures. The involvement of MDSC specifically in GvHD, IBD and cancer are of most importance to the studies presented further in this dissertation. Since their involvement in cancer has already been discussed above, what follows is what is known about their involvement in GvHD and IBD.

As mentioned before, GvHD is a significant hurdle that limits the use of BMTs as an effective treatment for diseases mediated by pathological immune function such as cancer, anemia or severe immunodeficiency syndrome. Similar to Tregs, MDSC suppressive function can be exploited to prevent GvHD. One such study examined the role of MDSC in preventing GvHD while preserving the GVL reactivity of donor lymphocyte infusions (DLI) in a murine model using a minor histocompatibility antigen-mismatched BMT. If the DLI was administered immediately after the BMT, the mice

would succumb to GvHD; but if 3 weeks passed before administering the DLI, GvHD did not occur while preserving GVL. The researchers suggested that this was mediated by MDSC that underwent a transient expansion peaking at 3 weeks after radiation and BMT, and whose suppressive function was dependent on INFγ and mediated by NO production[271]. Morecki *et al* showed that MDSC generated in and enriched from mice treated with CpG+IFA were capable of preventing GvHD when co-transferred with naive T cells[272]. Recently, another group, using G-CSF and GM-CSF, was able to generate MDSC *in vitro*. The suppressive activity of these MDSC was mediated by Arg-1 expression and enhanced by IL-13. These IL-13 enhanced MDSC protected mice from GvHD while also preserving the GVL effect of donor T cells. Furthermore, use of pegylated Arg-1 alone also resulted in significant GvHD reduction[273].

The role of MDSC in IBD and colitis has not been extensively studied, though the few existing studies clearly demonstrate that they may indeed play a role. One study used an antigen-specific mouse model of IBD mediated by $CD8^+$ T cell. While VILLIN-HA mice receiving a single transfer of HA-specific $CD8^+$ T cells exhibited weight lose and intestinal inflammation, mice that received repetitive transfer of splenocytes from CL4-TCR mice showed almost no signs of enterocolitis or weight loss. The repeated transfer of splenocytes caused an increase of MDSC in the SPL and intestine that produced high levels of NO and expressed functional Arg-1. Though, their suppression of $CD8^+$ T cell proliferation was dependent on NO production and not on Arg-1 activity. Furthermore, when co-injected with CL4-TCR splenocytes, MDSC isolated from mice with 3 transfers protected recipient mice from developing enterocolitis[274].

The Role of Tumor Suppressor, SHIP, in Regulating the Immune System

Discovery of SHIP. Src homology (SH) 2 domain containing 5' inositol phosphatase 1 (SHIP), a 145kDa protein, was first described in hematopoietic cell lines where stimulation with erythropoietin (Epo) induced its phosphorylation at a tyrosine residue and its subsequent association with Shc[275]. A few years later, in 1996, five research groups independently cloned SHIP by gene trapping[276] and by its binding to the SH3 domain of growth factor receptor-bound protein 2 (Grb2)[277], to the protein-tyrosine binding (PTB) domain of SH2-containing sequence protein (Shc)[277-279] and to the IgG Fc receptor, FcγRIIB[280]. Once identified, SHIP was been found to hydrolyze the 5' phosphate group in inositol-1,3,4,5–tetrakisphosphate (IP4) and in phosphatidylinositol-3,4,5-phosphate (PIP3)[279]. IP4, a soluble phosphoinositide, regulates store-operated Ca_2^+ channels in lymphocytes[281]. In modulating PIP3 levels, SHIP regulates the activity of signaling molecules, such as Tec kinases, Akt and PLCγ, downstream of phosphoinositide 3-kinase (PI3K), which in turn modulates several cellular pathways that drive proliferation, differentiation, apoptosis and migration[282]. Because the expression of SHIP is shared by most hematopoietic cells[277-279, 283], its role in modulating immune function and hematopoiesis has been extensively studied. The structure of SHIP, the factors that influence its activity and its role in T cells and myeloid cells specifically are reviewed below.

SHIP Structure and Functional Domains. In addition to the enzymatic domain, the 5' inositol phosphatase located at its core, SHIP's contains other regions, such as an SH2 domain in its animo terminus, and several NPXY and polyproline rich motifs in its carboxyl terminus, that mediate its interaction with a variety of other signaling molecules.

SH2 Domain. The SH2 domain allows SHIP to bind to phosphorylated tyrosine residues on activation or inhibitory motifs found on the intracellular tails of several receptors such as FcγRIIB on B cells[280], Ly49 receptors on NK cells[284], and the IgE receptor, FcεRI on mast cells[285]. In addition, the SH2 domain allows SHIP to interact with Shp-2[286, 287], an SH2 domain containing tyrosine phosphatase, Lyn[288], a member of the Src family of protein tyrosine kinase, and Shc[289], the SH2 domain containing transforming protein 1, when tyrosine residues within them are phosphorylated. Importantly, as a result of these interactions, SHIP becomes localized near the membrane and acquires enhanced function[290]. The phosphorylated tyrosine residue through which SHIP interacts with Shc is the same docking site for Grb2. Thus, when SHIP interacts with Shc via its SH2 domain, it prevents Grb2 from interacting with Shc and subsequently, from recruiting other protein complexes that activate the Ras and downstream mitogen-activated protein kinase (MAPK) pathways[283]. Similarly, the SH2 domain confers SHIP with a *masking function* pivotal in its ability to modulate other signaling pathways[291].

***5' Inositol Phosphatase*.** SHIP's enzymatic domain is pivotal to its ability to affect cell signaling. In order for it to recognize its substrates, the 5' inositol phosphatase requires a phosphate group, PO_4, positioned at the D3 location of the inositol ring. This limits its enzymatic activity to the following phosphoinositides, PIP3 and IP4, which are converted to $PI(3,4)P_2$ and $I(1,3,4)P_3$, respectively, by SHIP[279]. PI3K is responsible for the production of PIP3 from $PI(4,5)P_2$[292] and inositol 1,4,5-trisphosphate 3-kinases (IP3K) is responsible for the production of IP4 from $I(1,4,5)P_3$[293].

By decreasing PIP3 levels in the cell, SHIP can limit the activation of pathways downstream of PI3K, such as AKT, also known as Protein Kinase B (PKB)[294]. Specifically, AKT translocates to the site of PIP3 production, primarily at the plasma membrane, via its pleckstrin homology (PH) domain[295]. Here, it becomes activated by being phosphorylated at Threonine 308 and Serine 473[296, 297]. Activated AKT has been shown to phosphorylate and thereby inactivate pro-apoptotic proteins, such as BAD, which ultimately resulted in the inhibition of the intrinsic pro-apoptotic pathway[298]. Thus, SHIP activity indirectly leads to decreased proliferation and survival signaling. In addition, $PI(3,4)P_2$ has been shown to trigger qualitatively different PI3K effector pathways than those promoted solely by PIP3. In cells, $PI(3,4)P_2$ has been suggested to serve as a secondary messenger resulting in the activation of AP1 and AKT[299-301]. Thus, it can be said that in certain contexts, SHIP may amplify PI3K signals.

PIP3 levels dictate the progression of several other signaling pathways. For example, bruton tyrosine kinase (Btk), a Tec family kinase, is another downstream target whose membrane localization and activation is inhibited by decreased levels of PIP3. This results in decreased levels of PLCγ and a subsequent block in the influx of extracellular calcium. In addition, PLCγ generates the IP3K substrate, $I(1,4,5)P_3$ from $PI(4,5)P_2$. Thus, by indirectly causing a decrease in the IP3K substrate, SHIP limits the production of its own substrate, IP4, by IP3K[302]. Finally, decreased PIP3 levels have also been found to down-regulate gene transcription in myeloid cells mediated by the NF-κB pathway[303].

Importantly, recent studies have shown that SHIP's enzymatic activity can be allosterically regulated. Specifically, SHIP contains a C2 domain which enables it to associate with its product $PI(3,4)P_2$. This association was found to increase SHIP's catalytic activity[304]. In addition, cAMP-responsive PKA mediated phosphorylation of

SHIP on serine can also enhance SHIP's enzymatic activity[305]. Thus, availability of its own product and phosphorylation by PKAs, and perhaps other kinase, may determine the potential magnitude of SHIP's enzymatic activity.

***NPXY and PxxP Motifs*.** At the carboxyl terminus, SHIP has several NPXY motifs where NPXY represents the amino acids, arginine (N), proline (P), any amino acid (X), and tyrosine (Y). When SHIP becomes activated, these motifs are phosphorylated at the tyrosine residue forming a binding site for proteins containing PTB domains, such as Shc, Dok1 and Dok2[289, 306, 307]. The p85 subunit of PI3K can also bind directly to these NPXY motifs, suggesting another mode in which SHIP may control PI3K signaling[308, 309]. Although, studies suggest that the phosphorylation of NPXY motifs does not seem to be required for the function of the 5' inositol phosphatase[310]. Finally, polyproline rich regions found within the carboxyl terminus, allow for SH3 domain containing proteins to interact with SHIP[311].

Factors that Dictate SHIP Signaling. SHIP expression and recruitment, in addition to allosteric control, already discussed above, are all important factors dictating SHIP's involvement in cell signaling. Although expressed in all hematopoietic cells[279, 312], SHIP is differentially expressed in certain cell types[313]. In fact, its expression seems to contribute to the varying functional activities of cell subsets within specific lineages, such as within myeloid and NK cells[314, 315]. SHIP expression at the protein level can be controlled at multiple levels. For example, SMAD family transcription factors can induce its transcription[316], microRNA species[317] can target it posttranscriptionally, and ubiquitination and subsequent degradation can occur posttranslationally[318]. SHIP can also be truncated posttranslationally at the C-terminus, which would prevent its

recruitment by proteins that bind to its polyproline rich regions[319]. In addition, there is an intronic promoter within the SHIP gene that allows for the transcription of an 110kDa isoform, namely s-SHIP, found to be specifically expressed by stem cells, such as hematopoietic stem cells (HSC). This isoform lacks more than 200 amino acids at the amino terminus, thus missing the SH2 domain[312].

Recruitment of SHIP from the cytosol to the plasma membrane where signaling occurs, also determines SHIP's contribution in cell signaling. PI3K activity and the production of PIP3 occur primarily at the plasma membrane. Phee *et al* showed that when and where SHIP gets recruited to the membrane dictates the extent of its phosphatase effects. Specifically, membrane localization of SHIP, but not phosphorylation or receptor tyrosine engagement of SHIP, resulted in a significant reduction in the levels of cellular PIP3[290]. As mentioned earlier, SHIP contains functional elements, SH2 domains, NPXY motif and polyproline rich regions, that facilitate its recruitment[291]. Adapter proteins such as Shc, Grb2 and Dok3, and scaffold proteins such as Gab1 and Gab2 have been found to associate with these structural elements and recruit SHIP to sites of cell signaling[279, 312, 320-323]. Specifically, SHIP has been shown to be recruited directly or by these adaptor and scaffold proteins to the intracellular tail of various growth factor receptors and immunological receptors such as Fc receptors[280], the B cell receptor, T cell receptor, Ly 49 receptors, KLRG1, DAP10, DAP12, and 2B4[324-330]. Many of these associations require tyrosine phosphorylation / activation of SHIP, which as mentioned, was first discovered to occur as a consequence of Epo stimulation[275]. In fact, phosphorylation of SHIP is promoted following stimulation with other various cytokines including GM-CSF, G-CSF, Flt3-L, IL-3, IL-4, IL-2 and stromal cell derived factor-1 (SDF-1), among others[279, 289, 331-335]. Finally, SHIP's recruitment may also serve to compete with the recruitment of other key signaling

proteins, as seen in NK cells where SHIP prevented the recruitment of SHP1 to 2B4[336]. Furthermore, SHIP was also found to prevent the recruitment of PI3K to DAP10 and DAP12 on immune receptors[330].

Murine Models of SHIP-Deficiency. Several SHIP-deficient mouse models have been engineered that differ in the specific portion of the protein being deleted[337, 338], and in where, meaning specific tissue or cell type[339, 340], or when, as accomplished in inducible models[341], SHIP-deficiency is achieved. These mouse models have helped elucidate the role SHIP plays in hematopoiesis. Mice with germline SHIP-deficiency are viable[337]. Though, many abnormalities manifest as the mice develop, including hyper-responsive degranulation in mast cells[342], a reduction in $CD8^+$ T cells[337], a disrupted NK cell repertoire[325] and myeloproliferation[343, 344], which results in the expansion of the myeloid compartment in the BM and peripheral lymphoid organs. The myeloid cells in the periphery have also been found to be immunosuppressive[345], similar to MDSC found in tumor-bearing mice. This, in combination with the disrupted NK cell repertoire, provides the SHIP-deficient mouse with protection against GvHD and with the ability to accept a BMT from a completely allogeneic donor[325, 341, 345]. Though, if allowed to progress, this myeloproliferation has detrimental consequences that lead to consolidation of the lung due to macrophage infiltration and ultimately to death at 6-10 weeks of age[337].

Several mouse models of SHIP-deficiency were used in the studies presented here. Specifically, two different germline models, one inducible model and two different cell lineage, namely T cell[346] and myeloid cell[347], specific models of SHIP-deficiency were used. All models employed site-specific recombinase technology to mediate the functional deletion of SHIP[325]. Specifically, short DNA sequences called loxP sites are

inserted to flank, also termed as flox, the targeted nucleotide sequence. LoxP sites are recognized by Cre recombinase, which mediates the excision of the intervening DNA and recombination of the remaining DNA[348]. In one of the germline SHIP-deficient models indicated throughout as $SHIP^{-/-}$, the promoter and first exon of the SHIP gene were floxed[325], while in the other germline model indicated as $SHIP^{\Delta IP/\Delta IP}$, the exons encoding the enzymatic domain were floxed[340]. Embryonic stem cells that had the floxed gene properly integrated, namely $SHIP^{flox/flox}$ ES cells, were transiently transfected to express Cre recombinase thus resulting in the deletion of the floxed genomic sequence. These ES cells were then used to generate chimeric mice, which were then backcrossed to achieve germline transmission of SHIP-deficiency in the entire mouse[325].

In addition, $SHIP^{flox/flox}$ ES cells were used to generate mice that would allow for the targeted deletion of SHIP induced at any given time during development or in a given cell type, specifically T cells[339] and myeloid cells[347], depending on the promoter used to drive the expression of Cre recombinase. In order to achieve the inducible model of SHIP deletion, $SHIP^{flox/flox}$ mice were crossed with MxCre transgenic mice, which have the Mx1 promoter driving Cre recombinase expression, thus generating the $MxCreSHIP^{flox/flox}$ mouse[341]. The Mx1 promoter, usually inactive in healthy mice, becomes active in virtually all cells, notably in hematopoietic cells, upon elevated levels of interferon (INF). This can be induced by administering polyinosinic-polycytidylic acid (polyI/C) injections. In other words, upon polyI/C injections, interferon levels increase activating the Mx1 promoter and expression of Cre recombinase that then excises the floxed sequence[349], resulting in the deletion of SHIP. In order to achieve T cell specific SHIP deletion, $SHIP^{flox/flox}$ mice were crossed with LckCre transgenic mice to generate $LckCreSHIP^{flox/flox}$ mice. In these mice, the expression of Cre recombinase is controlled by the Lck promoter, which is active from the earlier stages of T cell development[346].

The myeloid specific SHIP-deficiency mouse model was generated similarly by using instead the LysCre transgenic mice[347]. The Lys (M lysozyme) promoter is exclusively active in cells of the myeloid lineage such as monocytes/MΦ and neutrophils.

SHIP and the Hematopoietic Compartment. As can be appreciated in the overall phenotype of SHIP-deficient mouse model, SHIP plays a significant role in the development and function of the hematopoietic compartment. Further detailed studies have shown that SHIP contributes significantly to cell signaling within virtually every hematopoietic cell including HSC, B cells, NK cells, T cells, myeloid cells, neutrophils, basophils, mast cells and eosinophils[291]. Important to the work presented here is SHIP's role in T cells and myeloid cells, as discussed specifically below.

SHIP in T Cells. The initial studies of SHIP in T cells demonstrated that upon TCR engagement, SHIP becomes phosphorylated and associated with Shc[289]. Further studies using leukemic T cell lines and PBLs provided evidence that SHIP contributes to the metabolism of PIP3 and thus can limit the activity of effectors downstream of PI3K[350]. SHIP was also found to interact with Tec, a member of the Tec family of protein-tyrosine kinases. Tec localizes to the membrane by the PI3K product, PIP3, and mediates PLCγ activation upon TCR engagement. By interacting with Tec, SHIP functionally inactivates Tec by dephosphorylating local PIP3 and thereby preventing its recruitment to the membrane[351]. Another study showed that SHIP is recruited to the killer cell lectin-like receptor G1 (KLRG1) expressed on specific subset of T cells and NK cells. Importantly, KLRG1 engagement was found to inhibit sub-optimal TCR signaling, which required its association with SHIP as well as SHP2[327]. Finally, upon TCR stimulation, SHIP has also been shown to associate with a multimolecular complex including downstream of kinase

1 (Dok1), Dok2, Grb2 and linker for activation of T cells (LAT), a membrane signaling scaffold protein. Notably, SHIP was required for the recruitment of Dok2 to this complex, which is critical in attenuating early TCR signals[324]. These *in vitro* studies suggest that SHIP could alter signals emanating from the TCR that may ultimately affect the development and function of T cells.

In vivo studies have provided further insight into SHIP's role in T cell signaling. Importantly, in SHIP-deficient mice, both germline and T cell specific, autoimmunity or neoplasm involving the T cell compartment have not been observed. In fact, the frequency of T cells is normal or reduced in these SHIP-deficient mice[325, 340, 344]. Furthermore, T cells isolated from the periphery of SHIP-deficient mice displayed reduced antigen specific IFN-γ production[352], thus pointing to defective, not hyperactive, TCR signaling in SHIP-deficient T cells. Also, reduced levels of SHIP expression is not commonly observed in cancer T cell lines, with the exception of Jurkat cells[350] as well as most T-ALL cancer cases[353]. Other cancer T cell lines exhibit phosphorylated SHIP, indicating its active participation in cell signaling[350].

Although it is not yet clear what role SHIP plays in modulating TCR signaling, it has become evident, by using mouse models of SHIP-deficiency, that SHIP plays a prominent role in the function, differentiation and accumulation of T cell subsets. Studies using T cell specific SHIP-deficient mice showed a direct role for SHIP in T_H1, T_H2, and $CD8^+$ T cell functions. Specifically, based on cytokine production and response to infection, T_H2 responses were compromised while T_H1 responses were normal or slightly enhanced. Intriguingly, the cytolytic response by $CD8^+$ T cells was more potent, possibly due to enhanced T_H1 support promoted by T cell specific SHIP-deficiency[339]. As shown by Kashiwada *et al*, SHIP-deficient mice exhibited an increased frequency of Tregs in peripheral tissues with normal *in vitro* suppressive capacity[354]. Subsequently, *in vitro*

studies performed by Locke *et al* suggest that SHIP skews T cell differentiation towards T_H17 cells versus Tregs[355]. The studies herein further examine the role of SHIP in the accumulation and suppressive function of Tregs.

SHIP in Myeloid Cells. SHIP also plays a critical role in regulating the myeloid compartment as simply demonstrated by the significant expansion of myeloid cells in the SPL, LN and BM of SHIP-deficient mice. Although this myeloproliferative syndrome does not develop into myeloid leukemia, it is thought to be significantly responsible for the consolidation of the lungs in SHIP-deficient mice[344]. This myeloid expansion is thought to be initiated by microenvironment cells, such as osteoblasts, that produce excessive amounts of myelopoietic growth factors, such as G-CSF. Consistently, osteoblasts exhibit defective maturation and G-CSF serum levels are increased by 400-500% in SHIP-deficient mice[356].

Several groups examining SHIP's role in modulating the myeloid compartment have found conflicting evidence showing that SHIP can promote and repress effector functions. For example, one study found that SHIP represses phagocytosis initiated by the Fcγ receptor and complement receptor signaling[357], while another study found that SHIP can promote phagosome maturation via PIP2 production[301]. SHIP has also been described as limiting the production of inflammatory cytokines and superoxide in oxidative burst[358]. Conversely, SHIP's product has been shown to promote oxidative burst by increasing early NADPH oxidase activity, which results in the generation of ROS[359]. This contradictory data has been obtained from studies performed primarily *ex vivo* on myeloid cells from SHIP-deficient mice. Thus, further *in vivo* studies may provide more definitive answers, especially if utilizing myeloid specific SHIP-deficient mice[291].

Importantly, both *in vitro* and *in vivo* data has been obtained showing that SHIP promotes the effector function of osteoclasts, which are mature tissue MΦ that mediate bone remodeling by engulfing bone forming osteoblasts. Consequently, SHIP-deficient mice are osteoporetic[338]. In addition, the use of myeloid specific SHIP-deficient mice has clearly shown that SHIP is required for the development of marginal zone MΦ *in vivo*[340]. Finally, *in vivo* data has shown that SHIP, whose upregulation is induced by LPS, is critical for endotoxin tolerance mediated by MΦ and their production of TGFβ[314]. Furthermore, SHIP expression in MΦ is also induced by TGFβ in a SMAD-4 independent manner, which along with endotoxin-mediated SHIP induction, can further amplify SHIP signaling during intense immune responses[291, 360].

Most important to the studies discussed herein is SHIP's role in limiting the accumulation of immunosuppressive myeloid cells, which will also be referred to as MDSC from here on. As mentioned before, these MDSC are thought to largely contribute to the ability of SHIP-deficient mouse to accept an allogeneic BMT while being protected from GvHD[325, 341, 345]. The mechanism by which this MDSC accumulation occurs is still not fully understood. This is further investigated in the studies described in the following chapters.

The Role of Tumor Suppressor, Rb, in Regulating the Immune System

Discovery of Rb. The retinoblastoma gene (Rb1), one of the first genes identified as a tumor suppressor gene[361], was initially described as the human genetic locus that predisposes to retinoblastoma[362], a name first coined by Verhoeff *et al* in 1926

and adopted by the American Ophthalmological Society to denote the malignant childhood ocular tumor[363]. As early as 1973, the existence of regulatory genes was proposed, now known as tumor suppressor genes, which were responsible for actively inhibiting transforming genes (potential oncogenes) that when active, promote cell growth. Thus, when the regulatory gene was inactivated, suppression was released and subsequent cellular transformation could ensue[364]. Rb1 was proposed and later validated to be such a regulatory gene. Furthermore, the onset of retinoblastoma required the inactivation of both Rb1 alleles, first described by the Knudson's two-hit hypothesis[365, 366]. This hypothesis also explained the difference between the hereditary and non-hereditary forms of retinoblastoma where the inheritance of a germline mutation in one of the Rb1 alleles dictated the severity of the disease. Specifically, children that are affected by unifocal retinoblastoma do not carry an Rb1 germline mutation, meaning that they do not inherit this mutation from one of their parents. On the other hand, children with multifocal and bilateral retinoblastoma did inherit a germline mutation in one of the Rb1 alleles[361]. In addition, children with Rb1 germline mutations are predominantly afflicted with retinoblastoma at a very early age and are predisposed to develop osteosarcoma, melanoma or soft tissue sarcoma later in life[367, 368].

Once discovered to reside on chromosome 13q 14[369], further investigation into the function and activity of Rb1 gene revealed that it was expressed in most tissues and underwent inactivation by loss of heterozygosity in many other cancers[370]. In fact, the majority of studied human cancers, including breast[371], bladder[372], prostate[373] and small cell lung carcinoma[374], exhibit Rb1 inactivation either directly with mutations in the Rb1 locus or indirectly with mutations affecting other proteins that regulate Rb1 cellular function.

Rb Structure and Functional Domains. The retinoblastoma gene encodes a phosphoprotein involved in numerous cellular processes, such as differentiation, apoptosis, cell cycle progression and as appreciated recently, DNA repair and cell cycle checkpoints[375]. Although cells contain two copies of the Rb1 gene, only one normal copy is needed to accomplish its function[376, 377]. The human Rb1 locus spans about 200 kilobases (kb) of genomic DNA, consisting of 27 exons from which a 4.8 kb mRNA species is transcribed[378]. The translated product in humans is a 928-amino acid (aa) protein; while in mice, Rb1 is 921 aa long with 91% homology to human Rb1[379]. The Rb1 protein consists of three distinct regions: the N-terminus, the central A/B "pocket" made of an A and B domain separated by a spacer, and the C-terminus. The Rb1 gene is a member of the Rb gene family along with two other members, p107 and Rb2/p130. All Rb gene family members are collectively known as 'pocket proteins' because they all share resemblance in the A/B pocket domain. Although in some instances, they play somewhat compensatory roles in the cell, they do differ in several aspects[375].

In order for Rb family members to associate with most of its binding partners, it is critical that the A/B pocket be structurally intact[380]. Numerous cellular and viral proteins harbor an LXCXE motif that allows them to bind to an LXCXE binding site within the A/B pocket. The LXCXE motif (where the L is leucine, C is cysteine, E is glutamine and X is any amino acid) was initially described in viral oncoproteins as critical for their interaction with Rb1[381]. Examples of Rb1-binding viral oncoproteins are the SV40 large-T antigen[382], the adenovirus EIA protein[383], and the human papilloma virus E7 protein[384]. The most documented activity of Rb family proteins is their interaction and repression of E2F family of transcription factors which contributes to Rb –mediated control over cell cycle progression and survival. Though E2F proteins do not possess any LXCXE domains, their interaction with Rb family proteins is also dependent on the A/B pocket

along with a portion of the C-terminus[385]. Other Rb-binding partners known to interact with Rb family proteins via the LXCXE binding site within the pocket domain are D-type cyclins[386], histone deacetylases (HDAC)-1, -2 and -3 recruited by the Rb-binding protein (RBP1)[387, 388], BRG1 of the human SWI/SNF chromatic-remodeling complexes[389], the polycomb group (PcG) protein HPC2[390], the histone methyltransferase Suv39H1[391], and the CtIP/CtBP transcriptional corepressor complex[392], among others. Some of these binding partners, such as the D-type cyclins and HDACs, also require a portion of the C-terminus to form a stable complex[393]. Importantly, Rb family proteins bind E2F proteins and several other binding partners concurrently since the binding sites are distinct from each other. In fact, transcriptional repression of E2F target genes requires this simultaneous interaction with E2F and the other Rb-binding partners just mentioned[394].

As mentioned, Rb1/p105, p107 and Rb2/p130 are all part of the Rb gene family. Rb2/p130 and p107 share 50% amino-acid homology, thus being more related to each other than they are to Rb1, which shares 30-35% homology. Functionally important, the length of the C-terminus differs among Rb family members. The C-terminus contains the nuclear localization signal (NLS) which controls the transport of Rb into the nucleus from the cytoplasm and also works as a carrier for E2F proteins. As mentioned, the C-terminus is also required for the binding of HDAC1 and cyclin/cdk complexes[394]. Although many studies show that these proteins serve partially compensatory roles in several instances, the use of murine models deficient in one or more of the Rb gene family members has exhibited different phenotypes demonstrating that this functional redundancy is not absolute. Their expression differs with cell status, specifically if quiescent, differentiated or proliferating. Their binding ability to E2F family members and to cyclin/cdk complexes, which fluctuates throughout the cell cycle, also differs. Analysis of cells deficient in the RB gene family members demonstrated that Rb and p107/p130

regulate different E2F target proteins. Specifically, p107/p130 deficiency promoted the deregulation of a much larger set of E2F target genes compared to Rb1. DNA microarray analysis has provided evidence suggesting that Rb1 regulates genes encoding DNA replication and cell cycle regulatory proteins, while p107/p130 regulate genes encoding proteins involved in cell growth and maintenance of the extracellular matrix and its signaling activities[395]. In addition, the Rb family members differ in their ability to elicit cell cycle arrest in specific cell types. Finally, they differ in their specific involvement in regulating differentiation and apoptosis, which also seems to depend on cell type[375]. Importantly, the literature reviewed below pertains specifically to Rb1 and not the other family members, unless otherwise noted specifically.

Regulation of Rb Expression and Activation. Various mechanisms and proteins have been described to regulate Rb1 expression at the transcriptional level. Firstly, several transcription factors, such as $p16^{INK4A}$, BRCA1, ICBP90, and YY1 have been found to regulate Rb1 transcription. One group showed that Rb1 mRNA levels were much higher in cell lines that lacked $p16^{INK4A}$ expression. The reestablishment of $p16^{INK4A}$ expression using an adenovirus vector resulted in a significant reduction in Rb mRNA levels[396]. BRCA1 also inhibits the expression of Rb1 and Rb family proteins. Interestingly, in order to mediate this transcriptional regulation of Rb, BRCA1 requires a functional LXCXE motif, allowing it to be in complex with Rb. Thus, Rb regulates its own transcription in this context[397]. ICBP90 (inverted CCAAT box binding protein of 90kDa), a transcriptional regulator of the topoisomerase II alpha gene, is also a negative regulator of Rb expression[398]. When overexpressed in lung fibroblasts, ICBP90 was found to down-regulate Rb mRNA levels with an increase in S and G2/M-phase cells fractions. ICBP90 was shown to bind to the Rb gene promoter when methylated[399].

Finally, YY1, a polycomb group protein and transcription factor associated with both positive and negative transcriptional regulation as well as initiation of transcription, has been shown to act as a repressor of Rb expression during myogenesis. Specifically, YY1 is present on the Rb promoter with GABP, a GA-binding transcription factor. Upon signals to differentiate, YY1 leaves the Rb promoter, while GABP along with the cofactor HCF-1, a chromatin associated heterodimeric complex, stays and activates Rb expression[400]. The Rb promoter also contains binding sites for two nuclear transcriptional factors, ATF and SP1, that regulate Rb expression and have been found to be mutated in hereditary retinoblastoma[401, 402].

Additionally, several studies suggest that Rb itself, Rb family members and other upstream member of the Rb pathway autoregulate Rb transcription. For example, the Rb promoter has an E2F binding site that when methylated, has been shown to recruit repressor complexes and down-regulate Rb transcription[403]. Furthermore, when overexpressed, E2F1 can activate Rb expression in some contexts[404]. Also, in cells that exhibit functional inactivation of Rb by phosphorylation, researchers have observed increased levels of Rb protein[405]. Another study showed that overexpression of Rb in P19 cells resulted in repression of an Rb reporter[406]. Because p107 and p130 also interact with E2F proteins, they may also regulate the Rb promoter. In fact, loss of p107 in mouse embryos has been shown to lead to an increase in Rb expression, although the exact mechanism is unclear[407]. More recently, Burkhart *et al* showed in various murine organs and tissues that Rb transcription is indeed regulated *in vivo* by members of the Rb and E2F families. Interestingly, they found that unlike other classical gene targets of E2F, Rb expression was not always upregulated during cell cycle progression[408].

Regulation of Rb activation is achieved posttranslationally in a cell-cycle dependent manner where gradual phosphorylation, mediated primarily by cdks[370], inactivates it. The hypophosphorylated Rb form is active, capable of binding E2F and thereby preventing cell cycle entry. Specifically, D-type cyclins coupled with cdk4 or cdk6 have been shown to phosphorylate Rb during early G1 phase. In late G1 phase, cyclin E and A in complex with cdk2 further phosphorylate and thereby inactivate Rb[409], leading to the disassociation of E2F factors. Rb remains hyperphosphorylated until late mitosis[410]. Phosphorylation, which can be reversed, occurs both on the A/B pocket and on the C-terminus[394]. In some instances, phosphorylation can expose a proteolytic cleavage site within Rb that can ultimately lead to its degradation[411]. Rb has also been shown to undergo other post-translational modifications, such as acetylation[412], sumoylation[413], ubiquitination[414] and methylation[415] in response to varying cell signals. These modifications alter the protein levels of Rb, as well as its ability to interact with its binding proteins.

Rb Signaling Pathways. As mentioned above, Rb1 plays a role in several cellular processes, such as regulating cell cycle entry, promoting and maintaining differentiation, protecting from cell death and maintaining genomic integrity.

Rb and the Cell Cycle. The cell cycle is a tightly orchestrated process in which cyclin/cdk complexes play distinct roles during each phase by phosphorylating specific target proteins in a coordinated manner that allows for cell cycle progression. Importantly, cyclin expression and cdk activity fluctuates throughout the cell cycle. Specifically, cyclins D and E mediate progression through G1/S phases, while cyclins A and B mediate progression through the S/G2/M phases. The expression of cyclin D is

rapidly induced by mitogens. With sustained mitogenic stimuli, cyclin D/cdk activity persists through the first and subsequent cycles. Cyclin E protein levels peak at the G1/S transition followed by an increase in cyclin A protein levels in S phase. At the G2/M boundary, cyclin B protein levels increase, thereby activating its partner, cdk1[394].

Normally, upon mitogenic stimuli, a cell decides to proceed through and complete the cell cycle only during a specific phase of its cycle known as the ‘‘restriction point’’, which is between early G1 phase and G1/S phase. Once past this ‘‘restriction point’’, cells become committed to DNA synthesis and subsequent cell division. Many studies have demonstrated that Rb1 functions as the ‘restriction point’ switch[416]. Specifically, one study showed that purified unphosphorylated Rb1 protein microinjected into cells during early G1 phase, caused reversible G1 arrest. When injected during late G1 phase of early S phase, Rb1 protein had no impact[417]. In resting G0 cells, the actively growth-suppressing hypophosphorylated Rb1 is predominate, capable of repressing E2F transcriptional activity. Rb proteins repress gene transcription by directly binding to the transactivation domain of E2F or by binding to the promoter of these regulated genes in complex with E2F and other proteins such as HDAC, SWI/SNK factors, polycomb group proteins or methyltransferases. As mentioned above, when the cell progresses through G1 phase, Rb1 is increasingly phosphorylated by cyclin/cdk complexes thereby no longer capable of binding to E2F and repressing the transcription of E2F target gene necessary for S phase progression[394].

There are six E2F family members that can be divided into two classes: activators (E2F1, E2F2, E2F3) and repressors (E2F4 and E2F5) of transcription[416]. E2F6 has been found to behave as a transcriptional repressor in an Rb-independent manner. Thus, it is considered independent and lacks several functional domains, such as the Rb-binding domain and the trans-activation domain[418]. Rb1 binds preferentially to

activator-E2Fs, while p130 binds to repressor-E2Fs and p107 only binds to E2F4, preferentially[419]. These proteins complexes exhibit precise expression patterns throughout the cell cycle with the Rb1/E2F complex being present in G1 phase, the p130/E2F complex being most evident in quiescent cells and the p107/E2F complex being detected in both G1 and S phase[416].

Rb proteins can also regulate transcriptional repression and cell cycle progression through E2F independent mechanisms. For example, Rb1 mutants not capable of binding E2F promoted the formation of promyelocytic leukemia (PML) nuclear bodies which led to transcriptional repression and was associated with entry into senescence, particularly in Soas-2 cells[420]. In addition, hLin-9 was also shown to cooperate with Rb1 to induce senescence is Soas-2 cells and promote Rb1-dependent transcriptional activation regardless of its ability to bind E2F[421]. Rb1 has also been shown to mediate cell cycle arrest by increasing the expression and stabilization of the cdk inhibitor (CKI) p27. By using timed Rb1 expression experiments, researchers found that the effects of E2F repression lagged behind the onset of G1 cell cycle arrest. Instead, p27 was found to accumulate much faster. Furthermore, disruption of p27 function or expression prevented Rb1 from causing G1 arrest. Non-E2F binding Rb1 mutants were capable of increasing p27 expression and stability. Importantly, these Rb1 mutants were also equally capable of causing G1 cell cycle arrest as WT Rb1[422].

Finally, more recent studies have demonstrated that control of cell cycle progression mediate by Rb1 is not limited to the 'restriction point' between early G1 phase and G1/S phase. For example, Rb family members have been shown to also regulate the earlier transition from G0 to G1[423]. During G0, hypophosphorylated Rb1 promotes low levels of RNA, characteristic of the G0 state, by inhibiting the expression of ribosomal and transfer RNA[394]. Exit of G0 and increase in RNA content is mediated

by the cyclin C/cdk3 complex that, in turn, phosphorylates Rb1. Consistently, cyclin C expression precedes that of cyclin D[424]. Another example comes from Mukherjee *et al* which demonstrated that Rb1 also functions to prohibit cell cycle progression during late G1. This process requires treatment with TGFβ, a potent inhibitor of cell proliferation, during late G1 (presumably past the 'restriction point') and does not involve inhibition of cyclin/cdk complexes. Instead, Rb1 mediates TGFβ arrest by directly targeting the activity of the minichromosome maintenance (MCM) helicase and thereby inhibiting the activation of the prereplication complex and initiation of DNA replication (the G1/S transition)[425].

***Rb and Differentiation*.** Embryos deficient in Rb1 die between gestation days 13 and 15 making it evident that Rb1 plays a role in development and differentiation[426]. Put simply, Rb proteins remove specific blocks set at certain stages of development to ensure the proper timing of differentiation. The role of Rb proteins in differentiation has been found to be highly dependent on tissue type while involving cell specific factors. A role for Rb proteins in development and differentiation has been established in several tissue types, including neuronal tissue, skeletal muscle, adipose tissue, and retina. Rb proteins also a role in the development of cells of the hematopoietic compartment, which is discussed further in its own subsection[416].

Rb knockout mice display defects in differentiation of neuronal cells and erythrocytes. These defects are, in part, promoted by the unrestricted activity of the inhibitor of differentiation Id2, a target of Rb that is required to maintain the proper sequencing in differentiation and that positively regulates cell cycle progression[427]. Rb interacts with and sequesters Id2, thereby preventing its activity[428]. This is supported by the fact that Id2 deficiency rescues Rb knockout embryos from these defects, prolonging

their life span[395]. Rb deficiency causes abnormal mitosis and apoptosis in the intermediate zones of developing neural tubes, and decreased expression of several neuronal markers. It can be concluded that Rb is critical following commitment to a neuronal fate[416]. Interestingly, extraembryonic tissues of the placenta exhibit extensive apoptosis making it possible that the defects seen in Rb $^{-/-}$ embryos could be due to functions external to the developing embryo. In fact, the presence of a normal placenta allows Rb$^{-/-}$ embryos to reach full term, at which time they instead die from severe skeletomuscular issues[429]. Recently, a mouse model with conditional Rb deficiency specifically in the cerebrum demonstrated the Rb does play a cell-autonomous role in neuronal migration[430].

RB mRNA and protein levels have been found to increase during muscular differentiation[431]. Rb has been found to enhance the activity of MyoD, a transcription factor important in suppressing cell cycle progression and in promoting differentiation in muscles. In fact, the transcription of certain myogenic genes promoted by MyoD also requires functional Rb[432]. Also, Rb inactivation inhibits myoblast and myotube differentiation in culture. Further studies have been able to tease apart the molecular mechanism underlying the exact role Rb plays during myogenesis. Specifically, studies performed by Delehouzee *et al* led them to propose a model where transcription factors GABP, YY1, and HCF-1, described above, all work in concert to promote the upregulation of Rb expression at day 2 of myogenesis[400]. Using mouse models where Rb is deleted before or after myogenic differentiation support this model. If Rb is deleted in myoblasts as achieved with Myf5CreRb$^{flox/flox}$ mice, the mice die at birth exhibiting extensive apoptosis and the absence of myofibers. In contrast, MCKCreRb$^{flox/flox}$ mice that have Rb deleted in differentiated fibers were viable with normal muscle development. Thus, in muscles, Rb is vital for the initiation and progression, but not for

the maintenance, of the differentiated state[433]. Different results are obtained when looking at Rb conditionally deleted in hair cells, demonstrating that the exact role and requirement of Rb during differentiation is dependent on cell or tissue type[434].

There are two major cell types with distinct functions that arise from adipocyte precursor cells; namely, white adipose, which stores energy, and brown adipose, which releases energy via thermogenesis[416]. In adipogenesis, the Rb family members have been found to play opposing roles. Rb1-deficient MEFs do not convert into adipocytes upon proper treatment with inducers known to be adipogenic, thus having defective differentiation potential[435]. With PPAR-gamma ligand treatment, Rb1-deficient MEFs preferentially differentiated into white adipocytes[436]. Though, when Rb1 is deleted in adult primary preadipocytes, their differentiation into white adipocytes is inhibited[437]. Conversely, MEFs deficient in p130 or p107 have an increased potential for differentiation[438]. Furthermore, in p107 deficient mice, a replacement of white adipose tissue with brown adipose tissue has been observed[437]. These studies suggest that Rb family members mediate the determination between brown or white adipocyte differentiation[416].

The retina consists of several different cell types present in proper proportions to each other and arranged specifically to dictate the overall size of the retina and ultimately, the quality of vision achieved. This functional arrangement is mediated by the precise coordination of cell cycle exit and cell fate specification; which when uncoupled, result in vision obstruction and tumor formation, as seen in retinoblastoma[416]. Specifically, Rb1 deficiency promotes ectopic cell proliferation in the retina made even more severe when in combination with p107 deficiency[439]. Studies suggest that Rb1-deficient retinal precursor cells are incapable of undergoing terminal differentiation and this is what leads to oncogenic transformation. Though, Rb1-deficient retinal cells are

also prone to growth arrest and thus must escape this propensity to develop into retinoblastoma[440]. In addition, the role that Rb1 plays is different among the cell types composing the retina. For example, in proliferating retinal progenitor cells, Rb1 mediates cell cycle exit; while in differentiating rod photoreceptors, Rb1 is necessary for proper maturation[441]. Also, in lens progenitor cells, Rb1 was found to interact with Pax6, a regulatory factor critical in the formation of the lens[442] and active in peripheral retinal progenitors at embryonic day 10. With Pax6 deficiency during retinal development, Rb1 becomes inactivated and thereby causing a decrease in ganglion and bipolar cells[416].

Rb and Apoptosis. Aside from their ability to induce expression of genes associated to cell-cycle progression, the E2F family of transcription factors also controls the expression of pro-apoptotic genes. In repressing E2F-target genes, Rb1 can thus, also block E2F-induced apoptosis[443]. Although, studies have shown that this depends of the type of apoptotic stimuli[444]. To illustrate its role in apoptosis, embryos deficient in Rb1 display widespread cell death in cells of the central nervous system (CNS). This can be mitigated by mutations in p53 and E2F1 which allow for prenatal development but that do not save the fetus from dying shortly after birth due to extensive apoptosis in skeletal muscles[445]. Dysfunctional Rb1 promotes the accumulation of p53 and subsequently the induction of apoptosis by p53. Specifically, with increased E2F1 activity, the expression of $p19^{ARF}$ is increased which in turn prevents MDM2, a p53 ubiquitin ligase, from degrading p53[446, 447]. In addition, E2F1 and p53 both control the expression of Apaf-1, a component of the apoptosome and a player in the pathway activating mitochondria-dependent apoptosis. Deletion of Apaf-1[448] and caspase-3[449] rescues the CNS and peripheral nervous system, respectively, from apoptosis in Rb1-deficient embryos, thus demonstrating that apoptosis mediated by the absence of Rb1

requires the apoptotic machinery. Interestingly, Rb1 contains several caspase-3 cleavage consensus sites which enhance its degradation upon transduction of the apoptotic signal[450]. In fact, in mice, the use of a caspase-resistant Rb1 mutant repressed apoptosis promoted by the type I tumor necrosis factor (TNF)-α receptor but not by the TNF receptor type II, whose expression is limited to the cells of the hematopoietic compartment[451]. This provides yet another example of how Rb functions differently in specific tissues. Rb1 has also been found to bind to and inhibit c-ABL, a tyrosine kinase[452], and JNK kinase[445], which are other pro-apoptotic proteins involved in apoptosis induced by stress.

Rb and DNA Repair. Maintaining the integrity of DNA and achieving its faithful replication is vital for all living organisms in order to avoid the onset of impaired cellular functions, apoptosis, irreversible growth arrest and cancer. Inevitably, DNA damage is mediated by both exogenous and endogenous sources, such as radiation, chemicals, and free radicals generated during metabolism. Lesions induced by these sources include oxidation, deamination, pyrimidine dimerization, depurination, single-strand breaks (SSB) and double strand breaks (DSB), this last one being the most harmful[375]. These lesion are known to inhibit transcription and to promote cell cycle arrest and apoptosis[453]. Accordingly, cells have the ability to deal with DNA damage mediated by several repair factors and DNA-repair mechanisms specific to the several types of DNA lesions. In order to employ the DNA-repair machinery, cells must initiate a response mechanism that includes halting cell cycle progression in order to provide time for DNA repair. This occurs at specific transition points named “cell cycle checkpoints”[375]. Studies have shown that, as a response to DNA damage, phosphorylation of Rb is inhibited and the accumulation of hypophosphorylated Rb is promoted, thereby

activating the Rb pathway. This is thought to be a downstream consequence of the p53/p21^{Cip1} pathway which becomes activated by DNA damage[454]. Upon DNA damage, Rb has been found to be recruited to certain initiation sites of replication presumably to inhibit replication from continuing aberrantly[455]. Studies have proposed that one of the ways Rb prevents replication is by disrupting the association of PCNA to chromatin[456]. In addition, the expression of several factors involved in DNA damage repair, such as PCNA, RPA2-3, and FEN1, among others, is influenced by Rb[375].

Rb has been implicated in the effectiveness of cell cycle checkpoints upon DNA damage. In summary, Rb mediates its contribution to the DNA damage checkpoint response by repressing the transcription of E2F-regulated genes, by inducing cell cycle arrest in the various phases and by inhibiting the accumulation of DNA DSB mediated by E2F1[457]. Specific to this last mechanism, if Rb becomes inactivated, E2F1 consequently becomes deregulated which promotes the induction of DNA DSB independent of Atm, p53, ROS, caspases, and apoptosis[458]. The following paragraphs describe how Rb induces arrest during each phase of the cell cycle.

Firstly, let's consider the G1 checkpoint. The presence of Rb in transcriptional repression complexes and its phosphorylation status serve as a measure for controlling G1 exit[375]. When DNA damage occurs during G1, it has been shown that the cell cycle arrest that follows is mediated by the p53/p21^{Cip1} pathway, which requires functional Rb. In fact, when Rb activity is lost, cells do not engage the G1 checkpoint and do not undergo G1 arrest in response to DNA damage regardless of p53/p21^{Cip1} activation[459]. Loss of p16^{INK4A} or overexpression of cyclin D1 or cdk4, also results in the disablement of this checkpoint[370].

In S phase, Rb has been found at sites of DNA replication and involved in causing S phase arrest when cells have encountered DNA damaging agents at high

doses. Upon DNA damage during S phase, Rb is dephosphorylated followed by complete termination of DNA synthesis, even when cells have already achieved S phase DNA content[460]. Furthermore, when rendered Rb-deficient, fibroblasts exposed to DNA damaging agents undergo hyper-replication and display hyperploidy[461]. Because it is known to interact with SWI/SNF complexes, Rb may also regulate S phase progression[375].

Another checkpoint exists in the G2 phase, allowing for cell cycle arrest in response to DNA damage. Although initiating the G2 phase checkpoint is independent of it, Rb has been shown to control the length of G2 arrest. The duration of G2 arrest is critical for allowing the time for DNA repair proportionate to the extent of DNA damage. Similar to that seen for G1 arrest, p53/p21^{Cip1} mediates the accumulation of hypophosphorylated active Rb, which in turn maintains G2 arrest[462]. In addition, G2 arrest induced by DNA damage is accompanied with the decreased expression of genes required during G2 and M phase, some of which have been shown to be downregulated by Rb2/p130 and p107 specifically[463]. Inactivation of Rb family members, of the p21/WAF1 pathway or of cdks prevents the cell from initiating and sustaining the G2/M arrest[375].

Murine Models of Rb Deficiency. Rb1 homologues have been described in several vertebrates including sharks, chicken, cats, and mice[464]. As mentioned before, mouse Rb shares 90% homology with human Rb and Rb deficiency in mice results in death *in utero* between embryonic days 13-15 due to defective neurogenesis and erythropoiesis[465]. Also, low expression of Rb1 achieved with an Rb1 transgene engineered into Rb$^{-/-}$ mice delays death until birth, which is caused by defects in muscular differentiation[466]. Mice heterogeneous at the Rb locus (Rb$^{+/-}$) are viable and do

not develop retinoblastoma but instead exhibit tumors in the pituitary gland[467]. When depleted in combination with p107, as seen in chimeric mice generated with Rb and p107 deficient ES cells, mice are prone to developing retinoblastoma[439]. This suggests that in mice, p107 may play a compensatory role when Rb is deleted. Furthermore, chimeric mice generated with Rb and p130 deficient ES cells develop lung neuroendocrine hyperplasia, retinoblastoma and pheochromocytoma[468]. Mice deficient in p107 alone, particularly in a Balb/cJ background, are viable and display increased proliferation and apoptosis of neural progenitor cells of the CNS, thickening of long bones, growth impairment and myeloproliferative disease[469]. Interestingly, on a Balb/cJ background, p130 deficiency is lethal at E11-13 with aberrant neural, muscular and cardiac development, while on the C57BL/6J background, mice are viable and fertile[470]. Several mouse models lacking the different pocket proteins in combination with other important binding partners, all exhibiting varying phenotypes, have also been generated. Examples are Rb1 deficiency in combination with deficiency in E2F1, -2, or -3, or Rb1 deficiency in combination with ARF, Id2, N-ras, K-ras, Casp3 or Apaf1. In addition, mouse models with similar deficiency combinations have been generated where Rb1 deficiency is instead heterozygous. Finally, the ability to conditionally ablate Rb1 in different tissues has provided greater insight into the role Rb1 plays in different cell types; a role which has both cell autonomous and non-cell autonomous aspects to it[395].

Important to the studies presented here is the MxCreRb1$^{flox/flox}$ mouse model, which is functionally similar to that described above for the MxCreSHIP$^{flox/flox}$ mouse model. In short, this model allows for the conditional deletion of Rb1 in cells responsive to INF, primarily cells of the hematopoietic system including HSC, upon injection of polyI/C, which causes an inflammatory response that includes elevated INF expression. Importantly, this can be achieved during adulthood, once development has occurred

normally[349]. Importantly, In MxCreRb$^{flox/flox}$ mice, when stable Rb1 deletion is achieved, the expression of p130 and p107 does not increase in compensation for Rb loss. Immediately following Rb1 deletion, MxCreRb1$^{flox/flox}$ rendered Rb1-deficient exhibited a mild and stable anemia. After 4 weeks post-deletion, MxCreRb1$^{flox/flox}$ mice displayed pan-leukocytosis with elevated levels of progenitor cells circulating in the peripheral blood. Twelve weeks after deletion, a myeloproliferative-like disease could be observed within the BM of MxCreRb1$^{flox/flox}$ mice, characterized by myeloid hyperplasia (mainly neutrophilia) while B-lymphopoiesis and erythropoiesis were inhibited. MxCreRb1$^{flox/flox}$ also displayed apparent changes in bone architecture and loss of trabecular bone, an important niche for HSCs within the BM. Consistently, HSCs were lost from the BM with extensive extramedullary hematopoiesis occurring in the SPL, which increased 5.5 fold in weight in Rb-deleted MxCreRb1$^{flox/flox}$ mice compared to controls[471]. The results obtained from the use of this mouse model as well as other cell type specific Rb deletion models have provided great insight to the role that Rb plays in the hematopoietic system which is discussed below.

Rb and the Hematopoietic Compartment. Cells of the hematopoietic compartment are constantly undergoing proliferation, differentiation and apoptosis. Thus, it is no surprise that Rb would play a role in such. Consistently, Rb has been shown to interact with several hematopoietic transcription factors, such as NF-IL-6, PU1 and EIF1[472]. As mentioned briefly earlier, a role Rb in the hematopoietic compartment was identified with the initial studies examining Rb$^{-/-}$ embryos which exhibit neural and hematopoietic defects, specifically in erythropoiesis[395]. Use of chimeric mice provided further insight into the fact that Rb also had functions that were not cell-autonomous. In chimeric mice, phenotypically normal peripheral blood erythrocytes were found to be Rb^{-}

$^{/-}$ while abnormal nucleated erythrocytes were WT[473]. Furthermore, studies demonstrated that when $Rb^{-/-}$ embryos are supplied by a WT placenta, they survived to term without exhibiting the neurological and erythroid defects, suggesting that the many defect exhibited by $Rb^{-/-}$ embryos are at least partly due to inadequate placental function. Though, these $Rb^{-/-}$ pups still succumbed to death due to severe skeletal muscle abnormalities[429]. Another more recent study showed that the abnormal erythropoiesis was instead mediated by defective fetal liver MΦ. This study found that Rb opposes the inhibitory function of Id2, a transcription factor that regulates differentiation, and thereby promotes macrophage differentiation. In $Rb^{-/-}$ embryos, Id2 is unrestrained in MΦ leading to the aberrant erythropoiesis. In fact, Id2 inactivation in $Rb^{-/-}$ embryos inhibits this erythroid defect[474]. Although these experiments only point to an extrinsic role for Rb in erythropoiesis, other studies have clearly demonstrated that Rb indeed plays an intrinsic role as well. By acutely deleting Rb1 *in vitro* in erythroblasts, Spike *et al* showed that Rb1 was intrinsically required for proper erythroblast expansion and red cell enucleation under stress conditions. By performing different hematopoietic reconstitution experiments using WT, $Rb^{+/-}$, $Rb^{-/-}$, or $Rb^{-/-}$ chimeric fetal liver as donor tissue, they also demonstrated a cell intrinsic role for Rb in maintaining hematopoietic homeostasis[475]. Another study showed that Rb also intrinsically controls cell cycle exit required for the differentiation of early erythroblasts to late erythroblasts. Rb deficiency was also found to inhibit mitochondrial biogenesis which is coupled to this differentiation block[476].

In addition, Rb has been shown to be involved in the differentiation of progenitor cells to cells of the monocytic and neutrophilic lineages. Specifically, in one study, human $CD34^{+}$ progenitor cells promoted to undergo monocytic differentiation with Flt3-L and IL-3, exhibited a high level of hypophosphorylated Rb. In contrast, when promoted

to undergo neutrophilic differentiation with G-CSF and SCF, a low level of hypophosphorylated Rb was observed. Furthermore, $CD34^+$ progenitor cells in liquid culture treated with antisense Rb oligonucleotides, which effectively reduced Rb expression, were inhibited from differentiating into monocytes, even in the presence of Flt3-L and IL-3, and instead preferentially differentiated into neutrophilic cells[472]. Consistently, a more recent study which described the expression levels of cell-cycle proteins during granulopoiesis *in vivo,* found that the expression of Rb and the other two pocket proteins was down-regulated from the myelocyte and metamyelocyte stages, which represent two neutrophil precursor populations, onward. There was no detectable phosphorylated Rb protein and very little nonphosphorylated Rb protein expressed in mature polymorphonuclear neutrophils. Similar results were obtained for p107 and p130 expression. Consistently, the expression of cdks and of cyclin D and A were also downregulated in the more mature neutrophil populations[477].

Lastly, the role of Rb in HSC self-renewal, quiescence and multilineage differentiation has been extensively studied. These studies have used conditional Rb knockout mouse models. In one study, whole BM from $MxCreRb^{flox/flox}$ mice was transplanted into WT congenic mice and once hematopoietic reconstitution was accomplished, Rb was deleted. The ability of $Rb^{-/-}$ HSCs to contribute to hematopoiesis in the peripheral blood and multilineage differentiation was unaffected, except for a mild onset of anemia. When analyzing the BM, for the most part, hematopoiesis was very similar between Rb-deficient and Rb-expressing cells. Only the number of Rb-deficient mature B cells per femur was significantly lower. In addition, the use of serial transplantation showed that HSC self-renewal is unaffected by loss of Rb. Notably, the expression of the other pocket proteins, p107 and p130 is not deregulated in HSC lacking Rb[478]. Another group examined the role Rb played in regulating the relationship

between HSC and their BM microenvironment. When Rb was deleted in MxCreRb$^{flox/flox}$ mice during adulthood, the development of myeloproliferative disease was promoted, accompanied by hematopoiesis in extramedullary sites. For example, the SPLs of MxCreRb$^{flox/flox}$ mice increased significantly in weight following Rb deletion due to an excess of megakaryocytes, erythroid cells and myeloid cells. In addition, Rb-deleted MxCreRb$^{flox/flox}$ mice displayed extensive peripheral mobilization of stem and progenitor cells that also exhibited increased differentiation. Though, these phenotypes were not observed when Rb-deficient HSC were in a WT microenvironment or when Rb deletion was myeloid-restricted. Transplantation experiments where myeloid specific Rb-deficient (LysCreRb$^{flox/flox}$) mice were donors and MxCreRb$^{flox/flox}$ were recipients demonstrated that myeloproliferative disease developed only when Rb1 was deleted in myeloid-derived cells and in the microenvironment, while still being expressed in HSC. This study suggests that Rb1 regulates HSC homeostasis in an extrinsic manner involving the BM microenvironment and its interaction with myeloid cells[471]. Though, this does not discount the possibility that the other Rb family members may be serving compensatory roles. A more recent study used Rb family triple knockout (TKO) mice in which deletion is achieved simultaneously in adulthood. The collective contribution of the Rb pocket proteins to the function and homeostasis of HSCs was examined. TKO mice developed a cell-intrinsic myeloproliferative disease originating from early hematopoietic progenitor cells undergoing hyperproliferation and from lymphoid progenitors undergoing increased cell death. TKO HSC displayed enhanced mobilization and could not mediate long-term reconstitution of hematopoiesis upon transplantation. Interestingly, the myeloproliferative disease was prevented when a single WT allele of p107 was genetically reintroduced. Using gene expression profiling, the researchers showed that TKO hematopoietic progenitor cells exhibited a gene

expression profile consistent with preferential myeloid development and decreased lymphoid development, as can be observed in TKO mice[479].

Chapter 2. Materials and Methods

Mice

SHIP$^{-/-}$ mice were created previously[325] and maintained by intercrossing SHIP$^{+/-}$ mice (F10 to the C57BL/6J background). The creation of SHIP$^{\Delta IP/\Delta IP}$ mice is described in Karlsson *et al*[340]. SHIP-deficient mice and WT littermates used were between 6 and 9 weeks of age. Mice with germline transmission of a SHIP^{flox} allele were previously created in our laboratory[325] and maintained by intercrossing with SHIP$^{flox/flox}$ mice (F10 to the C57BL/6J background). MxCre transgenic mice (Jackson Laboratory) were intercrossed with SHIP$^{flox/flox}$ mice to generate MxCreSHIP$^{flox/flox}$ on a C57BL/6J background. MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ littermates were generated by intercrossing MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ mice. LysCreSHIP$^{flox/flox}$, LckCreSHIP$^{flox/flox}$ mice and SHIP$^{flox/flox}$ littermates were generated in a similar fashion. Likewise, MxCre transgenic mice and Rb1$^{flox/flox}$ mice (National Cancer Institute) were intercrossed to generate MxCreRb1$^{flox/flox}$ and Rb1$^{flox/flox}$ littermates. Tumor-bearing mice were established by injecting 500,000 EL-4 thymoma cells or MC-38 tumor cells subcutaneously in the right flank of 6–8 wk old C57BL/6J mice (National Cancer Institute). Rag2$^{-/-}\gamma c^{-/-}$ mice on an H2d background were obtained from Hergen Spits[480] (Netherlands Cancer Institute). Severe combined immune deficient (SCID) mice (C57BL/6J background) were purchased from The Jackson Laboratory. Studies were performed in accordance with the guidelines and approval of the Institutional Animal Certification and Use Committee at the University of South Florida.

Conditional Deletion of SHIP or Rb1

SHIP or Rb1 was conditionally deleted in MxCreSHIP$^{flox/flox}$ mice or MxCreRb1$^{flox/flox}$ mice, respectively, by intraperitoneal injection of 625μg polyinosinic-polycytidylic acid (polyI/C; Sigma-Aldrich) on days 0, 3, and 6. SHIP$^{flox/flox}$ or Rb1$^{flox/flox}$ littermates were also injected simultaneously to serve as controls.

Cell Purification

Whole splenocytes from SHIP$^{-/-}$, SHIP$^{\Delta IP/\Delta IP}$, MxCreSHIP$^{flox/flox}$, and respective littermate controls were magnetically enriched for CD3$^+$ T cells using anti–CD3-phycoerythrin (PE), Miltenyi anti-PE microbeads, and an Automacs (Miltenyi Biotec, Auburn, CA) per the manufacturer's instructions. The positive fraction was stained for CD8, CD4, CD25, and viability (4′,6-diamidino-2-phenylindole dihydrochloride [DAPI]), then sorted for viable CD3$^+$CD4$^+$CD25$^-$CD8$^-$ and CD3$^+$CD4$^+$CD25$^+$CD8$^-$ T cells using a BD FACS (fluorescence-activated cell sorter) Aria cell sorter. Population purity was more than 95% as determined by post-sort analysis. Sorted cells were used for Western blot analysis, *in vitro* mixed leukocyte reactions (MLRs), or adoptive transfer.

Whole splenocytes and BM (BM) cells from naïve (phosphate-buffered saline (PBS) treated) mice, and from MC-38 or EL-4 tumor-bearing mice (3-weeks after injection) were processed into a single cell suspension. MC-38 tumors were extracted and digested with collagenase D (400U/ml, Roche) to process into a single cell suspension. Splenic and intratumoral (MC-38) MDSC were isolated by staining the single cell suspensions with fluorescent antibodies against CD11b and Gr1 and with DAPI. The stained cells were sorted using BD FACSAria cell sorter to isolate viable CD11b$^+$Gr1$^+$ and CD11b$^-$Gr1$^-$ populations. Population purity was more than 95% as determined by post-sort analysis. Sorted cells were lysed and the lysates used for

Western blot analysis. MDSC or G-MDSC from EL-4 tumor-bearing mice were magnetically isolated from SPL or BM (BM) cells by staining the single cell suspension with biotin conjugated antibody specific for either Gr1 (BD Biosciences) for MDSC isolation, or Ly6G (Miltenyi Biotec) for G-MDSC isolation. Labeled cells were then subjected to magnetic isolation using Miltenyi anti-biotin microbeads, a MidiMacs separator and an LS column (Miltenyi Biotec) per the manufacturer's instructions. Purity of the MDSC or G-MDSC population was more than 90% as determined by flow cytometry using an LSRII (BD Biosciences). Isolated cells were used for Western blot analysis, RT-PCR analysis and *in vitro* culture assays.

Flow Cytometry

For phenotypic analysis and quantitation of viable T cells or MDSC, splenocytes, mesenteric LN cells, or thymocytes were Fc-blocked and stained using fluorescent-conjugated antibodies. For T cells, antibodies against the following surface markers were used: CD3, CD4, CD25, CD103, GITR, OX40, CD127, and CD16/32 (R&D Systems). Importantly, when staining for CD16/CD32, Fc block was not used. For intracellular FoxP3 expression, cells were stained as mentioned above, then permeabilized and fixed using the eBioscience Fixation/Permeabilization kit (eBioscience), and stained with anti-FoxP3 (FJK-16a). For MDSC, antibodies against the following surface markers were used: CD11b, Gr1, Ly6G, and Ly6C; and DAPI was used for analysis of viability. All samples were analyzed on an LSRII (BD Biosciences). All antibodies except CD16/32 were purchased from BD Biosciences or eBioscience.

In Vitro Culture

All cell cultures were done using complete media consisting of RPMI (Cellgro) with 10% FBS (Atlas Biologicals) and 1% antibiotics (Invitrogen), except for MC-38 colon carcinoma tumor cells which were cultured in complete media with DMEM (Invitrogen), 10% FBS and 1% antibiotics. In MDSC cultures, the complete media was supplemented with 10ng/ml GM-CSF (Invitrogen) and changed every 2-3 days. EL-4 tumor explant supernatant (TES) was generated by obtaining a tumor mass with no ulceration from a tumor-bearing mouse ($6x10^5$ EL4 cells were subcutaneously injected about 3 weeks prior). The tumor was mechanically separated, incubated in collagenase D (2mg/ml in RPMI) for 1 hour and strained through a cell strainer to obtain a single cell suspension. Cells were then plated at 2 million cells per ml of complete media. The supernatant media (TES) was then collected and filter sterilized after two days in culture. When culturing with TES, MDSC were plated in complete media supplemented with GM-CSF, as mentioned above, and with TES as 20% of the final volume. When using HDAC inhibitor (HDACi) treatment, the following final concentrations were used: Trichostatin A (TSA) was used at 20nM or 100nM (Cell Signaling Technology); valproic acid (VPA) was used at 1mM (Sigma-Aldrich), suberoylanilide hydroxamic acid (SAHA) was used at 3μM (Cayman). The DMNTi, 5-aza-2'-deoxycytidine (AZD) was used at 1μM or 2μM (Sigma-Aldrich).

BrdU Incorporation

To measure proliferation, the BrdU incorporation assay was performed using BD Pharmigen BrdU flow kit. Simply, cells were plated for 2 days, pulsed with 10uM of BrdU for 4 hours before harvesting for staining of surface markers, CD11b, Ly6G, and Ly6C (BD Biosciences). After 20 minutes of staining, cells were washed with staining media

and fixed using the provided Cytofix/Cytoperm buffer for 30 minutes at room temperature (RT). Cells were then washed and stored in freezing media (10% dimethylsulfoxide, DMSO, in FBS) until further use. Once cells were thawed, they were washed again and incubated in the Cytofix/Cytoperm buffer for 5 minutes. Cells were treated with DNase for 1 hour at 37°C to expose incorporated BrdU, washed then stained with fluorescent anti-BrdU antibody for 20 minutes at RT. After washing, DAPI at 1µg/ml (Invitrogen) was added to stain the DNA for DNA content and cell cycle analysis. Samples were run on an LSRII (BD Biosciences) and analyzed on FlowJo software.

Mixed Leukocyte Reaction

A total of $1x10^5$ cells/well irradiated (2000rad) Balb/cJ splenocytes (stimulators) were plated in quadruplicate with $1x10^5$/well WT or SHIP-deficient $CD3^+CD4^+CD25^-CD8^-$ T cells (responders) in 96-well U-bottom plates (Corning Life Sciences) containing RPMI 1640 complete media. After 3 days of culture, proliferation of responder T cells was determined by quantifying overnight incorporation of [^{3}H] thymidine (1.0µCi per well; MP Biomedicals). Results were expressed as the mean counts per minute (cpm) of quadruplicate wells plus or minus SEM (standard error). To assess suppressive ability, WT $CD3^+CD4^+CD25^+CD8^-$ Tregs, SHIP-deficient $CD3^+CD4^+CD25^+CD8^-$ Tregs, or SHIP-deficient $CD3^+CD4^+CD25^-CD8^-$ T cells were added at the indicated ratios to each MLR well containing $1x10^5$ irradiated BALB/C splenocytes and $1x10^5$ WT $CD3^+CD4^+CD25^-CD8^-$ responder T cells.

Western Blotting

Protein lysates were prepared from magnetically isolated or FACS–sorted cells by resuspending cell pellet in a modified TNE lysis buffer (50mM Tris-HCl, 1% Nonidet

P-40, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 1mM phenylmethylsulfonyl fluoride, 1mM NaOV, 1mM NaF, and protease inhibitors) and incubating for 30 minutes on ice. For SHIP and FoxP3 protein analysis, equal cell equivalents were resolved on a 4% to 12% Bis-Tris gel (Invitrogen) and transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare). Blots were blocked with Odyssey blocking buffer (LI-COR Biosciences), probed with antibodies against SHIP1 (P1C1, 1:200) or FoxP3 (eBio7979, 1:500) and β-Actin (C-11, 1:500) followed by a fluorochrome-tagged secondary. Probed blots were developed on a LI-COR Odyssey imager to quantitate and normalize SHIP levels or FoxP3 levels to β-Actin, displayed as arbitrary fluorescence units (AFU). For Rb1 and E2F detection, 50ng of protein were run on an 8% SDS page gel at 90V for 25 minutes. Voltage was increase to 120V once loading buffer passed the stacking buffer and proteins were run until 25kD protein ladder ran off the gel. Samples in the gel were transferred to a methanol pre-activated PVDF (Millipore) membrane at 20V overnight at 4°C. Membrane was blocked with 5% Milk PBS-T (1% Tween-20 in PBS) for 2 hours at RT with agitation. Membrane was then incubated with the primary antibody in 5% milk PBS-T. For Rb1 detection, membrane was incubated with the primary antibody (BD Biosciences) overnight at 4°C. For Actin (Santa Cruz Biotechnologies) and E2F (Santa Cruz Biotechnologies) detection, membrane was incubated with the primary antibody for 2 hours at RT with agitation. This was followed by probing with a secondary antibody conjugated to HRP (Santa Cruz Biotechnologies). Protein levels were detected using Pierce ECL Plus Western Blotting Substrate (Thermo Scientific) and autoradiography film (Midwest Scientific).

Quantitative Real-Time PCR (qRT-PCR)

RNA extraction was performed using Trizol (Invitrogen) and Qiagen RNeasy columns. Simply, Trizol was added to the cell pellet and incubated at RT while vortexing frequently. Chloroform (1/5 of Trizol amount) (Fisher Scientific) was added and mixed well. The sample was centrifuged at 12,000xg for 15 minutes at 4°C to separate phases. The top layer was mixed equal parts with 70% ethanol and transferred to an RNeasy Mini spin column. The rest was performed according to manufacturer's instructions, including DNase I (Qiagen) treatment to remove traces of DNA. 0.5µg of total RNA was used to synthesize 20ul of cDNA using the High-Capacity Reverse Transcription kit (Applied Biosystems), following manufacturer's protocol. RT-PCR was performed with 2µl cDNA, 10µl of 2X TaqMan gene expression master mix and TaqMan primers specific for one of the following target genes, Rb1 (Mm00485586_m1), β-Actin (Mm00607939_s1), and 18S (4319413E) (Applied Biosystems). Standard curve (absolute quantitation) assays were done for each gene using the Applied Biosystems 7900HT Fast Real-Time PCR System and corresponding software. For each sample, the amount of Rb1 was normalized against the amount of control gene (β-Actin and/or 18S) in the same sample. Relative fold changes in target gene expression were then calculated among the samples being compared.

Chromatin Immunoprecipitation

The Acetyl-Histone H3 Immunoprecipitation (ChIP) assay kit (Millipore) components were used throughout. 7-10x10^6 freshly sorted or cultured cells that were harvested were washed with ice cold PBS and cross-linked immediately by incubating for 10 minutes with 0.4% formaldehyde (in PBS) with gentle stirring at RT. To stop cross-linking, 0.125 M glycine was added and the cells in solution were incubated with

continued stirring for an additional 5 minutes at RT. Cells were then washed twice with ice cold PBS and pelleted by centrifugation at 400xg for 10 minutes at 4°C. The cell pellet was resuspended in 500µls of SDS lysis buffer (with added protease inhibitors) and incubated for 10 minutes on ice. Lysates were sonicated to shear DNA to lengths between 200 and 1000 basepairs (bp) (confirmed by electrophoresis) using a Bioruptor™ from Diagenode. Making sure to keep samples ice cold, a cycle of eight pulses of 30 seconds with 30 seconds rest time was repeated eight times at a frequency of 20 KHz. After centrifugation at 16,000xg for 10 minutes at 4°C, the supernatant was collected, and kept at -80°C. An aliquot of 25µls was removed to analyze DNA fragmentation. Crosslinking was reversed with NaCl treatment overnight followed by RNase and Proteinase K treatment to eliminate RNA and protein contamination. The same amount of each sample was run on a 1% agarose gel with Ethidium Bromide to visualize DNA. The average intensity of the bands was measured and used to calculate how much of lysate should be used among samples being compared to start immunoprecipitation with approximately equal amounts of DNA. Each sample was aliquoted three ways to contain approximately equal amounts of DNA in 100µls of sonicated cell supernatant for each antibody specific immunoprecipitation, no-antibody immunoprecipitation and input. The sonicated cell supernatant was then diluted 10 fold in ChIP Dilution Buffer (with added protease inhibitors) for a final volume of 1ml in each immunoprecipitation condition. To reduce nonspecific background, samples were pre-cleared with Salmon Sperm DNA/Protein A Agarose-50% Slurry (Catalog #16-157C) for one hour at 4°C with agitation. After brief centrifugation, the collected supernatant was immunoprecipitated with Acetyl-Histone H3 specific antibody overnight at 4°C with rotation. For the negative control, a no-antibody immunoprecipitation was performed by incubating the supernatant fraction with Salmon Sperm DNA/Protein A Agarose-50%

Slurry for one hour at 4ºC with rotation. Antibody/histone complexes were recovered by adding Salmon Sperm DNA/Protein A Agarose Slurry for one hour at 4ºC with rotation. The protein A agarose/antibody/histone complexes were washed once with the Low Salt Immune Complex Wash Buffer, once with the High Salt Immune Complex Wash Buffer, once with LiCl Immune Complex Wash Buffer and twice with the TE Buffer. The solution was incubated during each wash for 5 minutes with rotation at 4°C. The histone complexes were then eluted from the antibody by adding 150µls of freshly prepared elution buffer (1%SDS, 0.1M NaHCO3), vortexing and incubating at RT for 15 minutes with rotation. This was repeated twice and the supernatant fractions (eluate) were combined. NaCl (12µls of 5M NaCl) was added to the combined eluates (300µls total) to reverse histone-DNA crosslinks by heating at 65ºC for 4 hours. Reversal of cross linkage was also performed on the input/starting material. 6µl of 0.5M EDTA, 12µl 1M Tris-HCl, pH 6.5 and 1.2µl of 10mg/ml Proteinase K were added to the combined eluates and incubated for one hour at 45ºC. To purify DNA, the Qiagen DNA purification kit was used. RT-PCR was performed on the purified DNA using Rb1 promoter specific primers (forward: 5'-TACTTGGGTTCGAGTCCTCTGCCAG-'3, reverse: 5'-AGTTGGCCGTGTTCATGCG-'3) on a CFX96 RT-PCR thermal cycler (Bio-Rad) and analyzed using Bio-Rad CFX Manager software.

G-CSF Neutralization and SDF1 ELISA

SHIP was conditionally deleted in MxCreSHIP$^{flox/flox}$ mice by administering an intraperitoneal injection of 625µg polyinosinic-polycytidylic acid (polyI/C)) (Sigma-Aldrich) on days 0, 3, and 6 as described in Paraiso *et al.* SHIP$^{flox/flox}$ mice were treated similarly to serve as control mice. On day 2, polyI/C treated MxCreSHIP$^{flox/flox}$ mice and SHIP$^{flox/flox}$ control mice were also injected subcutaneously with monoclonal anti-mouse G-CSF

antibody (R&D systems, 10µg/animal/day) or PBS (diluent) for one week. Then MDSC and Tregs in the SPL and mesenteric LNs were quantitated by flow cytometry (as described above).

Adoptive Transfer of T cells for Colitis and GvHD Induction

In the syngeneic colitis model, to assess Treg function, C57BL/6J SCID hosts received $4x10^5$ sorted $CD3^+CD4^+CD25^-CD8^-$ T cells from WT C57BL/6J donors along with or without $7x10^4$ sorted $CD3^+CD4^+CD25^+CD8^-$ Tregs from WT or $SHIP^{-/-}$ C57BL/6J donors by intraperitoneal injection on day 1. In parallel, a control group of C57BL/6J SCID hosts received a PBS injection. In the allogeneic GvHD colitis model, to assess allogeneic T cell response, $Rag2^{-/-}\gamma c^{-/-}$ mice (on an H2d background) received 10^5 sorted $CD3^+CD4^+CD25^-CD8^-$ T cells from WT or $SHIP^{-/-}$ C57BL/6J donors by retro-orbital injection. In parallel, a control group of $Rag2^{-/-}\gamma c^{-/-}$ mice hosts received a PBS injection.

Clinical and Histologic Examination of Colitis

Recipient mice were weighed and monitored for colitis-associated appearance 3 times per week. Recipient mice were kept in a pathogen-free barrier room for 8 to 12 weeks after cell transfer. When premoribund (lost ≥ 10% or more of its starting body weight) or after 8 to 12 weeks after cell transfer, recipient mice were killed and given a disease activity index (DAI). The colon was obtained from each mouse and fixed in 10% formalin in PBS. Paraffin-embedded sections were cut and stained with hematoxylin and eosin for histologic examination and scoring. Histology micrographs were taken using a Leica DMLB microscrope (N PLAN 20x/0.40, total magnification x200, at RT, and a SPOT Insight QE Model 42 camera with Spot Advanced acquisition software

(Diagnostic Instruments). The DAI represents the sum of 2 scores: the clinical assessment score (CAS) and the histopathology score (HPS). The CAS is determined on a scale from 0 to 4 as follows: 0 indicates no signs; 1, bristled fur; 2, bristled fur with hunched posture, and/or reduced activity; 3, all of the above and change in stool consistency (for example, soft, sticky); 4, rectal prolapse. The HPS was determined by grading the histologic appearance of the colon using the following criteria: grade 0 indicates an unaffected proximal colon; grade 1 indicates mild leukocyte infiltration of the lamina propria (not shown); grade 2 indicates moderate leukocyte infiltration of the lamina propria, mild reduction of goblet cells, and mild crypt epithelial regenerative hyperplasia; grade 3 indicates marked leukocyte infiltration beyond the muscularis mucosa into a thickened submucosa, goblet cell depletion, and epithelial regenerative hyperplasia with atypia; and grade 4 indicates marked transmural leukocyte infiltration deep into a thickened submucosa and tunica muscularis with increased vascular density, marked goblet cell loss, and epithelial regenerative hyperplasia with atypia. Histopathology grading was performed in a blinded fashion by Dr. Robert Engelman.

Vascularized Heart Rejection Model

C57BL/6J, polyI/C-treated MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ mice received hearts from adult Balb/cJ donors. Fourteen days after the initiation of SHIP deletion, vascularized heart transplantations were performed following the procedure of Corry *et al*[481]. Monitoring of transplant function was assessed by daily palpation of the graft. Moderate rejection was detected by a sclerotic graft, final rejection by missing heartbeats.

Statistics

In vitro experiments are representative of at least 3 independent analyses. MLR, flow cytometry results, weight change, and HPS results were analyzed with the 2-tailed Student *t* test or the Mann-Whitney U test using Prism 4 software (GraphPad Software). Differences were considered significant at *P* values less than 0.05. Comparisons of graft or mouse survival were done using the Kaplan-Meier log-rank test using Prism 4 software.

Chapter 3. SHIP Limits Immunoregulatory Capacity in the T Cell Compartment

Note to Reader

The work presented in this chapter has been previously published (Collazo *et al*, 2009)[482] and are utilized with permission of the publisher.

Introduction

Tregs actively mediate self-tolerance and thus control autoimmunity[52, 483]. Tregs also limit antitumor T cell responses and deleterious allogeneic T cell responses that cause GvHD[158, 159] and solid organ allograft rejection,[484] making them valuable therapeutic targets. We previously found that donor and host allogeneic responses are compromised in SHIP-deficient hosts, which exhibit significantly reduced acute rejection of MHC-mismatched BM grafts and GvHD[325, 341]. Thus, an immunosuppressive environment prevails in SHIP-deficient hosts. We also consistently observe a profound expansion of MDSC in SHIP-deficient mice[325, 341, 345, 485]. Because host and donor Tregs limit GvHD[159, 486] and $CD11b^{+}Gr1^{+}$ cells, similar to $SHIP^{-/-}$ MDSC, expand Tregs in tumor and GvHD models[233, 487]; we considered that the Treg compartment in SHIP-deficient hosts may also be expanded. In addition, SHIP-deficiency could intrinsically affect Treg homeostasis and function. SHIP can oppose PI3K signaling pathways triggered by engagement of costimulatory and cytokine receptors critical for the suppressive function, survival, and expansion of Tregs, such as CD25, IL-7R, and OX40[488-490].

Because Tregs were initially characterized as $CD4^+$ T cells coexpressing CD25, most Treg studies focus on this phenotype, which is shared with activated $CD4^+$ T cells[54]. To distinguish between Tregs and activated T cells, molecular markers correlated with or obligate for Treg function have been identified, such as the transcription factor FoxP3, as well as surface markers CD103, GITR and OX40, among others. FoxP3 functions as the master regulator in the development and suppressive ability of Tregs[60]. CD103 expression among $CD4^+CD25^+$ Tregs distinguishes an effector/memory-like subset that displays an inflammation-seeking phenotype and exhibits greater suppressive capacity[491, 492]. In addition, CD103, which binds E-cadherin, mediates the retention of $CD103^+$ lymphocyte in epithelial compartments[492], which are major sites of GvHD. Thus, $CD103^+$ Tregs might have a prominent role in the prevention against GvHD. GITR and OX40, members of the tumor necrosis factor receptor superfamily of receptors, are costimulatory molecules known to play key roles in promoting the homeostasis, expansion, and suppressive capability of Tregs[493, 494]. Furthermore, $CD4^+CD25^+OX40^+$ Tregs represent a mature population that does not require preactivation or stimulation to suppress antigen-specific T cell responses[495].

Analysis of CD103, GITR, and FoxP3 expression has allowed the identification of a Treg population among "naive" $CD4^+CD25^-$ T cells. Specifically, $CD4^+CD25^-CD103^+$ T cells display regulatory activity in both an *in vitro* proliferation assay and *in vivo* disease models, such as colitis and antigen-induced arthritis[491, 496]. Additionally, $CD4^+CD25^-GITR^+$ T cells express IL-10, TGFβ , and intracellular CTLA-4, are anergic, suppress T cell proliferation, and can prevent wasting disease, colitis, autoimmune myocarditis, diabetes, and multiorgan inflammation[497, 498]. Thus, when assessing the entire Treg compartment, one should also consider these immunoregulatory subsets within the so-called "naive" $CD4^+CD25^-$ T cell compartment.

Using 3 murine genetic models of SHIP-deficiency, we show here that the frequency of $CD4^+CD25^+FoxP3^+$ Tregs and $CD4^+CD25^-FoxP3^+$ T cells is increased in SHIP-deficient mice. We find that the suppressive capacity of SHIP-deficient $CD3^+CD4^+CD25^+$ Tregs ($CD25^+$ Tregs) is equal to that of WT $CD25^+$ Tregs. Interestingly, the SHIP-deficient $CD3^+CD4^+CD25^-$ T cell ($CD25^-$ T cell) compartment displays significant immunosuppressive capacity *in vitro* and *in vivo*, possibly because of the increase in $FoxP3^+$ T cells. Furthermore, the surface expression of CD103, GITR, OX40, and FcγRII/III is significantly increased in SHIP-deficient $CD25^-$ and $CD25^+$ $CD4^+$ T cell compartments. These qualitative changes possibly increase the survival and immunosuppressive capacity of the SHIP-deficient T cell compartment and contribute to the reduced host-versus-graft and graft-versus-host responses observed in SHIP-deficient hosts.

Results

Mice with Germline SHIP-Deficiency have Increased Numbers of Conventional Tregs. The expansion of Tregs in SHIP-deficient mice would be consistent with their relative resistance GvHD[325, 341]. Thus, we examined the Treg compartment in peripheral lymphoid organs of mice from 2 different genetic models of SHIP-deficiency. Both models are germline SHIP-deficient, one having the promoter and first exon of SHIP deleted ($SHIP^{-/-}$) and the other having the exon encoding the enzymatic domain of SHIP deleted ($SHIP^{\Delta IP/\Delta IP}$). Consistent with our hypothesis, we observed a significantly increased frequency and absolute number of $CD25^+FoxP3^+$ Tregs in the SPL and LN of both SHIP-deficient strains relative to their WT littermates

(Figure 1). In neither mutant strains is the frequency of total $CD3^+$ T cells increased in the SPL or LN. In fact, the frequency of splenic $CD3^+$ T cells is significantly decreased in both strains, whereas LN $CD3^+$ T cell frequency remains unchanged (Figure 2). Interestingly, FoxP3 expression levels appear to be greater in splenic SHIP-deficient Tregs compared to their WT counterparts as determined by flow cytometry and Western blot analysis of sorted splenic $CD25^+$ Tregs (Figure 3). Thus, germline SHIP-deficiency promotes a preferential expansion and/or accumulation of conventional Tregs that have increased expression of FoxP3.

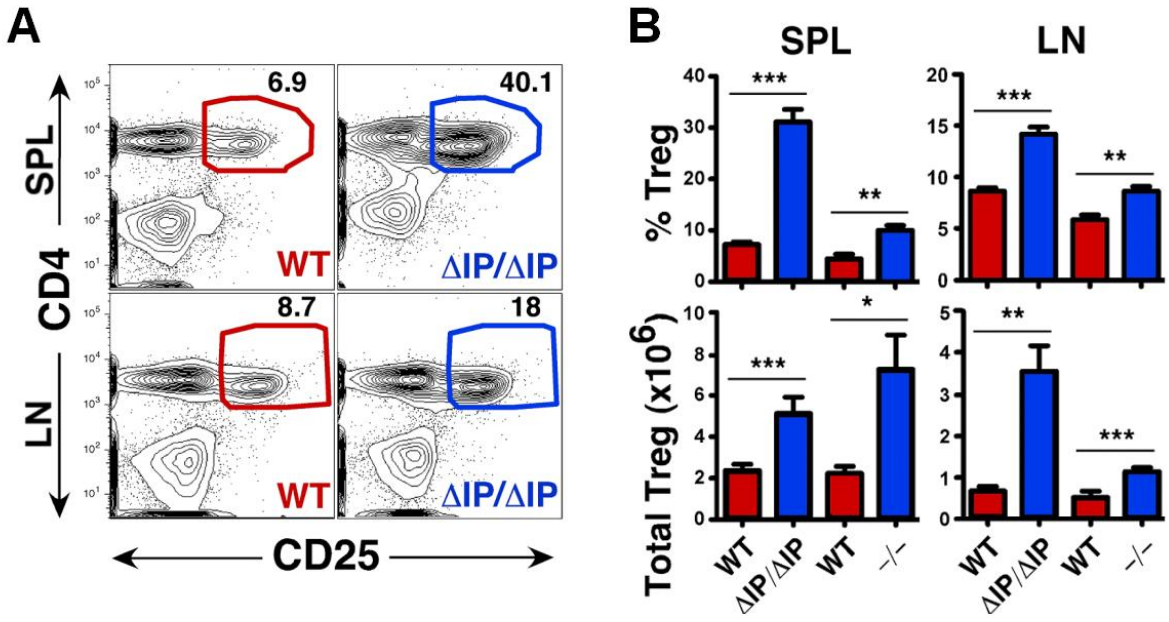


Figure 1. Mice with germline SHIP-deficiency have an expanded $CD25^+$ Treg compartment in peripheral lymphoid organs compared to WT counterparts.
(A) Representative CD4 vs. CD25 staining after gating on $CD3^+$ T cells for SPL and LN of $SHIP^{\Delta IP/\Delta IP}$ and WT littermates. (B) Percentage frequency of $CD4^+CD25^+FoxP3^+$ Tregs after gating on $CD3^+$ T cells, and total absolute $CD3^+CD4^+CD25^+FoxP3^+$ Treg numbers in the SPL and LN of the indicated genotype. For $SHIP^{\Delta IP/\Delta IP}$ mice: n = 12 and littermate control: n = 10. For $SHIP^{-/-}$ mice: n = 6 and littermate controls: n = 6. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

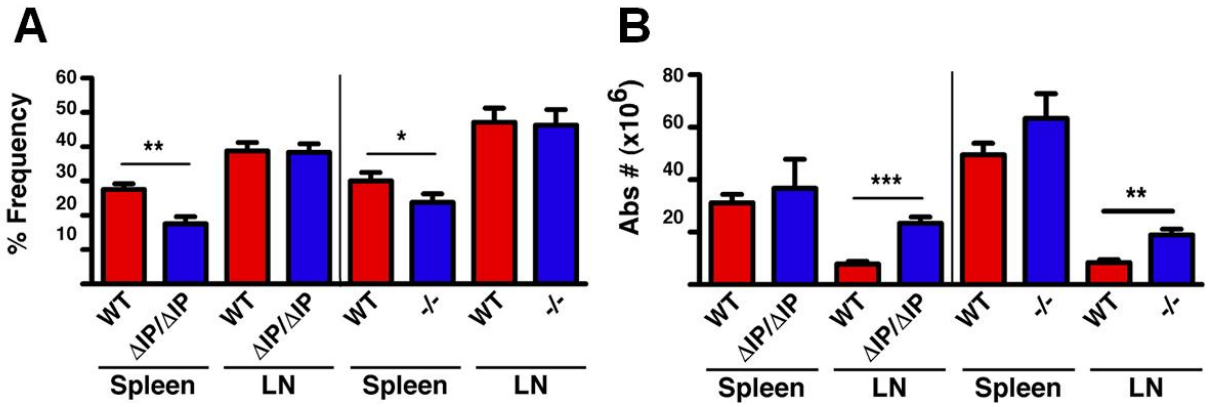


Figure 2. The frequency and absolute number of CD3$^+$ T cells in mice with germline SHIP-deficiency compared to WT counterparts.
Percentage frequency (A) and absolute number (B) of CD3$^+$ T cells after gating on viable cells in the SPL and LN of the indicated genotype. For SHIP$^{\Delta IP/\Delta IP}$ mice: n = 12 and littermate control: n = 10. For SHIP$^{-/-}$ mice: n = 6 and littermate control: n = 6. (*$P \leq$ 0.05, **$P \leq$ 0.01, ***$P \leq$ 0.001)

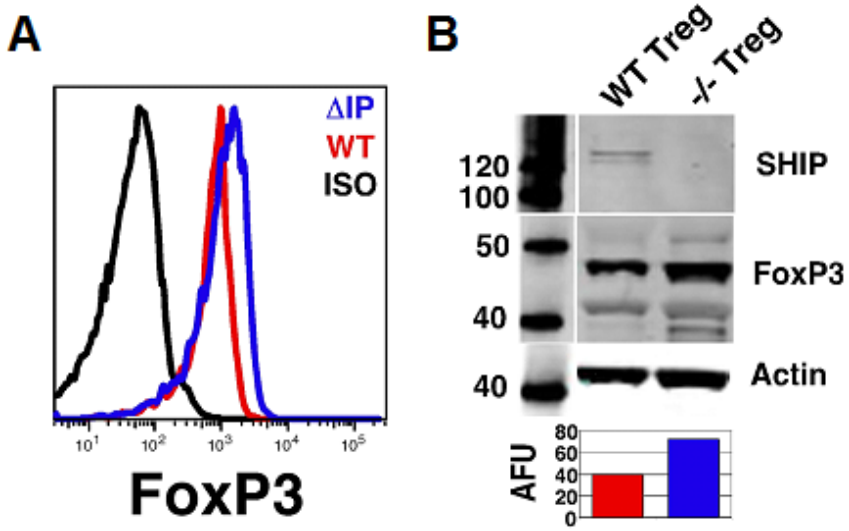


Figure 3. FoxP3 expression levels are greater in splenic SHIP-deficient Tregs compared to WT counterparts.
(A) Representative FACS analysis of FoxP3 expression in CD3$^+$CD4$^+$CD25$^+$ Tregs from the SPL of the indicated genotype. (B) Western blot analysis of SHIP, FoxP3, and β-Actin expression in lysates prepared from sorted CD3$^+$CD4$^+$CD25$^+$ Tregs of the indicated genotype. AFU values for FoxP3 expression in SHIP$^{-/-}$ ($^{-/-}$ Treg) and WT Tregs are displayed below the corresponding band in the bar graph.

We then considered whether peripheral expansion of the Treg compartment might be partly the result of increased thymic production of $CD25^+$ Tregs. To examine this, we assessed the thymic content of $CD25^+FoxP3^+$ Tregs in both SHIP-deficient strains compared with their WT littermates. We found a significant increase in the frequency of thymic $FoxP3^+$ with $CD25^-$ and $CD25^+$ T cell compartments in both SHIP-deficient models relative to their WT littermates (Figure 4). However, because SHIP-deficient mice have smaller thymuses, the absolute numbers of these $FoxP3^+$ Tregs are not significantly different from their WT littermates. Thus, increased thymic output does not account for the expanded peripheral Treg compartment. On a side note, the ratio of $CD4^+CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$ T cells in the thymus of SHIP-deficient mice is not significantly different from that seen in WT mice (data not shown).

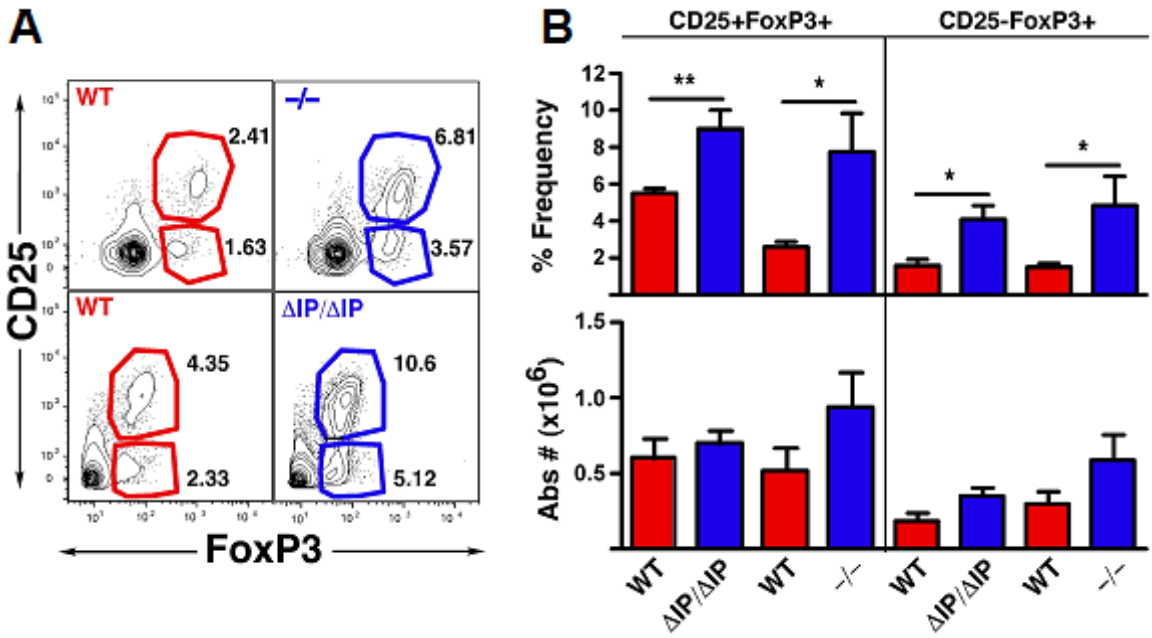


Figure 4. Mice with germline SHIP-deficiency have higher percentages of thymic $CD4^+FoxP3^+$ Tregs than WT counterparts.
(A) Representative CD25 vs. FoxP3 staining after gating on $CD4^+CD8^-$ thymic cells from $SHIP^{\Delta IP/\Delta IP}$ or $SHIP^{-/-}$ and WT littermate controls. (B) Percentage frequency of $CD25^+FoxP3^+$ and $CD25^-FoxP3^+$ Tregs after gating on $CD4^+CD8^-$ T cells and total

absolute number of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^+$ Tregs in the thymus of the indicated genotype. For SHIP $^{\Delta IP/\Delta IP}$ mice: n = 5 and littermate control: n = 5. For $SHIP^{-/-}$ mice: n = 4 and littermate control: n = 4. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

SHIP Regulates Treg Compartment Size during Normal Adult Physiology.

We previously found that, when SHIP-deficiency is induced in adulthood, the MDSC compartment expands rather rapidly, indicating that SHIP regulates MDSC numbers in peripheral lymphoid tissues in response to homeostatic signals present in adult physiology[341]. To test whether this is also the case for Tregs, we examined the T cell compartment in peripheral lymphoid organs in adult $MxCreSHIP^{flox/flox}$ mice after inducing SHIP-deficiency. The results obtained are strikingly similar to that observed for germline SHIP-deficiency. The frequency and absolute number of $CD25^+FoxP3^+$ Tregs are significantly increased in both the SPL and LN of $MxCreSHIP^{flox/flox}$ mice with induced SHIP-deficiency compared with $SHIP^{flox/flox}$ mice (Figure 5). Thus, SHIP-deficiency induced during normal adult physiology promotes the abnormal accumulation of $CD25^+FoxP3^+$ Tregs in the periphery, leading to a pronounced bias in the T cell compartment toward immunosuppressive cells.

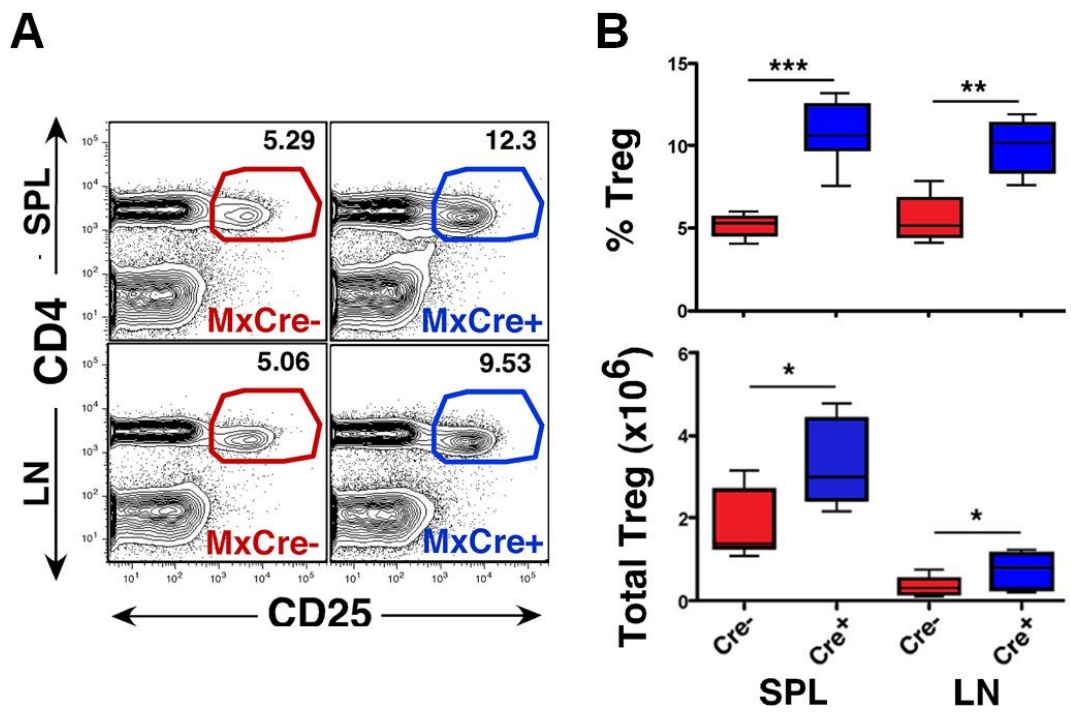


Figure 5. Induction of SHIP-deficiency expands the CD25$^+$FoxP3$^+$ Treg compartment in peripheral lymphoid organs.
(A) Representative CD4 vs. CD25 staining in SPL and LN from MxCreSHIP$^{flox/flox}$ (Cre$^+$) and SHIP$^{flox/flox}$ (Cre$^-$) mice after poly(I/C) administration. (B) Percentage frequency of CD4$^+$CD25$^+$FoxP3$^+$ Tregs after gating on CD3$^+$ T cells, and total absolute CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$ Treg numbers in the SPL and LN of the indicated genotype. For MxCreSHIP$^{flox/flox}$ (Cre$^+$): n = 6 and SHIP$^{flox/flox}$ (Cre−): n = 9. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

SHIP-Deficiency Alters the Expression of Key Receptors in the CD4$^+$ T Cell Compartment. The expression of specific receptors by Tregs, such as CD103, GITR, and OX40, has been associated with their regulatory function, activation status, trafficking, and retention in specific organs. We find that the percentage of CD103$^+$ cells in CD25$^-$ and CD25$^+$ T cell subsets is significantly increased in the SPL and LN of SHIP$^{\Delta IP/\Delta IP}$ mice compared with WT littermates (Figure 6A). Examination of GITR expression on CD25$^+$ Tregs from SHIP$^{\Delta IP/\Delta IP}$ mice shows that the surface density, as

determined by mean fluorescent intensity (MFI) obtained from flow cytometric analysis, is also significantly greater than that seen on WT $CD25^+$ Tregs in the SPL and LN. In addition, $SHIP^{\Delta IP/\Delta IP}$ mice exhibit a significantly larger representation of $CD25^-GITR^{hi}$ T cells in the SPL and LN compared with WT littermates (Figure 6B). Similar to GITR, in $SHIP^{\Delta IP/\Delta IP}$ mice, there is an increased surface density of OX40 on $CD25^+$ Tregs. In addition, the frequency of $OX40^+$ cells among $CD25^-$ T cells is significantly higher in the SPL and LN of $SHIP^{\Delta IP/\Delta IP}$ mice compared with WT littermates (Figure 6C).

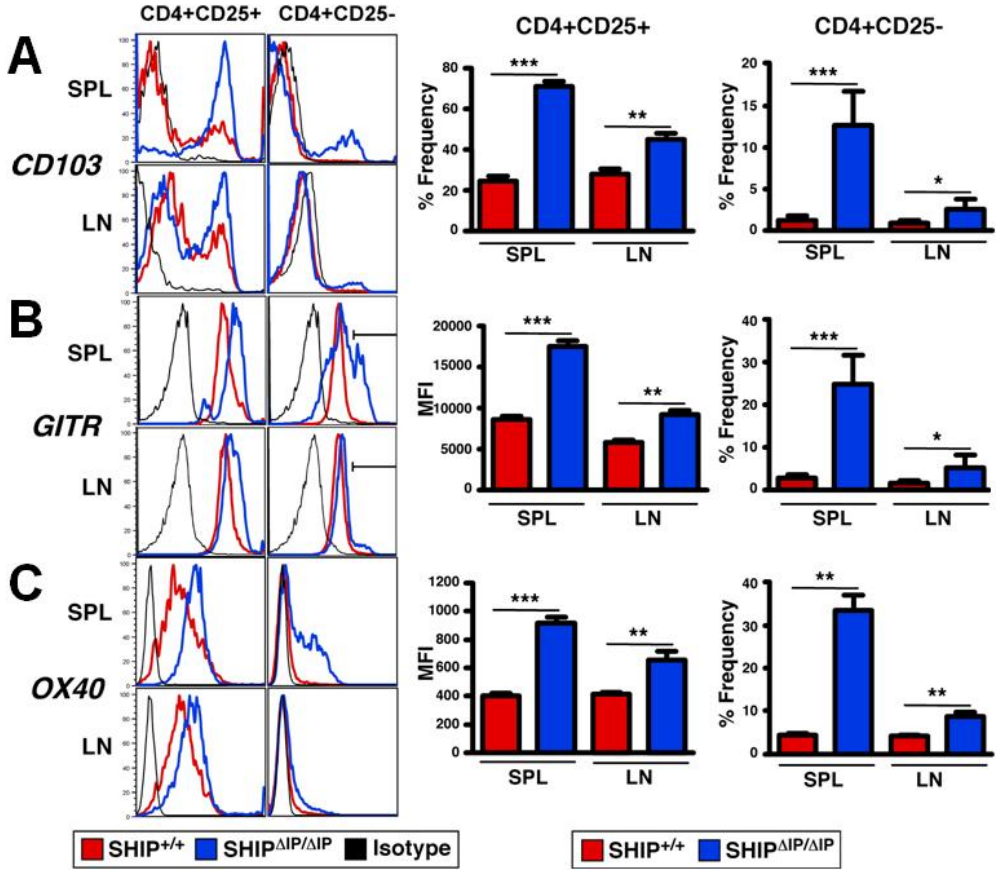


Figure 6. SHIP-deficiency promotes altered expression of surface markers, CD103, GITR and OX40 in the $CD4^+$ T cell compartment.
(A) Representative histogram of CD103 expression levels on viable $CD3^+CD4^+CD25^+$ ($CD25^+$) or $CD3^+CD4^+CD25^-$ ($CD25^-$) T cells from SPL and LN of the indicated genotype. Bar graphs representing percentage frequency of $CD103^+$ T cells among viable $CD25^+$

or $CD25^-$ T cells from the SPL and LN of the indicated genotype. (B) Representative histograms of GITR expression on viable T cells as in panel A. Bar graphs representing MFI of GITR expression on viable $CD25^+$ T cells from SPL and LN of the indicated genotype. Bar graph representing percentage frequency of $GITR^{hi}$ (as determined by depicted gate in histogram) T cells among viable $CD25^-$ T cells. (C) Same as in panel B, but for OX40 expression. For $SHIP^{\Delta IP/\Delta IP}$: n = 6, and for WT littermates: n = 6. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

Recent studies attempting to define surface markers that improve purification of viable Tregs showed that Tregs express lower levels of the IL-7 receptor α chain, CD127, than do activated effector T cells[499]. Although IL-7R signaling, like IL-2R and IL-15R signaling, is required for Treg development. In $SHIP^{\Delta IP/\Delta IP}$ mice, we observed that $CD25^+$ Tregs in SPL and LN express higher levels of CD127 (~2-fold higher MFI value) compared with WT littermates (Figure 7A).

Furthermore, we examined the expression levels of these markers (CD103, GITR, OX40, and IL7R) among $CD4^+CD8^-CD25^-$ and $CD4^+CD8^-CD25^+$ T cells in the thymus. We found no significant difference comparing $SHIP^{\Delta IP/\Delta IP}$ thymocytes to WT thymocytes (data not shown), suggesting that the increased expression of these markers is acquired in the periphery.

As seen in other cell lineages, such as NK cells, receptors that recruit SHIP and whose activity is regulated by SHIP may be deregulated in SHIP-deficient T cells. For example, T cells are thought to not express Fcγ receptors, such as FcγRIIb (CD32b) and FcγRIIIa (CD16), which are regulated by SHIP and expressed by most other hematopoietic cells[500]. When examining the expression levels of FcγRIIb (CD32b) and FcγRIIIa (CD16) by flow cytometry using an antibody that recognizes both, we find that WT $CD25^-$ T cells and $CD25^+$ Tregs express these FcγRs at low levels compared with appropriate isotype controls (Figure 7B). Interestingly, in SPL and LN of SHIP-deficient

mice, we observed that both $CD25^-$ T cells and $CD25^+$ Tregs express significantly elevated levels of FcγRIIb/FcγRIIIa compared to WT controls.

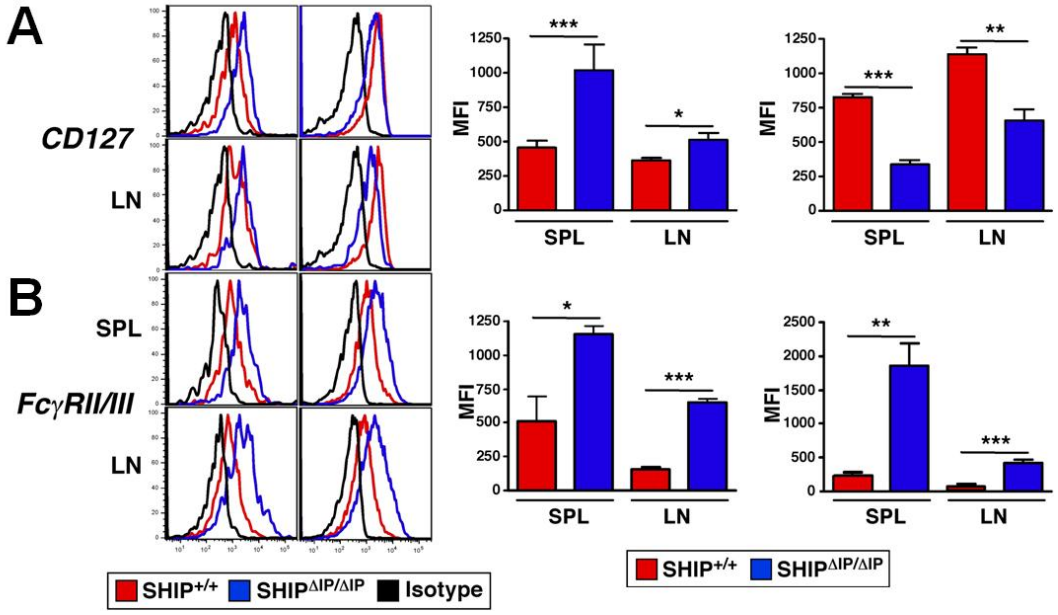


Figure 7. SHIP-deficiency promotes altered expression of surface markers, CD127 and FcgRII/III in the $CD4^+$ T cell compartment.
(A) Representative histogram of CD127 (IL-7R) expression on viable T cells as in panels A to C from Figure 6. Bar graphs representing MFI of CD127 expression on viable $CD25^+$ or $CD25^-$ T cells from SPL and LN of the indicated genotype. (B) Same as in panel A, but for FcγRII/FcγRIII expression. For $SHIP^{\Delta IP/\Delta IP}$: n = 6, and for WT littermates: n = 6. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

SHIP-Deficiency Promotes the Accumulation of $CD4^+CD25^-$ "Naive" T Cells that Express FoxP3 and have Suppressive Function. Researchers have shown that analysis of surface marker expression, such as CD103, GITR, and of intracellular FoxP3 identifies an immunosuppressive subpopulation within the $CD25^-$ T cell compartment. Consistent with the increase in $CD25^+FoxP3^+$ Treg numbers, we find a significant expansion of $CD25^-FoxP3^+$ T cells in the periphery of $SHIP^{-/-}$, $SHIP^{\Delta IP/\Delta IP}$, and polyI/C-

treated MxCreSHIP$^{flox/flox}$ mice, as detected by flow cytometry and Western blot analysis (Figure 8). As shown in Figure 6, when analyzing the expression of surface markers associated with Tregs, such as CD103, GITR, and OX40, we found an enrichment of CD25^{-} T cells that expressed these markers in SHIP$^{-/-}$ SPL and LNs compared with WT littermates. The majority of these cells also coexpressed FoxP3 (data not shown). Thus, these CD25^{-}FoxP3^{+} T cells may represent an immunoregulatory subset that contributes to the immunosuppressive environment in SHIP$^{-/-}$ mice.

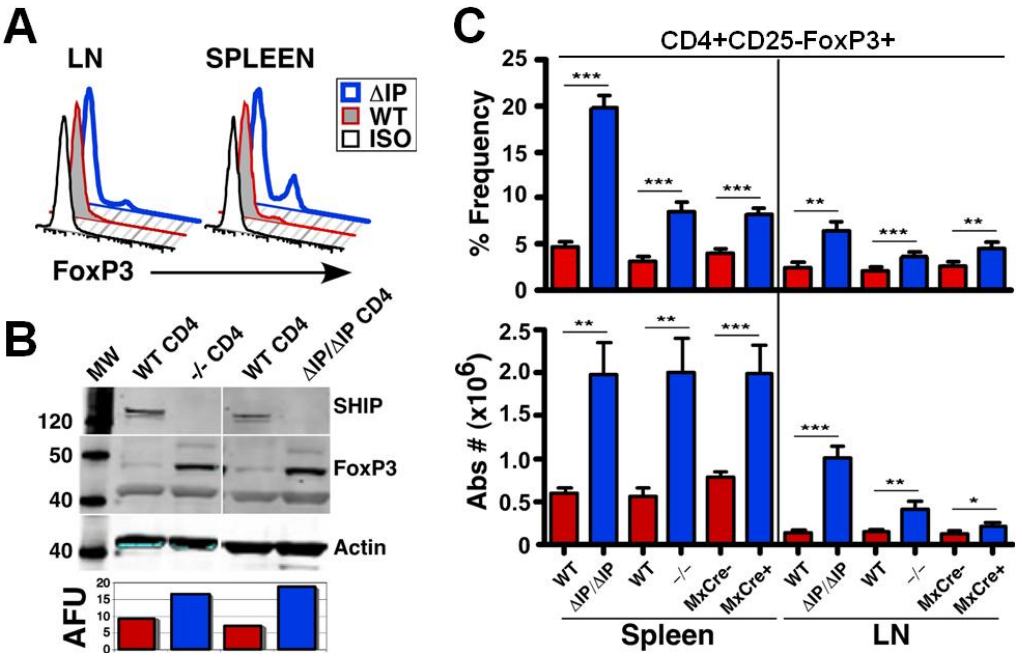


Figure 8. SHIP-deficiency promotes the expansion of a FoxP3^{+} subset amongst CD4^{+}CD25^{-} "naïve" T cells.
(A) Representative histograms of FoxP3 expression levels in fixed CD25^{+} T cells from SPL and LN of the indicated genotype. (B) Western blot analysis of FoxP3 protein expression in FACS-purified CD25^{-} T cells from the indicated SHIP-deficient strain and WT counterpart. (C) Bar graphs representing the percentage frequency of FoxP3^{+} T cells among CD25^{-} T cells and absolute numbers of CD25^{-}FoxP3^{+} T cells in the SPL and LN of the indicated genotype. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

Consistent with their increased FoxP3 expression, $CD25^-$ T cells from either SHIP-deficient strain are unresponsive to MHC-mismatched stimulators, and also demonstrate significant suppressive capacity on other $CD4^+$ T cells (Figure 9). When placed in an MLR with WT effector $CD25^-$ T cells at a 1:1 ratio, the suppressive capacity of SHIP-deficient $CD25^-$ T cells was comparable with that of conventional $CD25^+$ Tregs placed in an MLR with WT effector $CD25^-$ T cells at a 1:8 ratio (Figure 9B). This coincides with the fact that 15% to 20% of SHIP-deficient $CD25^-$ T cells are $FoxP3^+$. Thus, the $FoxP3^+$ cells in the $SHIP^{\Delta IP/\Delta IP}$ $CD25^-$ T cell population are at approximately a 1:8 ratio with WT effector $CD25^-$ T cells, suggesting that $SHIP^{\Delta IP/\Delta IP}$ $CD25^-FoxP3^+$ T cells have suppressive capacity comparable with that of conventional WT Tregs.

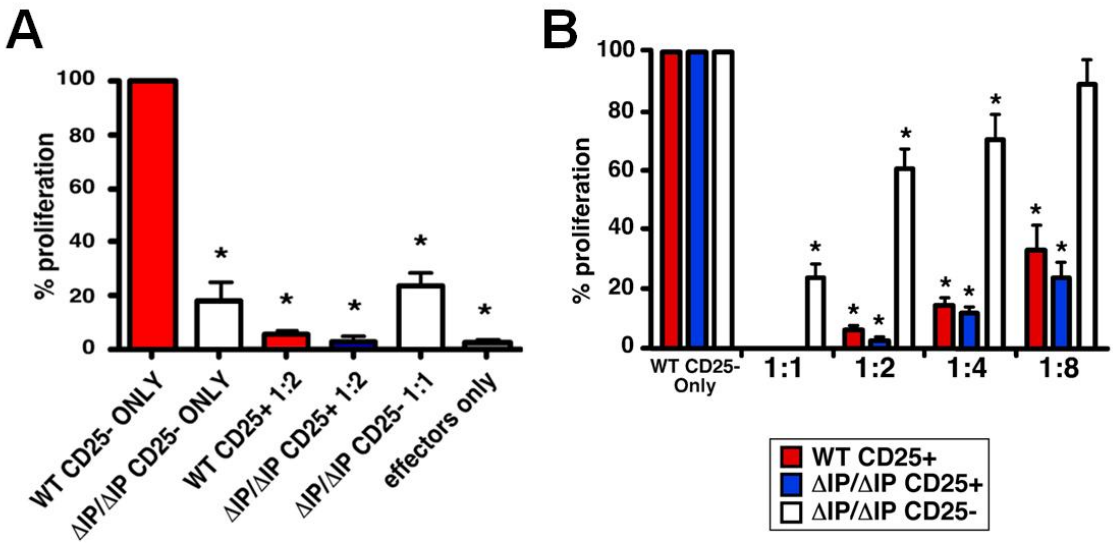
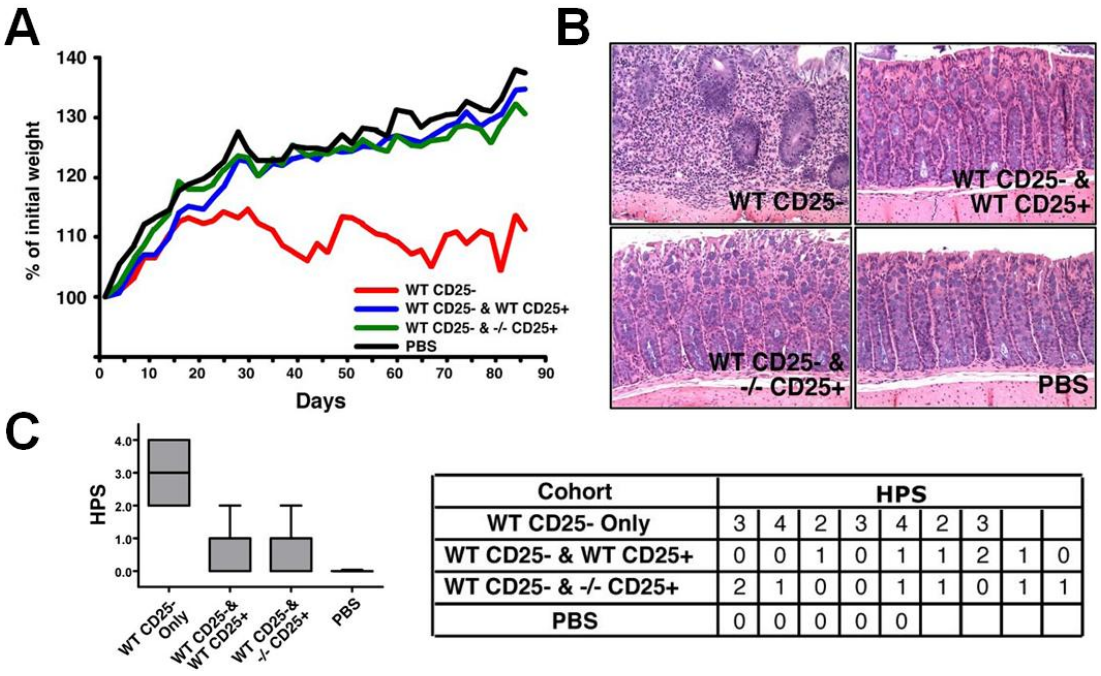


Figure 9. SHIP-deficient $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells are suppressive *in vitro.*

(A) In the first two bars, C57BL/6J WT (WT $CD25^-$) or SHIP-deficient $CD4^+CD25^-$ T cells ($\Delta IP/\Delta IP$ $CD25^-$) were mixed with irradiated Balb/cJ splenocytes in a one-way MLR. Subsequent bars are as described in (B). (B) C57BL/6J WT $CD4^+CD25^+$ Tregs (WT $CD25^+$, red), SHIP-deficient $CD4^+CD25^+$ Tregs ($\Delta IP/\Delta IP$ $CD25^+$, blue) or $CD4^+CD25^-$ T cells ($\Delta IP/\Delta IP$ $CD25^-$, white) were mixed with C57BL/6J WT $CD4^+CD25^-$ T cells at the indicated ratios (suppressors:effectors) and with irradiated Balb/cJ splenocytes in a MLR. (*$P \leq 0.05$)

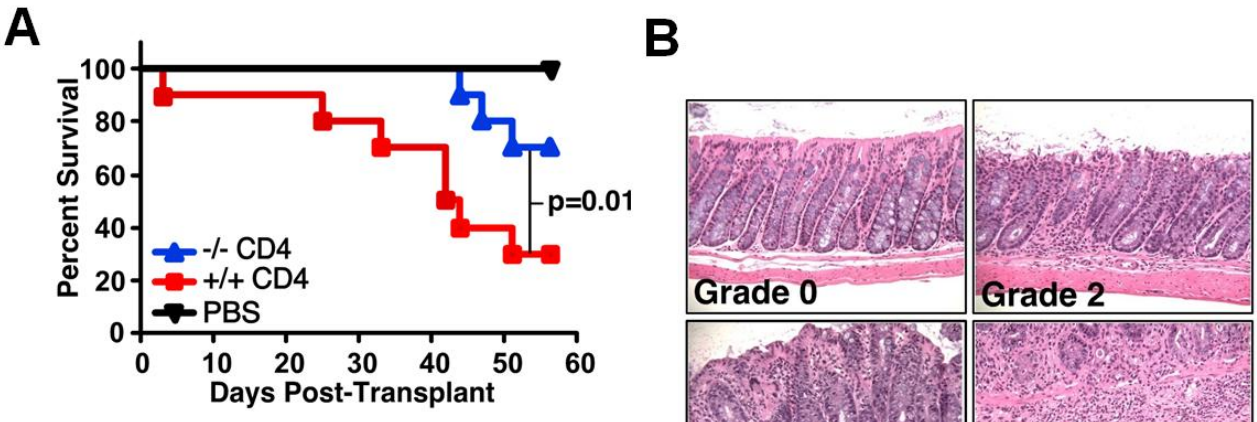
SHIP-Deficient CD4$^+$CD25$^+$ Tregs are as Suppressive as WT Tregs. The altered phenotype described herein suggests that SHIP$^{\Delta IP/\Delta IP}$ Tregs may be more suppressive than WT Tregs. To test this hypothesis *in vitro*, we directly compared the suppressive capacity of conventional SHIP-deficient and WT CD25$^+$ Tregs at different ratios. Multiple comparisons indicated SHIP-deficient Tregs are equally potent at suppressing an MLR compared with WT Tregs on a per-cell basis (Figure 9). To confirm that SHIP-deficient Tregs are as suppressive as WT Tregs, we used the *in vivo* syngeneic colitis model, which assesses the ability of Tregs to control autoreactive T cells. We found that SHIP$^{\Delta IP/\Delta IP}$ CD25$^+$ Tregs can protect C57BL/6J SCID hosts from WT CD25$^-$ T cell–induced colitis just as well as WT CD25$^+$ Tregs, as assessed by weight change and colon histopathology (Figure 10). Weight change analysis showed hosts receiving either WT or SHIP$^{\Delta IP/\Delta IP}$ Tregs, along with the WT effector CD25$^-$ T cells, gained approximately 35% more of their initial weight by the end of the study, similar to the control group that received PBS only (Figure 10A). Hosts receiving WT effector CD25$^-$ T cells gained approximately 10% of their initial weight, significantly less than the hosts receiving PBS or SHIP$^{\Delta IP/\Delta IP}$ or WT Tregs. Comparison of the colon histopathology for hosts in each cohort further supports that those hosts receiving either SHIP$^{\Delta IP/\Delta IP}$ or WT Tregs were equally protected from colitis (Figure 10B). The appearance and health of the colon in these hosts were very similar to that observed for hosts injected with PBS (Figure 10C). The colon histopathology of hosts injected with WT effector CD25$^-$ T cells only was the most severe compared with the hosts of the other cohorts (Figure 10B, C). Thus, SHIP$^{\Delta IP/\Delta IP}$ Tregs appear to have comparable regulatory capacity to their WT counterparts.



Cohort	HPS								
WT CD25- Only	3	4	2	3	4	2	3		
WT CD25- & WT CD25+	0	0	1	0	1	1	2	1	0
WT CD25- & -/- CD25+	2	1	0	0	1	1	0	1	1
PBS	0	0	0	0	0				

Figure 10. SHIP-deficient $CD4^+CD25^+$ Tregs exhibit normal immunoregulatory capacity *in vivo.*
C57BL/6J SCID hosts received $4x10^5$ $CD3^+CD4^+CD25^-$ T cells from WT C57BL/6J donors by intraperitoneal injection on day 1 [n = 7]. Where indicated, the WT effector $CD3^+CD4^+CD25^-$ T cells were coinjected with $7x10^4$ $CD3^+CD4^+CD25^+$ Tregs from WT or $SHIP^{-/-}$ C57BL/6J donors into C57BL/6J SCID hosts [n = 9]. In parallel, a control group of C57BL/6J SCID hosts received a PBS injection [n = 5]. Disease and weight were monitored every other day. (A) Analysis of the rate of weight change over the course of the study (3 months) in the cohorts that received WT effector $CD3^+CD4^+CD25^-$ T cells only (labeled WT $CD25^-$), or effector WT $CD3^+CD4^+CD25^-$ T cells with WT or $SHIP^{-/-}CD3^+CD4^+CD25^+$ Tregs (labeled WT $CD25^-$ & WT $CD25^+$ or WT $CD25^-$ & -/- $CD25^+$, respectively), or PBS. The weight change was determined by converting each actual weight to a percentage of that mouse's initial weight. Each line depicts the average weight change for the specified cohort ($P \leq 0.001$, WT $CD25^-$ vs. WT $CD25^-$ & WT $CD25^+$, WT $CD25^-$ vs. WT $CD25^-$ & -/- $CD25^+$, and for WT $CD25^-$ vs. PBS; $P > 0.1$ for WT $CD25^-$ & WT $CD25^+$ vs. WT $CD25^-$ & -/- $CD25^+$, WT $CD25^-$ & WT $CD25^+$ vs. PBS, and for WT $CD25^-$ & -/- $CD25^+$ vs. PBS). (B) Representative histological appearance of the colon (hematoxylin and eosin, X200) from a mouse in the WT $CD25^-$ (top left; HPS = 3), WT $CD25^-$ & WT $CD25^+$ (top right; HPS = 1), WT $CD25^-$ & -/- $CD25^+$ (bottom right; HPS = 1), and PBS (bottom left; HPS = 0) cohorts. (C) Box and whisker plots and table summarizing the histopathology scores (HPS) given to the hosts within each cohort. The HPS was determined by scoring the most affected area of the proximal colon on a scale of 0 to 4 according to the degree of inflammatory cell infiltration, goblet cell depletion, reactive mucosal epithelial hyperplasia, and thickness of the colon wall, as described further in Materials and Methods. ($P \leq 0.001$, WT $CD25^-$ vs. WT $CD25^-$ & WT $CD25^+$, WT $CD25^-$ vs. WT $CD25^-$ & -/- $CD25^+$, and for WT $CD25^-$ vs. PBS; $P > 0.5$ for WT $CD25^-$ & WT $CD25^+$ vs. WT $CD25^-$ & -/- $CD25^+$)

Evidence for Enhanced T Lymphoid Immune Regulation of Allogeneic Responses *In Vivo*. SHIP-deficient $CD25^-$ T cells exhibited reduced allogeneic T cell responses *in vitro* and approximately 15% of them express FoxP3 (Figure 8-9). Thus, we assessed their capacity to mediate allogeneic responses in an *in vivo* model of GvHD-induced colitis using MHC-mismatched (H2d) $Rag2^{-/-}\gamma c^{-/-}$ hosts[480]. As expected, WT $CD25^-$ T cells mediate robust colitis and lethal GvHD in MHC-mismatched hosts, resulting in only 30% survival, whereas 70% of $Rag2^{-/-}\gamma c^{-/-}$ hosts survived that received an equivalent number of SHIP-deficient $CD25^-$ T cells. This indicates that SHIP-deficient $CD25^-$ T cells have significantly less capacity for lethal GvHD (Figure 11A), consistent with their reduced response in the one-way MLR assay (Figure 9). Mice receiving SHIP-deficient $CD25^-$ T cells also have less evidence of colitis based on both the assessment of clinical symptoms (CAS) and histopathology (HPS) in the colon summarized in the DAI (Figure 11C). Specifically, the severity of colitis was approximately 2-fold less in mice receiving SHIP-deficient $CD4^+$ T cells (average DAI, 2.5) than mice receiving WT $CD4^+$ T cells (average DAI, 4.6). Thus, SHIP-deficiency reduces allogeneic $CD4^+$ T cell responses that can mediate colitis, GvHD, BM graft rejection, and organ graft rejection.



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Group	CAS	HPS	DAI
-/-	1.7	0.8	2.5
+/+	2.7	1.9	4.6
PBS	0	0	0

Figure 11. Reduced alloreactivity of SHIP-deficient $CD4^+CD25^-$ effector T cells *in vivo.*
$Rag2^{-/-}\gamma c^{-/-}$ hosts on an H2d background received $4x10^5$ $CD3^+CD4^+CD25^-$ T cells from $SHIP^{-/-}$ or WT C57BL/6J (H2b) donors by retro-orbital injection on day 1. In parallel, a control group of $Rag2^{-/-}\gamma c^{-/-}$ hosts received a PBS injection. Disease was monitored on a daily basis. Data represent 2 separate studies that were combined, each with an n = 5 per treatment group, resulting in an n = 10 per each treatment group. (A) Kaplan-Meier step functions that show survival for the indicated $Rag2^{-/-}\gamma c^{-/-}$ cohorts [n = 10]. $P \leq 0.01$ for $SHIP^{-/-}$ (labeled -/- CD4) vs. WT $CD25^-$ (labeled +/+ CD4) T cell injected cohorts. (B) Histopathologic appearance (hematoxylin and eosin, x200) of the proximal colon of $Rag2^{-/-}\gamma c^{-/-}$ mice after transfer of $SHIP^{-/-}$ or WT $CD4^+CD25^-$ T cells. These are representative examples that show grading to determine the HPS, using the criteria described in Figure 10. Histology micrographs were taken as described in Figure 10. (C) Table summarizing the assessment of disease in $Rag2^{-/-}\gamma c^{-/-}$ mice cohorts receiving $CD25^-$ T cells from the indicated genotype or sterile PBS control based on the clinical assessment score (CAS), the HPS and the disease activity index (DAI = CAS + HPS). The CAS score is determined on a scale from 0 to 4 according to the occurrence of bristled fur, hunched posture, reduced activity, change in stool consistency, and rectal prolapse as described in Materials and Methods.

To further assess immunoregulatory T cell function *in vivo* in SHIP-deficient hosts, we tested whether induction of SHIP-deficiency in adults could delay or prevent rejection of allogeneic organ grafts, a known function of Tregs[501]. We induced SHIP-

deficiency in MxCreSHIP$^{flox/flox}$ recipients by polyI/C injection. Fourteen days after the first polyI/C injection, vascularized heart grafts from Balb/cJ donors were placed in MxCreSHIP$^{flox/flox}$ and SHIP$^{+/+}$ C57BL/6J cohorts that received the same polyI/C regimen and unmanipulated SHIP$^{flox/flox}$ controls (Figure 12A). As expected with such cardiac allografts[481], the SHIP$^{flox/flox}$ and C57BL/6J control cohorts both rejected the Balb/cJ grafts within 7 days (Figure 12B). However, most MxCreSHIP$^{flox/flox}$ recipients demonstrated a significant delay in rejection, specifically within 9 days ($P < 0.01$; Figure 12B). These findings suggest that SHIP-deficiency induced in the adult transplantation host can delay allogeneic organ graft rejection consistent with the expanded number and function of immunoregulatory T cells in SHIP-deficient hosts.

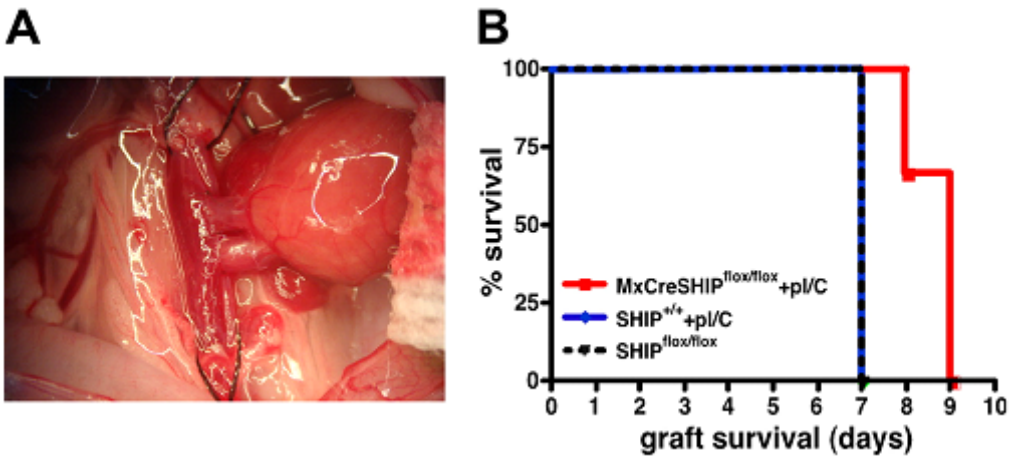

Figure 12. Induced SHIP-deficiency delays rejection of MHC-mismatched, vascularized heart allografts.
(A) Example of an anastomosed heart early after reperfusion. The heart was located in the right lower abdomen of the recipient mouse, and contractions could be palpated through the abdominal wall after closure. (B) Kaplan-Meier step-functions for graft survival in MxCreSHIP$^{flox/flox}$ mice after induction of SHIP-deficiency (red), SHIP$^{flox/flox}$ controls (dashed black) and C57BL/6J mice (blue). The latter group was treated with an identical polyI/C (labeled pI/C) regimen as that given to MxCreSHIP$^{flox/flox}$ mice. $P < 0.01$ for MxCreSHIP$^{flox/flox}$ vs. SHIP$^{flox/flox}$ graft survival. $P < 0.05$ for MxCreSHIP$^{flox/flox}$ vs. C57BL/6J graft survival.

Discussion

Here we provide evidence that germline or induced systemic SHIP-deficiency promotes an increased frequency of $CD25^{+}FoxP3^{+}$ T cells and $CD25^{-}FoxP3^{+}$ Tregs in secondary lymphoid tissues. Furthermore, we find that SHIP-deficiency promotes a significant increased expression or representation of CD103, GITR, and OX40, markers associated with Tregs, and FcγRII/III among $CD25^{+}$ Tregs and $CD25^{-}$ T cells. SHIP-deficiency does not compromise Treg function because SHIP-deficient Tregs are as suppressive as WT Tregs. Finally, SHIP-deficient $CD25^{-}$ T cells are unresponsive to allogeneic stimulus *in vitro* and *in vivo* and suppress allogeneic T cell responses *in vitro*. Because FoxP3 expression in murine T cells confers suppressive capacity[60], it is probable that the $FoxP3^{+}$ subset among SHIP-deficient $CD25^{-}$ T cells is responsible for the observed immunosuppressive capacity of SHIP-deficient $CD25^{-}$ T cells *in vitro* and reduced GvHD *in vivo*.

The increased representation of $FoxP3^{+}$ Treg populations within $CD25^{+}$ and $CD25^{-}$ T cell compartments could be due to by several extrinsic or intrinsic effects caused by SHIP-deficiency. SHIP-deficiency in T-lineage cells could alter intracellular signaling pathways important in thymic selection or peripheral differentiation or survival. Alternatively, SHIP-deficiency could promote an immunosuppressive environment that preferentially promotes the generation, expansion, and/or survival of Tregs. Tarasenko *et al*[339] found that T cell–specific SHIP-deficiency does not promote the increased development or representation of $FoxP3^{+}$ Tregs in the thymus or in peripheral lymphoid tissues. Furthermore, they showed that SHIP does not regulate signaling through the

TCR. These results, when paired with our findings, suggest that SHIP-deficiency promotes Treg expansion via a mechanism that is extrinsic to $CD4^+$ T cells, although the possibility exists that both a SHIP-deficient environment and a SHIP-deficient T cell are required for the increased accumulation of Tregs that we observe.

Here we show that the SHIP-deficient environment promotes the expression of receptors, CD103, GITR, and OX40, which have been associated with Treg function as well as with activated T cells. Because of this, others have concluded that T cells exist in an activated state in SHIP-deficient mice[354]. The data presented in this study further characterize T cells in SHIP-deficient mice, suggesting that expression of these receptors may be representative of an activated/effector T cell that also has immunosuppressive behavior. Although Tregs can suppress in an antigen nonspecific manner, Tregs must undergo antigen-specific activation to suppress[502]. Thus, SHIP-deficient Tregs in both $CD25^+$ and $CD25^-$ compartments may have an increased probability of suppressing other T cells because they exist in larger numbers and a larger proportion of them exist in an activated state. Although we found that SHIP-deficient Tregs are as equally potent at suppressing effector $CD4^+$ T cells as are WT Tregs, SHIP-deficient Tregs may still have more potent suppressive activity when in a SHIP-deficient environment. In addition, as done by Lehmann *et al*[491], the number of Tregs coinjected along with WT effector $CD25^-$ T cells can be titrated to compare the smallest ratio of either SHIP-deficient or WT Tregs to effector T cells at which protection from colitis is compromised or lost. Regardless, we show that SHIP-deficient Tregs are at least as suppressive as WT Tregs as assayed *in vitro* and *in vivo*.

In WT mice, GITR expression in the $CD25^-$ and $CD25^+$ T cell compartments represents immunoregulatory T cells capable of preventing autoimmune myocarditis, multiorgan inflammation, and murine inflammatory bowel disease.[497] These studies

propose that GITR expression may be a better Treg surface marker than CD25. Similarly, in WT mice, $CD25^-$ and $CD25^+$ T cells that express CD103 also represent a Treg subset capable of protecting mice from colitis in the SCID model *in vivo*[491]. Coincidentally, most $GITR^+$ and $CD103^+$ T cells within $CD25^+$ and $CD25^-$ $CD4^+$ T cell compartments in SHIP-deficient mice also coexpress FoxP3. When using GITR or CD103 in addition to FoxP3, instead of CD25 and FoxP3, as the markers to determine the frequency of Tregs, there is a more pronounced increase in the representation of $CD4^+GITR^+FoxP3^+$ Tregs or $CD4^+CD103^+FoxP3^+$ Tregs than of $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral lymphoid organs of SHIP-deficient mice compared with that in WT mice (data not shown).

As mentioned, both GITR and CD103 expression identifies a immunoregulatory T cell subset within the $CD25^-$ T cell compartment, although OX40 has not been shown to do so. Streeter *et al* demonstrated that $CD4^+CD25^-OX40^+$ T cells are proliferative on alloantigen stimulation whereas $CD4^+CD25^+OX40^+$ T cells are suppressive.[503] In SHIP-deficient mice, even though there is approximately a 6-fold increase in the representation of $CD25^-OX40^+$ T cells compared with WT littermates, the SHIP-deficient $CD25^-$ T cell compartment as a whole was not more proliferative on alloantigen stimulation as demonstrated *in vitro* and *in vivo*. Indeed, almost half of the $CD25^-OX40^+$ T cells in SHIP-deficient mice are also $FoxP3^+$, whereas only approximately one-fourth of WT $CD25^-OX40^+$ T cells are $FoxP3^+$ (data not shown). Thus, in SHIP-deficient mice, the expression of OX40 among $CD25^-$ T cells is more closely associated with T cells with immunoregulatory capacity instead of T cells capable of alloantigen-induced proliferation.

Engagement of costimulatory and cytokine receptors, such as OX40, GITR, CD103, IL-2R, and IL-7R, has been shown to confer survival and proliferative signals.[494,

[504-506] The increased density and expression levels of these markers on $CD25^-$ and $CD25^+$ Tregs in SHIP-deficient mice may thus provide a survival advantage. SHIP is known to oppose the activation of the PI3K pathway that can be activated by IL-2R, IL-7R, or OX40 signaling[504, 507]. Indeed, inhibition of PI3K signaling prevents IL-2 and other common gamma chain cytokines, IL-7 and IL-15, from supporting maximal suppression by Tregs[507]. Thus, the survival and proliferative signals emanating from these receptors may be stronger when SHIP is not present to oppose PI3K. The possibility of enhanced signaling from these receptors in combination with their increased surface expression could contribute to the increased representation of Tregs in peripheral lymphoid organs of SHIP-deficient mice.

SHIP is also known to regulate signals emanating from both FcγRIIb, an inhibitory receptor, and FcγRIIIa, an activating receptor[280, 508]. FcγR family members recognize the immunoglobulin Fc portion of antibodies, allowing free antibodies, immune complexes, and/or opsonized cells to fine-tune decisions between activation and suppression of an immune response. FcγRs are expressed by almost all types of hematopoietic cells. Previous studies examining whether T cells express FcγRs or not have been contradictory[500]. Here we show that T cells in WT mice do express low levels of FcγRIIb/FcγRIIIa. Intriguingly, SHIP-deficient $CD25^-$ T cells and $CD25^+$ Tregs express FcγRIIb/FcγRIIIa at significantly higher levels than their WT counterparts. This suggests that SHIP-deficient $CD4^+$ T cells may be responsive to antibody, immune complexes and/or opsonized cells as SHIP is known to limit signals from both of these FcγRs[280, 508]. Further analysis is needed to distinguish whether unopposed signals from these FcγRs occur in SHIP-deficient T cells and promote the preferable expansion of $CD25^-FoxP3^+$ T cells and $CD25^+FoxP3^+$ Tregs. Consistently, serum levels of certain

IgG isotypes are increased in SHIP$^{-/-}$ mice, providing increased ligands for deregulated FcγRs present on SHIP$^{-/-}$ CD4^{+} T cells[509].

In addition to the increased expression of costimulatory molecules on Tregs, a SHIP-deficient environment promotes other immunologic changes that promote the accumulation of Tregs as well as protect against GvHD, specifically increased MDSC numbers,[341, 345] and increased granulocyte colony-stimulating factor (G-CSF) expression[356]. G-CSF promotes an immunosuppressive environment and thus protects against GvHD via many mechanisms. Importantly, one study showed that *in vivo* G-CSF exposure promotes the acquisition of Treg properties by naive CD4^{+} T cells after T cell receptor ligation *in vitro*[510]. Consistently, donors treated with pegylated G-CSF exhibited an increased generation of IL-10 producing Tregs and transplantation tolerance[511]. Furthermore, MacDonald *et al* showed that G-CSF and derivatives protected against GvHD by promoting the expansion of a CD11b^{+}Gr1^{+} subset, similar to MDSC, that mediate the expansion of IL-10 secreting Tregs[487]. This mechanism is plausible in a SHIP-deficient host, which is protected from GvHD, where increased G-CSF levels promote the expansion of MDSC that in turn mediate expansion of the peripheral Treg compartment. Thus, SHIP may play both intrinsic and extrinsic roles to limit the accumulation of Tregs.

The *in vivo* models described here provide further support that targeting SHIP could facilitate transplantation across MHC barriers. We show that, in addition to the MDSC expansion, SHIP-deficiency promotes the accumulation of Tregs, which are known to play a pivotal role in transplantation immunology[159, 512]. In a model of GvHD-induced colitis, we show that isolated SHIP-deficient CD25^{-} T cells inefficiently mount an immune response to cause colitis. This outcome can be mediated by one of the following mechanisms. First, because SHIP-deficient CD25^{-} T cells could not cause

colitis in a $Rag2^{-/-}\gamma c^{-/-}$ mouse, which is not SHIP deficient, SHIP may play an intrinsic role in T cells that allows robust participation in allogeneic responses. Alternatively, the SHIP-deficient environment could have led to the irreversible differentiation of some or all of the SHIP-deficient $CD25^{-}$ T cells into an unresponsive and/or immunosuppressive cell. Consistently, two important observations can also be made from the vascularized heart transplantation model. First, a significant increase in Treg and MDSC accumulation is observed in adult mice after only 8 days of induced SHIP-deficiency (data not shown). Second, induced SHIP-deficiency in adulthood promotes a significant delay in graft rejection. This study further characterizes the cells that make up the immunosuppressive environment promoted by SHIP-deficiency, providing further support for targeting of SHIP to improve allogeneic transplantation procedures.

When considering cancer treatment, BMT can be employed to treat patients with certain forms of cancer, such as leukemia, lymphoma and multiple myeloma. In addition, BMT can be used after chemotherapy and radiation, which can effectively kill cancer cells but also destroy the patient's bone marrow. Though, BMT exhibit significant clinical disadvantages, which can be fatal, primarily mediated by host versus graft and graft versus host immune responses. Thus, improving its efficacy, especially across MHC barriers, would greatly impact the applicability and feasibility of BMT in cancer treatment.

Chapter 4. SHIP has Lineage Extrinsic and Intrinsic Control on the Accumulation of Immunoregulatory Cells

Introduction

A role for SHIP in limiting immunoregulatory processes was initially revealed when it was found that a SHIP-competent, T cell replete BM graft failed to mount a robust GvH response in MHC-mismatched, SHIP-deficient hosts resulting in improved transplant survival[325]. We subsequently found that the number of MDSC[341, 345] and Tregs[482] were profoundly increased in secondary lymphoid tissues of SHIP$^{-/-}$ mice and after ablation of SHIP expression in adult MxCreSHIP$^{flox/flox}$ mice. GvHD and organ graft rejection are primed by host APC present in secondary lymphoid organs[513-516]. MDSC and Tregs can antagonize this process resulting in reduced GvHD[158, 341, 345, 517] or acceptance of allogeneic organ grafts[484]. Thus, a more complete understanding of how SHIP limits the numbers of these two key immunoregulatory cells *in vivo* might have important implications for clinical transplantation.

Several different hematolymphoid defects have been reported in SHIP-deficient mice[325, 338, 340, 343, 344, 518-520]. In several cases these genetic phenotypes have been shown to result from a cell autonomous role for SHIP in the affected cell type[336, 485, 519-521]. However, instances have been documented where altered function on the part of a SHIP-deficient cell type impairs the function or development of a different cell type or lineage[356, 522]. This appears to be due in large part to the altered production of key cytokines, growth factors or chemokines that occurs in SHIP-deficient mice. SHIP$^{-/-}$ mice

exhibit profoundly increased production of IL-6[519] and G-CSF[356] and greatly diminished SDF1/CXCL12 production[356], all of which can contribute to altered function or localization of other cells, as seen specifically with HSC[356, 523, 524]. The cellular and molecular basis of over- or under-production of soluble factors in SHIP$^{-/-}$ mice remains to be undefined, although studies with SHIP$^{-/-}$ mast cells, for example, indicate hyper-activation of NF-κB contributes to their increased production of IL-6[519].

Studies have revealed that as anticipated, the primary role of SHIP in cell signaling is recruitment to receptor-signaling complexes where it can then oppose activation of PI3K effector kinases such as Akt by hydrolysis of the PI3K substrate, PI(3,4,5)P_3[325, 343, 525]. However, SHIP can also, in certain signaling contexts, 'mask' cytoplasmic motifs on certain receptors and in so doing prevent the inappropriate recruitment of other phosphatases (for example SHP1) or PI3K[336]. This non-enzymatic role should be considered when attempting to decipher the role SHIP plays in signaling by a given receptor. Also, although SHIP is largely considered to only exert a negative impact on cell function, survival or proliferation, in some contexts, SHIP can also promote cellular functions. For example, analysis of NK cells in SHIP$^{-/-}$ mice indicated that SHIP is essential for these cells to perform efficient target cytolysis and secretion of γ-IFN[325, 336, 485, 526], the two major NK effector functions. Consistently, recent reports have found that SHIP promotes macrophage effector function[301] and cancer cell survival[527] by synthesis of its product, PI(3,4)P_2, which is known to recruit GTPase Irgm1[301]and activate Akt[527-529].

We have shown that in SHIP-deficient hosts, a significant number of T cells of the naïve phenotype CD4$^+$CD25$^-$ express FoxP3 and are suppressive, suggesting T-lineage intrinsic control of FoxP3 expression and suppressive function by SHIP. Uncertainty remains as to how SHIP-deficiency promotes the expansion of these

$CD4^+CD25^-FoxP3^+$ T cells, as well as of conventional $CD4^+CD25^+FoxP3^+$ Tregs, and MDSC numbers. In the present study, we examine this question further and find evidence for lineage intrinsic control of MDSC numbers and function and that SHIP's control of G-CSF production plays a role in this regulation. However, SHIP exerts control over $CD4^+CD25^-FoxP3^+$ T cells and conventional $CD4^+CD25^+FoxP3^+$ Treg numbers through both lineage extrinsic and intrinsic mechanisms.

Results

Myeloid-Restricted Ablation of SHIP Expression Increases the Frequency of MDSC and Tregs in Peripheral Lymphoid Tissues. Several groups have used the LysCre transgenic mouse to create myeloid-restricted deletion of floxed gene loci[347]. We generated $LysCreSHIP^{flox/flox}$ mice to determine if myeloid-restricted deletion of SHIP would lead to increased numbers of MDSC in peripheral lymphoid tissues and, in turn, promote the expansion of Tregs. $LysCreSHIP^{flox/flox}$ mice appeared to have an essentially normal life span as they typically live well over a year with no apparent health complications as opposed to germline $SHIP^{-/-}$ mice that typically succumb within 6-10 weeks of life[344, 345]. $LysCreSHIP^{flox/flox}$ mice also failed to develop the myeloproliferative disease (MPD) reported in germline $SHIP^{-/-}$ and $MxCreSHIP^{flox/flox}$ mice[341, 344] as MPD-associated splenomegaly in $LysCreSHIP^{flox/flox}$ mice was not observed (data not shown).

Analysis of SPL and LN showed that the frequency of MDSC was significantly increased in both tissues in $LysCreSHIP^{flox/flox}$ mice relative to $SHIP^{flox/flox}$ controls (Figure 13A). This increase was not pan-lineage, as developing myeloid cells that possess the

same $CD11b^{+}Gr1^{+}$ phenotype as MDSC in the periphery, were not significantly increased in the BM of $LysCreSHIP^{flox/flox}$ relative to $SHIP^{flox/flox}$ littermates (Figure 13B).

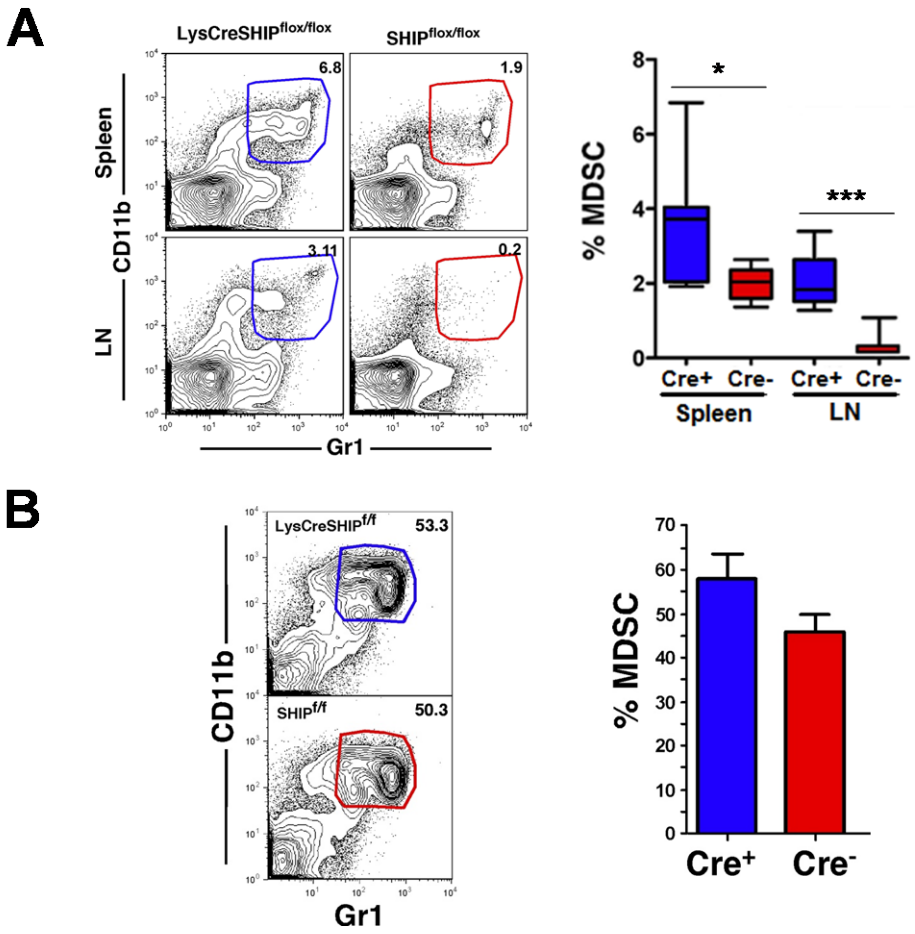

Figure 13. The frequency of myeloid immunoregulatory cells is increased in mice with myeloid-restricted ablation of SHIP expression.
(A) Representative contour plots of CD11b vs. Gr1 staining of viable SPL and LN cells from $LysCreSHIP^{flox/flox}$ (labeled as Cre^{+}) and $SHIP^{flox/flox}$ controls (labeled as Cre^{-}). The percentage of $CD11b^{+}Gr1^{+}$ MDSCs in spleen and LN from $LysCreSHIP^{flox/flox}$ (Cre^{+}) and $SHIP^{flox/flox}$ controls (Cre^{-}) are shown as box-and-whisker plots representing median with the maximum and minimum value range of n=12/genotype. (B) Representative FACS contour plots for CD11b vs. Gr1 staining of BM cells from Cre^{+} and Cre^{-}. Quantitation of immature $CD11b^{+}Gr1^{+}$ myeloid in the BM of Cre^{+} and Cre^{-} controls. Data are shown as a bar graph representing the mean + SEM of n=4/genotype. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

In addition, a significant decrease in the frequency of $CD3^+$ T lymphocytes in SPL and LN (Figure 14A) was observed, although the absolute number of $CD3^+$ T cells did not change (Figure 14B). In change, the frequency and absolute number of Tregs was significantly increased in both tissues (Figure 14D-F). We confirmed that the T cell compartment in $LysCreSHIP^{flox/flox}$ mice expressed normal levels of SHIP protein (Figure 14C). Thus, the Treg increase cannot be attributed to lineage-inappropriate ablation of SHIP expression in $LysCreSHIP^{flox/flox}$ mice.

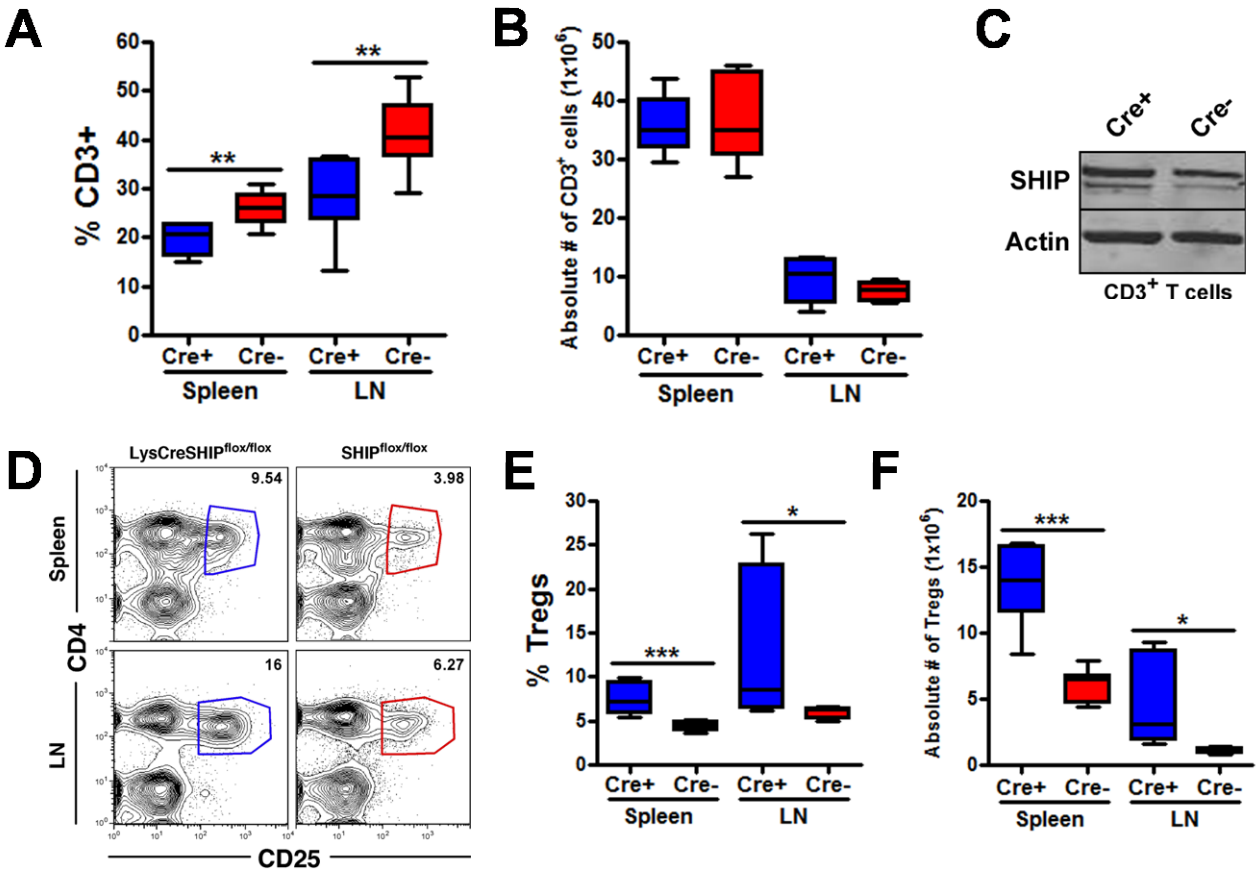

Figure 14. The frequency of T lymphoid immunoregulatory cells is increased in mice with myeloid-restricted ablation of SHIP expression.
(A, B) The percentage (A) and absolute number (B) of $CD3^+$ T cells in spleen and LN from Cre^+ and Cre^- as indicated are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=8/genotype. (C) Western blot analysis of SHIP expression in $CD3^+$ T cells sorted from Cre^+ mice and Cre^- controls. (D)

Representative contour plots of CD4 vs. CD25 staining of viable $CD3^+$ splenocytes and LN cells from Cre^+ and Cre^- controls. (E, F) The percentage (E) and absolute number (F) of $CD4^+CD25^+FoxP3^+$ Treg cells in spleen and LN as indicated are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=8/genotype. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

The increase in frequency in both myeloid and T lymphoid immunoregulatory cell numbers observed was physiologically relevant, as both $LysCreSHIP^{flox/flox}$ splenocytes (Figure 15A) and LN cells (Figure 15B) exhibited significantly reduced capacity for priming of allogeneic T cell responses by MHC-mismatched responder cells relative to SHIP-competent $SHIP^{flox/flox}$ controls. A similar increase in the immunoregulatory capacity of peripheral lymphoid organs was also observed in germline $SHIP^{-/-}$ mice[345], $MxCreSHIP^{flox/flox}$ mice after genetic ablation[341] and following treatment with a SHIP1 inhibitor[527]. As anticipated, myeloid-restricted ablation of SHIP expression in $LysCreSHIP^{flox/flox}$ mice promoted an expansion of the MDSC compartment in secondary lymphoid tissues demonstrating that lineage intrinsic control of the production and/or survival of MDSC is regulated by SHIP. The increased Treg frequency also observed in these tissues indicates that a myeloid cell that expresses SHIP limits the frequency of Tregs in peripheral lymphoid tissues in a lineage extrinsic fashion. Following cytokine administration or tumor formation, others have previously reported MDSC can induce a significant increase in peripheral Treg numbers[233, 487]. Our findings demonstrate SHIP also limits this function of the myeloid compartment.

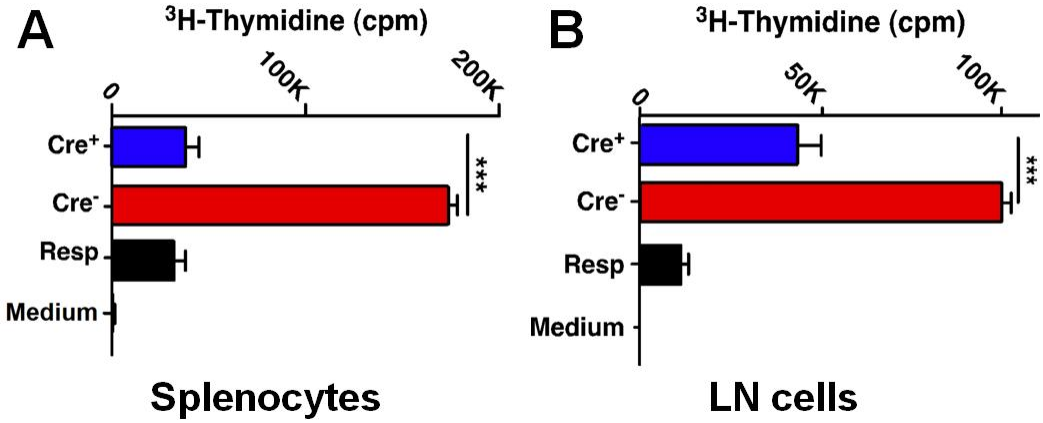

Figure 15. Peripheral lymphoid cells from mice with myeloid-restricted SHIP-deficiency exhibit reduced capacity for priming allogeneic T cell responses. (A,B) One-way MLR analysis of allogeneic T cell (BALB/c) priming by irradiated Cre^+ or Cre^- splenocytes (A) and LN (B) cells. Data are shown as mean + SEM of n=4. These results are representative of two independent experiments for both tissues. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

MDSC Isolated from Naïve or Tumor-Bearing Mice Lack Detectable Expression of SHIP Protein.

The expansion of MDSC observed in LysCreSHIP$^{flox/flox}$ mice suggests that SHIP expression in these cells might oppose signals in these cells that limit their proliferation and/or survival *in vivo*. As an initial test of this hypothesis, sorted MDSC from SHIP$^{+/+}$ SPL were analyzed for SHIP expression by Western blot (Figure 16A). Surprisingly, SHIP protein expression was essentially undetectable in naïve MDSC purified from SPL. In addition, conventional MDSC purified from the SPLs and from the tumors of mice bearing MC-38 tumors (Figure 16A) were analyzed. MC-38 tumors are known to harbor large numbers of potent MDSC[530]. As with naive MDSC, these tumor-promoted MDSC also lacked detectable expression of SHIP. These findings indicate that MDSC expansion in LysCreSHIP$^{flox/flox}$ mice is unlikely to be the result of cell-autonomous signaling by SHIP in MDSC since they lack expression of SHIP. Thus, control of MDSC formation and/or survival by SHIP must be mediated by

another cell type within the myeloid lineage that expresses SHIP. SHIP protein levels in immature $CD11b^{+}Gr1^{+}$ cells present in BM, as well as naïve T cells, Tregs and $CD8^{+}$ T cells from normal mice were further examined (Figure 16B). Although all T cell lineages express SHIP, the immature myeloid cells in BM also lacked detectable expression of SHIP.

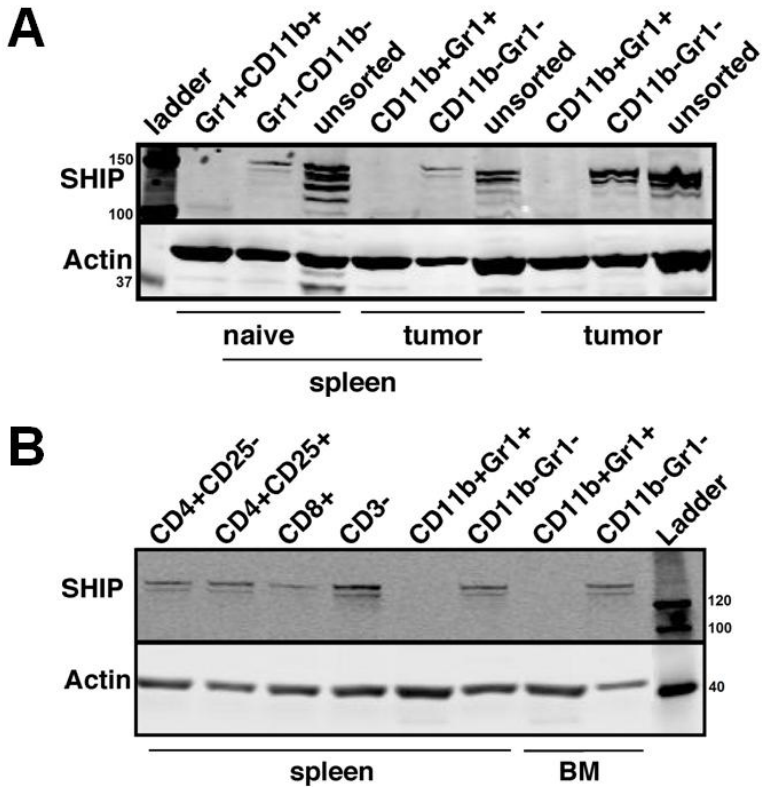

Figure 16. MDSC purified from either naïve or tumor-bearing mice do not express SHIP.
(A) Western blot analysis of SHIP and β-Actin expression in whole cell lysates prepared from sorted $CD11b^{+}Gr1^{+}$ cells from SPL of naïve mice, SPL of mice bearing MC-38 tumors or directly from MC-38 tumors harvested from C57BL/6J hosts. (B) Western blot analysis of SHIP and β-Actin expression in whole cell lysates prepared from the indicated FACS sorted cell populations prepared from the SPL or BM of naive C57BL/6J mice. **Note:** SHIP protein products commonly exhibit size variations with the longest isoform being SHIPα (145kDa), followed by SHIPβ (135kDa), SHIPγ (125kDa), and SHIPδ (110kDa)[310]

T Cell-Restricted Mutation of SHIP Leads to Expansion of Treg Numbers, but not MDSC. Efficient ablation of floxed loci in a T-lineage restricted fashion can be achieved with a Cre recombinase transgene driven by the Lck proximal promoter[531-534]. LckCreSHIP$^{flox/flox}$ mice were thus developed to assess whether T-lineage restricted ablation of SHIP expression also promotes expansion of Tregs and whether these Tregs might in turn facilitate an increase in the frequency of MDSC in peripheral lymphoid tissues. As anticipated, the T cell compartment in LckCreSHIP$^{flox/flox}$ mice exhibited significantly reduced expression of SHIP expression in T-lineage cells (Figure 17A). We found a significant reduction in the frequency of splenic CD3^{+} T cells (Figure 17B) and of CD4^{+} and CD8^{+} T cells in the peripheral blood of LckCreSHIP$^{flox/flox}$ mice relative to SHIP$^{flox/flox}$ controls (Figure 17D). The frequency of CD3^{+} T cells in LN was unchanged relative to SHIP$^{flox/flox}$ littermates (Figure 17B). Additionally, the absolute number of CD3^{+} T cells does not change in the SPL and LN of LckCreSHIP$^{flox/flox}$ mice relative to SHIP$^{flox/flox}$ controls (Figure 17C). In spite of this decrease in total splenic and circulating T cells in both the CD4 and CD8 lineages, a significant increase in the frequency and absolute number of Tregs in both the SPL and LN of LckCreSHIP$^{flox/flox}$ mice relative to SHIP$^{flox/flox}$ controls was observed (Figure 17E).

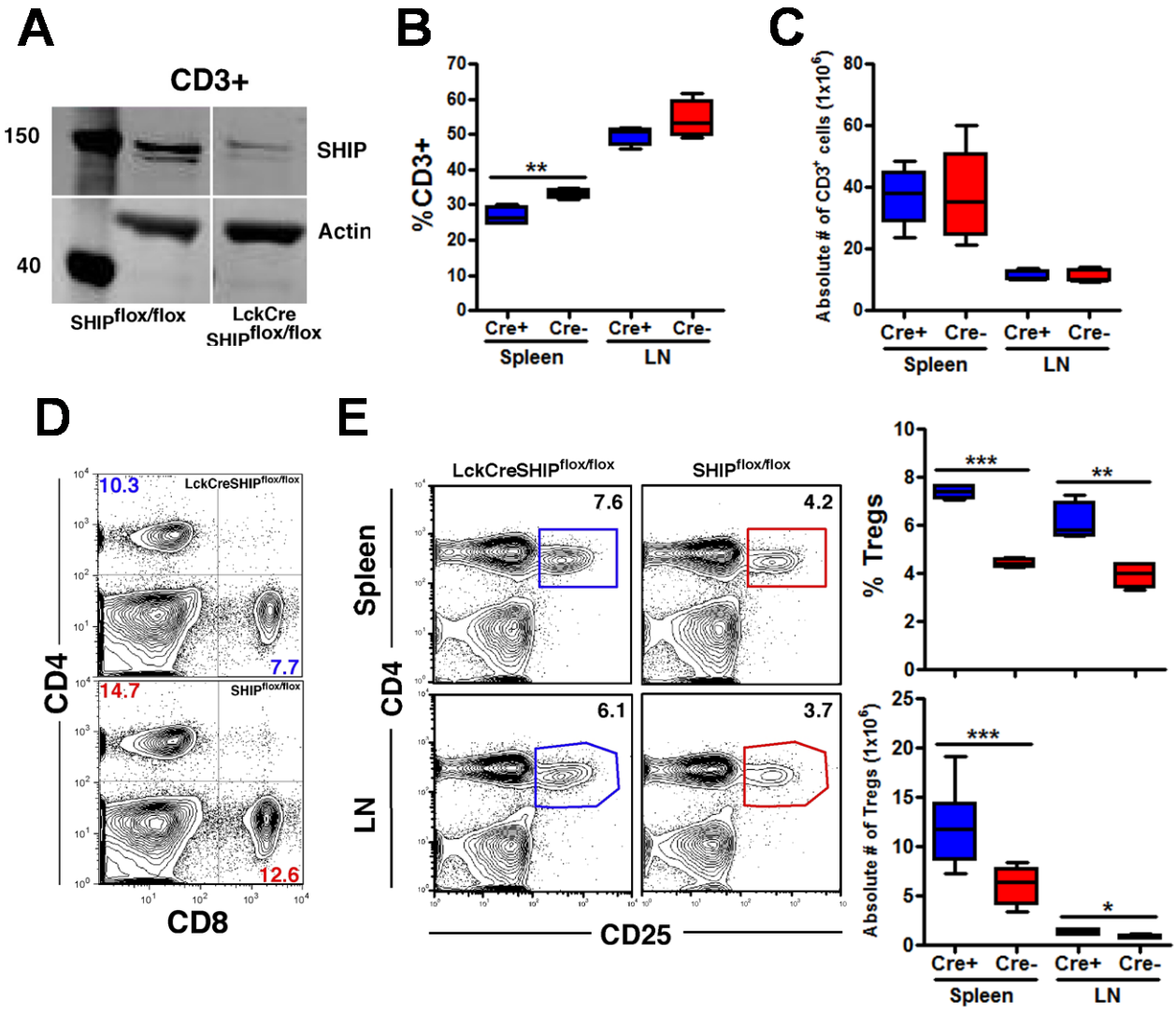

Figure 17. T cell-specific SHIP-deficiency promotes increased Treg numbers in peripheral lymphoid tissues.
(A) Western blot analysis of SHIP expression in $CD3^+$ T cells sorted from the indicated genotypes. (B,C) The percentage (B) and absolute number (C) of $CD3^+$ T cells in spleen and LN from $LckCreSHIP^{flox/flox}$ (Cre^+) and $SHIP^{flox/flox}$ (Cre^-) controls are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=6/genotype. (D) Representative contour plots for CD4 vs. CD8 staining of viable PBMC for the indicated genotypes (E) Representative contour plots for CD4 vs. CD25 staining of viable SPL and LN cells for the indicated genotypes. The percentage and absolute number of $CD4^+CD25^+FoxP3^+$ Treg cells in spleen and LN of the indicated genotype are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=6/genotype. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)

In addition, there was a significant increase in the number of T cells of the naïve $CD4^+CD25^-$ phenotype that expressed FoxP3 (Figure 18). These results indicate SHIP limits the expression of FoxP3 by $CD4^+CD25^-$ T cells and formation of $CD4^+CD25^+$ Tregs in a T-lineage intrinsic fashion.

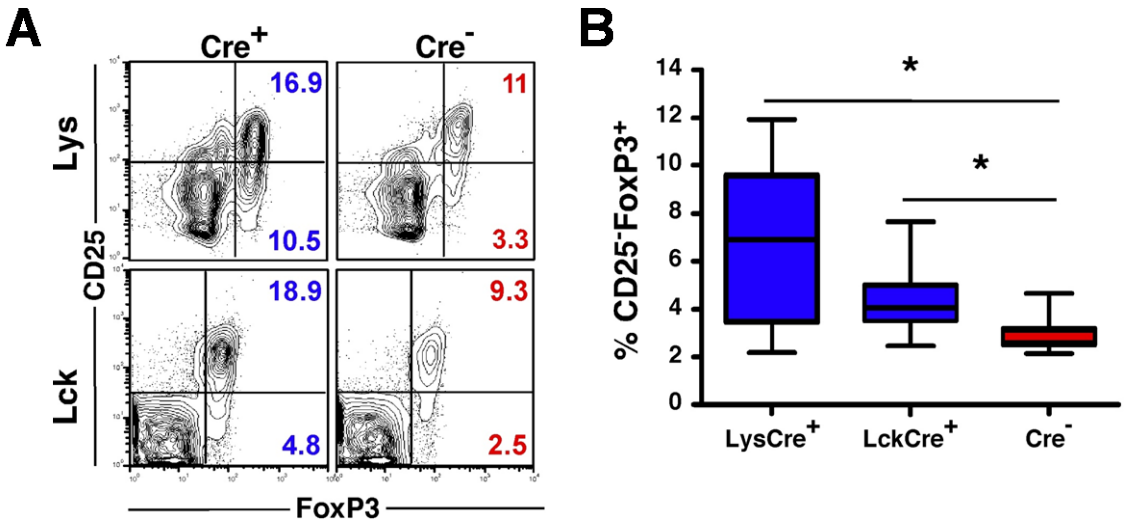

Figure 18. T cell-specific SHIP-deficiency promotes increased numbers of $CD4^+CD25^-FoxP3^+$ T cells in peripheral lymphoid tissues.
(A) Representative contour plots of intracellular FoxP3 vs. CD25 staining for splenic $CD4^+$ T cells of LysCreSHIP$^{flox/flox}$ (Lys Cre^+) or LckCreSHIP$^{flox/flox}$ (Lck Cre^+) and their respective littermate SHIP$^{flox/flox}$ controls (Cre^-) (B) The percentage of $CD4^+CD25^-FoxP3^+$ cells among splenic $CD4^+$ T cells for the indicated genotype are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=8 for LysCre+, n=6 for LckCre+ and n=14 for Cre-. (*$P \leq 0.05$)

To determine if SHIP expression by a myeloid lineage cell might also limit expression of FoxP3 expression by $CD4^+CD25^-$ T cells, the same analysis in LysCreSHIP$^{flox/flox}$ mice and SHIP$^{flox/flox}$ littermate controls were performed, which showed that myeloid-restricted SHIP-deficiency also promoted FoxP3 expression by $CD4^+CD25^-$ T cells (Figure 18). This suggests that SHIP limits conversion of naïve T cells to $FoxP3^+$ Tregs or FoxP3 expression in both a T-lineage intrinsic and extrinsic fashion, with the

latter regulation pathway mediated by SHIP-expressing myeloid cell. However, this increased frequency of FoxP3^{+} CD4^{+}25^{-} T cells and CD4^{+}CD25^{+} Tregs in LckCreSHIP$^{flox/flox}$ mice did not promote a corresponding increase in MDSC frequency in peripheral lymphoid tissues (Figure 19). Thus, the ability of SHIP-deficient immunoregulatory myeloid and T lymphoid cells to influence each other's homeostasis and formation is unidirectional, with only SHIP-deficient myeloid lineage cells capable of *trans-lineage* control of immunoregulatory cell numbers in another lineage.

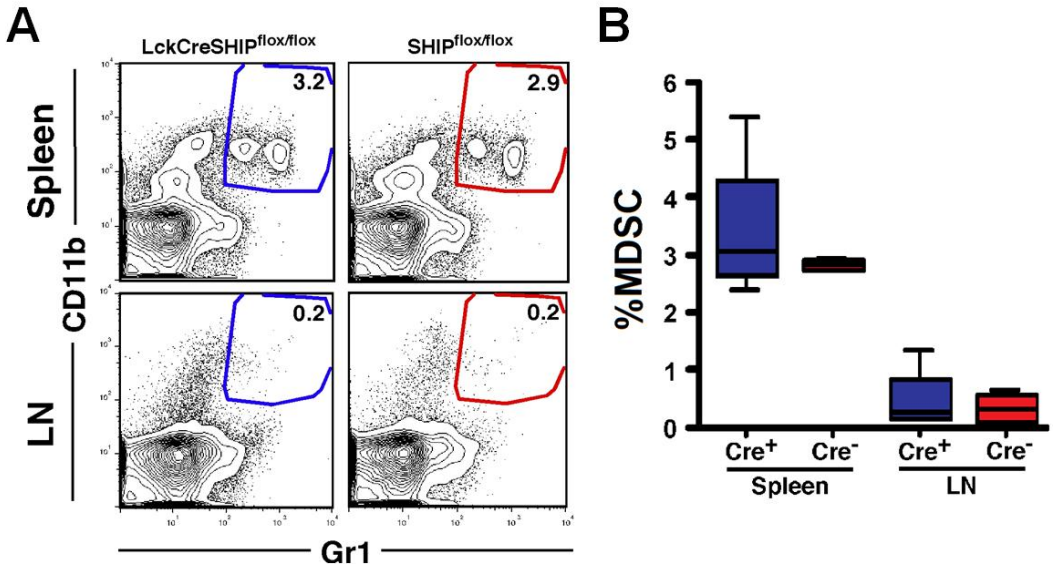

Figure 19. T cell-specific SHIP-deficiency does not affect MDSC numbers in peripheral lymphoid tissues.
(A) Representative contour plots of CD11b vs. Gr1 staining of viable SPL and LN cells from mice of the indicated genotype. (B) The percentage of CD11b^{+}Gr1^{+} MDSCs in spleen and LN of the indicated genotype are shown as box-and-whisker plots representing the median with the maximum and minimum value range of n=6/genotype.

G-CSF Promotes Expansion of MDSC in Mice Rendered SHIP-Deficient. We previously showed that there is a profound increase in production of the myelopoietic growth factor G-CSF in SHIP$^{-/-}$ mice[356]. This myelopoietic growth factor might promote

the MDSC expansion and consequently expansion of Treg numbers. To test this, mice were treated with neutralizing anti-G-CSF antibodies for one week after ablating SHIP expression using the MxCreSHIP$^{flox/flox}$ mouse model[341]. Consistent with this hypothesis, anti-GCSF treatment prevented the expansion of splenic MDSC after induction of SHIP-deficiency (Figure 20A, B). However, the splenic Treg expansion was still observed (Figure 20C, D). Thus, expansion of MDSC in the SPL, but not in the LN of SHIP-deficient mice (data not shown) is dependent upon increased G-CSF production. However, the Treg expansion that occurs in peripheral lymphoid tissues following induction of SHIP-deficiency in adults[482] is not prevented by G-CSF neutralization and thus is not dependent upon increased G-CSF production or expanded numbers of MDSC. In MxCreSHIP$^{flox/flox}$ mice where SHIP-deficiency is systemic, the Treg expansion likely results from the T-lineage intrinsic effects of SHIP-deficiency, increased production of another soluble or cell-bound ligand expressed by SHIP-deficient myeloid cells that is required for Treg expansion, but which does not impact MDSC numbers.

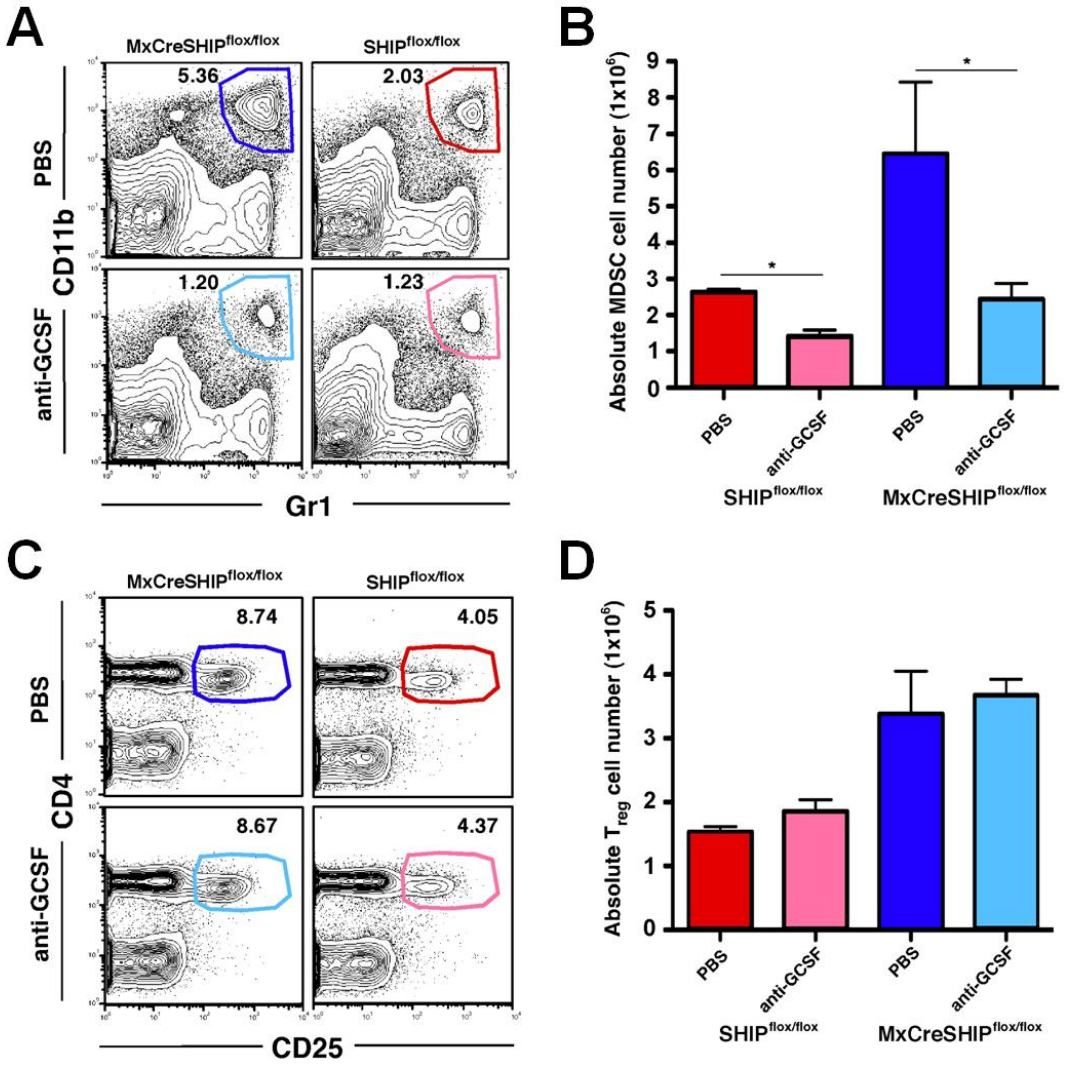

Figure 20. G-CSF is required for the expansion of splenic MDSC numbers, and not of Treg numbers, in SHIP-deficient hosts.
(A) Representative contour plots of CD11b vs. Gr1 staining of viable splenocytes from poly-I/C treated MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ mice after administration of anti-G-CSF or PBS as indicated. (B) Bar graphs indicating the absolute number of CD11b^{+}Gr1^{+} MDSC in SPL of mice of the indicated genotype and treatment. (C) Representative contour plots of CD4 vs. CD25 staining of viable splenocytes from mice as in (A). (D) Bar graphs indicating the absolute number of Tregs in SPL of mice of the indicated genotype and treatment. All data are shown as mean + SEM of n=8 and are pooled from 2 experiments. (*$P \leq 0.05$)

G-CSF can also suppress SDF-1/CXCL12 production by osteoblasts, which is critical for their support of quiescent HSC in the endosteal BM niche[535-537]. We previously found that BM niche expression and plasma levels of SDF1/CXCL12 are substantially diminished in SHIP$^{-/-}$ mice and MxCreSHIP$^{flox/flox}$ mice following ablation of SHIP expression[356]. G-CSF neutralization might then prevent suppression of SDF-1/CXCL12 production following induction of SHIP-deficiency. However, suppression of SDF-1/CXCL12 following induction was not prevented by G-CSF neutralization (Figure 21). These results indicate suppression of SDF-1/CXCL12 production following SHIP-deficiency is not a result from increased G-CSF production and may reflect a direct role for SHIP in promoting expression of SDF1/CXCL12.

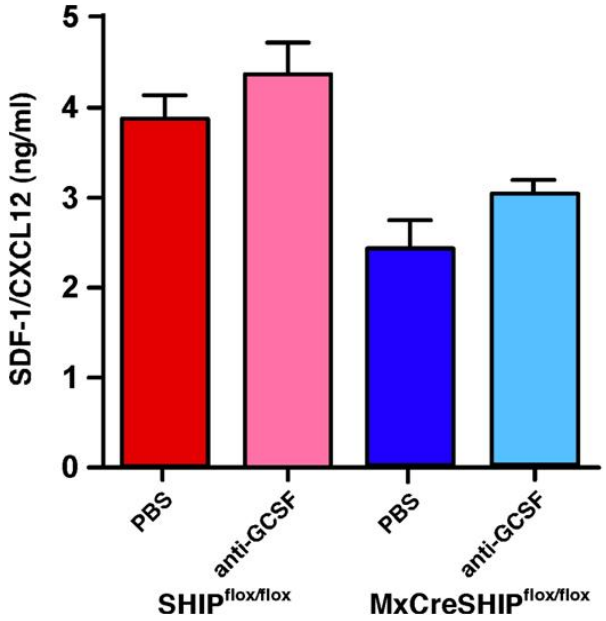

Figure 21. G-CSF neutralization does not reverse down-modulation of SDF-1/CXCL12 production in SHIP-deficient hosts.
Plasma concentration of SDF-1/CXCL12 measured by ELISA in MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ mice after deletion of SHIP and administration of anti-G-CSF or PBS as indicated. Data are shown as mean + SEM of n=8 and are pooled from 2 experiments.

Discussion

By selectively ablating SHIP expression in either myeloid or T lymphoid lineage cells, we show here that SHIP exerts both lineage intrinsic and extrinsic control over the peripheral immunoregulatory cell compartment. Myeloid-restricted ablation of SHIP expression causes a significant expansion of MDSC and SHIP-competent FoxP3^{+} T cells in both spleen and LN. Consistently, priming of allogeneic T cells responses by these tissues is significantly compromised. However, we failed to find evidence for reciprocal regulation of MDSC numbers by SHIP-deficient T cells despite a significant increase in Treg and CD4^{+}CD25^{-}FoxP3^{+} T cell frequency in LckCreSHIP$^{flox/flox}$ mice. Thus, SHIP possesses both lineage intrinsic and extrinsic control over peripheral Treg accumulation; and lineage-intrinsic control over MDSC accumulation when specifically manipulating SHIP in the T cell and myeloid compartments (see model, Figure 22).

Surprisingly, we found that MDSC from naïve mice or tumor-bearing mice lack detectable levels of SHIP protein expression. This suggests that regulation of MDSC formation occurs via a lineage-intrinsic mechanism mediated by another SHIP-expressing myeloid cell that perhaps produces a soluble factor that promotes peripheral MDSC expansion. Consistently, neutralization of G-CSF, which is significantly increased in SHIP-deficient mice, blocked splenic MDSC expansion of mice with induced SHIP-deficiency. Furthermore, immature CD11b^{+}Gr1^{+} myeloid progenitors in the BM lack SHIP expression, suggested that the myeloid cell that limits MDSC accumulation is a differentiated myeloid cell in the periphery. In addition, as seen with tissue MΦ[301], activation of SHIP expression may be required for myeloid cell differentiation and acquisition of effector function. Inability to activate SHIP expression may cause SHIP-deficient myeloid cells to default to cells that possess immunoregulatory rather than

effector function. Conversely, inappropriate activity, such as increased production of G-CSF, by other SHIP-deficient myeloid cells may also promote MDSC accumulation.

Previous studies have indicated a potential role for MDSC in promoting expansion of Tregs[341, 345, 482 233]. Although, our G-CSF neutralization studies suggest otherwise, MDSC-mediated Treg expansion cannot be conclusively dismissed as a potential mechanism at play in our model of induced SHIP-deficiency. Specifically, only splenic MDSC expansion, and not MDSC expansion in the LN, was prevented after G-CSF neutralization. Possibly, MDSC in the LN mediated Treg expansion in these mice. Otherwise, another myeloid cell population may also be responsible for this effect on the Treg compartment via a soluble factor or cell bound ligand other than G-CSF, since G-CSF neutralization only prevented splenic MDSC expansion, and not Treg compartment expansion (see model, Figure 22). Also, the unaffected increase in peripheral Tregs may have been due to their ineffective recruitment to their BM reservoir by SDF-1/CXCL12, which is suppressed in the BM of SHIP-deficient mice and was not reversed by G-CSF neutralization. Treg migration to tissue sites, including the BM reservoir, is controlled by SDF-1/CXCL12[538, 539].

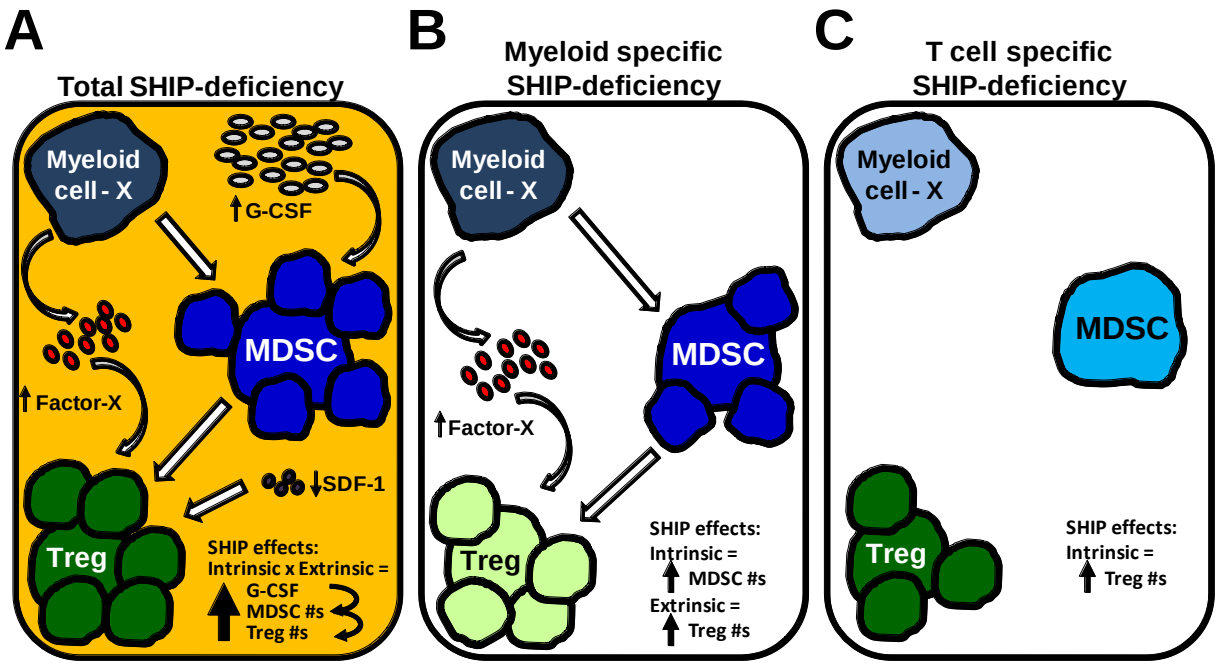

Figure 22. Proposed model of the lineage intrinsic and extrinsic effects SHIP-deficiency has on immune regulatory cells. (A) The combination of intrinsic and extrinsic effects mediated by total SHIP-deficiency promotes the highest increase in MDSC and Treg numbers. Extrinsic factors such as increased production of G-CSF and perhaps the effect of another unknown myeloid cell, promote the expansion of MDSC in peripheral lymphoid organs. Extrinsic factors that promote the increase in Treg numbers include MDSC-mediated Treg expansion, decreased levels of SDF-1 and perhaps another unknown factor produced by an unknown myeloid cell. (B) With myeloid-restricted SHIP-deficiency, a significant increase in MDSC and Treg is also observed. Due to the lack of SHIP expression by MDSC, another unknown SHIP-expressing myeloid cell may instead promote their expansion. Alternatively, perhaps myeloid differentiation is affected by myeloid-specific SHIP-deficiency such that myeloid cells default to cells with suppressive capacity capable of also promoting the expansion of Tregs. Again, Treg expansion may also be promoted by another unknown factor produced by an unknown myeloid cell. (C) T cell-specific SHIP-deficiency only affects the size of the Treg compartment suggesting that SHIP also plays an intrinsic role in Treg development independent of its extrinsic role as exhibited in the context of full SHIP-deficiency and myeloid-restricted SHIP- deficiency.

Tarasenko *et al* also examined SHIP's intrinsic role on the T cell compartment and reported contradictory results. Specifically, using CD4CreSHIP$^{flox/flox}$ mice, they found that T cell-specific deletion of SHIP had no affect on T cell development, activation

state, or Treg numbers[339]. The discrepancy may be because deletion of SHIP in CD4CreSHIP$^{flox/flox}$ mice may occur at a different time point during T cell development compared to SHIP deletion in LckCreSHIP$^{flox/flox}$ mice. Nonetheless, the only supporting data provided was a table depicting the percentage of splenic FoxP3+ cells without distinguishing CD4 and CD25 expression, thus not being an accurate representation of the Treg compartment. Furthermore, it was reported that 19-21% of splenocytes in CD4CreSHIP$^{flox/flox}$ mice were FoxP3^{+} which is an abnormally high percentage for splenic Tregs. The more precise Treg phenotype analysis presented here indicates that SHIP does indeed intrinsically limit the size of the Treg compartment *in vivo*. In addition, experiments performed by Locke *et al* further support SHIP's intrinsic role in Treg development. Specifically, they showed that a significantly higher proportion of SHIP-deficient CD4^{+}FoxP3^{-} T cells acquired FoxP3 expression compared to WT T cells in an *in vivo* adoptive transfer experiment[355].

Because of SHIP's potential to influence many PI3K-mediated signaling pathways during the lifespan of a T cell, SHIP may control the Treg compartment size by limiting, for example, the acquisition of FoxP3 expression by peripheral T cells (Treg induction) and/or limiting the survival or proliferation of existing Tregs in the periphery (Treg homeostasis). Consistently, NF-κB and NFAT, transcription factors whose activity can be limited by SHIP[520, 540], have recently been identified as critical for promoting FoxP3 expression during T cell development[541]. CD28 engagement activates the PI3K/Akt pathway, which SHIP opposes[542], to promote FoxP3 expression in peripheral T cells. In addition, others have found that Akt can promote iTreg formation independent of CD28 signals[543]. Furthermore, Treg numbers and function are compromised in p110delta PI3K mutant mice[544] and increased in mice with enforced expression of Akt/PKB[543]. Although, it must be noted that Tregs show reduced Akt phosphorylation[355]

and specifically require limited Akt activation to induce, but not maintain, FoxP3 expression[545, 546]. Furthermore, SHIP's function in T cells may be more than just negative regulation of PI3K and instead also include activation of qualitatively different PI3K effector pathways. Specifically, it has been shown that the catalytic product of SHIP, $PI(3,4)P_2$, recruits different signaling proteins from those recruited by PIP_3 and the PTEN product, $PI(4,5)P_2$[547].

The findings presented here and our previous studies of MDSC, Tregs and NK cell in SHIP-deficient mouse models [325, 336, 341, 345, 482, 485, 548] indicate that SHIP-deficiency promotes an immunosuppressive environment that preferentially impairs cell-mediated immunity, particularly those involving MHC-mismatched responses. Recently, a SHIP-1 selective inhibitor that also increases MDSC numbers *in vivo* and fosters a potent immunosuppressive environment in peripheral lymphoid tissues was identified[527]. Thus, pharmacological targeting of SHIP1 activity *in vivo* could be used to improve the efficacy and utility of allogeneic organ and bone marrow transplantation. We propose that down-regulation of SHIP expression serves as a molecular switch to promote an immunosuppressive state in peripheral lymphoid organs[549]. Such a "*SHIP switch*" is certainly plausible as SHIP expression is readily modulated post-transcriptionally by miR155[317] and post-translationally by ubiquination[550]. Indeed, SHIP expression varies significantly within both the myeloid (data not shown) and NK cell lineages [315] suggesting that SHIP regulation occurs during normal fluctuations in immune status and differentiation. Rather than acting as a global immune suppressor, the increased MDSC and Treg numbers promoted by a "SHIP switch" may act to dampen low affinity T cells responses or self-reactive T cell response during intense immune responses allowing more focused, and thus more effective, immune responses to major pathogen challenges. Intriguingly, tumor-bearing mice, similar to SHIP-deficient mice, exhibit an

increase in Tregs and MDSC. Perhaps, tumors may also exploit this "SHIP switch" to their advantage in order to promote tumor progression. This would require further studies.

A recent study performed by Kerr *et al* describing an interesting phenotype in SHIP-deficient mice provides further argument that SHIP does not simply act as a global immune suppressor. In addition, it shows that SHIP-deficiency has diverse effects in different anatomical compartments. Specifically, this study showed that SHIP-deficient mice exhibited ileitis similar to the enteric pathology seen in Crohn's disease. Within the small intestine of SHIP-deficient mice, both $CD4^+$ and $CD8^+$ T cells were scarce while neutrophils were significantly increased in numbers compared to WT controls. Furthermore, this phenotype was mediated specifically by the hematopoietic compartment since reconstitution of SHIP-deficient mice with WT bone marrow corrected ileitis and reconstitution of WT mice with SHIP-deficient splenocytes did not transfer ileitis[551]. This occurs as a paradox, where total SHIP-deficiency promotes an immune suppressive environment mediated by the accumulation of Tregs and MDSC in the periphery while also promoting immune hyper-activation in the small intestine mediated most likely by the over-abundance of neutrophils and lack of T cells. How a SHIP- deficient compartment promotes this phenotype is still unknown. Due to the lack of T cells in the intestine, perhaps T cells, including Tregs, are required to control the over-active neutrophilic response. SHIP-deficiency may have an effect on T cell homing and/or survival. Importantly, Tregs are also known to control IBD. If SHIP-deficiency does affect T cell homing, perhaps SHIP-deficient Tregs, regardless of their increased abundance, cannot properly home to the small intestine and protect the mice from CD. Unfortunately, the presence of ileitis was not investigated when performing the studies

presented above using the myeloid-specific SHIP-deficient mouse model. Clearly, these studies and others are required to fully clarify this paradox.

Chapter 5. Rb1 Controls the Differentiation of Myeloid Derived Suppressor Cells Subsets

Introduction

MDSC are a heterogeneous myeloid population of immature precursors and pathologically activated cells that accumulate significantly in several pathological settings, such as cancer and chronic infection, inflammation, and stress. The main defining characteristic of MDSC is that they effectively suppress several facets of the resulting immune response in these various settings. In particular, MDSC have been studied extensively in cancer where their suppressive function has been clearly appreciated to contribute significantly to cancer progression. In virtually every tumor model and in every type of human cancer that has been examined for them, MDSC have been shown to be expanded[242, 552]. Moreover, when MDSC are depleted in tumor-bearing mice and in cancer patients, enhanced immune responses and in some cases, a direct anti-tumor response are achieved[553]. Thus, understanding the mechanisms driving their accumulation can be translated into therapeutic application by directly targeting these mechanisms[554, 555].

Initially, MDSC in cancer were simply identified as $Gr1^+CD11b^+$ cells with potent suppressive capacity[204]. Further characterization of their phenotype according to morphology, the expression of surface markers, and modes of suppression has led to the identification of two major subsets within the overall MDSC population[219, 233-236]. One subset is granulocyte-like ($CD11b^+Ly6G^{hi}Ly6C^-$), thus called granulocytic MDSC (G-

MDSC); and the other is monocyte-like ($CD11b^{+}Ly6G^{-}Ly6C^{+}$), thus called monocytic MDSC (M-MDSC). G-MDSC suppressive capacity is primarily reliant on significantly high productions of ROS and close cell-cell contact with T cells. In turn, M-MDSC rely on the activity of iNOS, Arg-1 and several suppressive cytokines to effectively suppress T cell responses independent of antigen specificity[240, 242]. In many tumor models examined, there is a preferential accumulation of G-MDSC over M-MDSC in peripheral lymphoid organs, thus G-MDSC represents the majority of MDSC[241]. Consistently, these two subsets also differ in their differentiation and proliferative capacity[244, 556]. In culture supplemented with GM-CSF, G-MDSC that remain, essentially preserve their phenotype and morphology. Instead, M-MDSC differentiate further acquiring CD11c, F4/80 markers, which are DC and macrophage specific markers. Additionally, G-MDSC are non-proliferative while M-MDSC are highly proliferative both *in vitro* and *in vivo*. Conversely, M-MDSC preferentially acquired the phenotype of G-MDSC with decreased acquisition of DC and macrophage specific markers in culture with TES. Furthermore, the majority of M-MDSC transferred into tumor-bearing mice acquired the phenotype of G-MDSC, suggesting that G-MDSC accumulation may be a result of the proliferation and preferential differentiation of M-MDSC to G-MDSC in cancer[556]. Further studies are required to understand the molecular mechanisms driving this process.

The retinoblastoma protein (Rb) is well known for its involvement in a variety of cellular processes such as the cell cycle, differentiation, apoptosis and DNA repair. Its most well-documented form of regulation is through its association with E2F family transcription factors which leads to transcriptional inhibition of E2F response genes. In addition, Rb-E2F complexes act as active repressors by associating with several other chromatin-modifying complexes including histone deacetylases and methylase, DNA methyltransferases and ATP-dependent chromatin-remodeling enzymes[375].

Because hematopoietic cells are constantly proliferating, differentiating and dying, Rb activity within the hematopoietic compartment is critical. In fact, Rb is known to interact with several hematopoietic transcription factors[472]. Rb-mediated regulation is both extrinsic and intrinsic in hematopoietic cells. For example, Rb indirectly controls erythropoiesis by interacting with Id2, a transcription factor that regulates differentiation in fetal liver MΦ[474]. In change, under stress condition, Rb was intrinsically required for proper erythroblast expansion and red cell enucleation[475]. In addition, Rb has been shown to regulate monocytic and neutrophilic lineage commitment. When Rb expression is decreased using antisense Rb oligonucleotides in progenitor cells, they preferentially differentiate into neutrophilic cells even under monocytic-promoting culture conditions[472]. Consistently, Rb expression is down-regulated during granulopoiesis *in vivo*, in which neutrophil precursor cells express high level of Rb while more mature polymorphonuclear neutrophils express much less Rb[477]. Finally, when Rb is deleted conditionally during adulthood, as accomplished with MxCre$Rb^{flox/flox}$ mice, myeloproliferative disease develops. This phenotype was not achieved when Rb was deleted only in HSC. In change, the deletion of Rb in myeloid cells and in the BM microenvironment was sufficient to promote myeloproliferative disease suggesting an extrinsic effect of Rb on HSC homeostasis and differentiation[471]. Though, when all Rb family members are conditionally deleted in mice, myeloproliferation disease develops in a cell-intrinsic manner and reintroduction of a single p107 allele prevented this. Furthermore, hematopoietic progenitor cells with all Rb family proteins deleted exhibited a gene expression profile consistent with the preferential myeloid development observed[479].

In this study, the role of Rb1 in the accumulation and differentiation of MDSC subsets in cancer was examined. We found that M-MDSC and G-MDSC express

different levels of Rb1 at both the mRNA and protein level. Specifically, M-MDSC expressed high levels whereas G-MDSC expressed undetectable levels. In culture with GM-CSF, M-MDSC Rb1 expression levels stayed relatively the same while increasing in G-MDSC over time, which is accompanied by an increase in histone acetylation at the Rb1 promoter. Further, when treated with an HDAC inhibitor (HDACi), the increase in Rb1 expression in G-MDSC was enhanced with increased acetylation at the Rb1 promoter. Treatment of M-MDSC with HDACi, which presumably promotes sustained Rb1 expression, abrogated their ability to differentiate into G-MDSC, even in the presence of TES. Finally, analysis of these subsets in a model of induced Rb1-deficiency exhibited a preferential expansion of myeloid cells with a phenotype similar to that of G-MDSC. These results suggest that Rb1 indeed plays a role in the accumulation of MDSC, particularly G-MDSC in cancer.

Results

MDSC Express Low Levels of Rb1 Compared to Other Differentiated Cells.

Several studies have suggested that Rb1 plays a role in the differentiation and/or accumulation of myeloid cells. In cancer, there is a significant accumulation of MDSC, a heterogeneous population of pathologically activated myeloid precursors. As an initial test to see if Rb1 may be playing a role in tumor-associated accumulation of MDSC, the expression of Rb1 mRNA and protein in MDSC compared to non-MDSC isolated from the same SPL of a tumor-bearing mouse were examined. Surprisingly, Rb1 protein expression was essentially undetectable in MDSC. Rb1 mRNA expression was about five times greater in non-MDSC compared to MDSC (Figure 23A). Rb1 expression

levels in MDSC compared to other more differentiated myeloid cell types, such as dendritic cells (DC) and MΦ were further investigated. Again, DCs and MΦ expressed much higher levels of Rb1 protein and mRNA than MDSC (Figure 23B).

MDSC are known to survive and further differentiate in culture supplemented with GM-CSF[209, 557]. Thus, we wanted to examine if Rb1 expression changed over time *in vitro.* When placed in culture with GM-CSF, Rb1 mRNA and protein expression levels in MDSC increased with time. The increase in Rb1 mRNA levels occurred quickly, within the first day in culture and remained high for the duration of the experiment (Figure 23C). The increase in Rb1 protein level did not peak until after 3 days in culture with GM-CSF. TES has been shown to delay MDSC differentiation into DCs and MΦ[209, 557]. Interestingly, when tumor explant supernatant (TES) was also added to the culture, the increase in Rb1 protein level seen over time with GM-CSF only was delayed (Figure 23D).

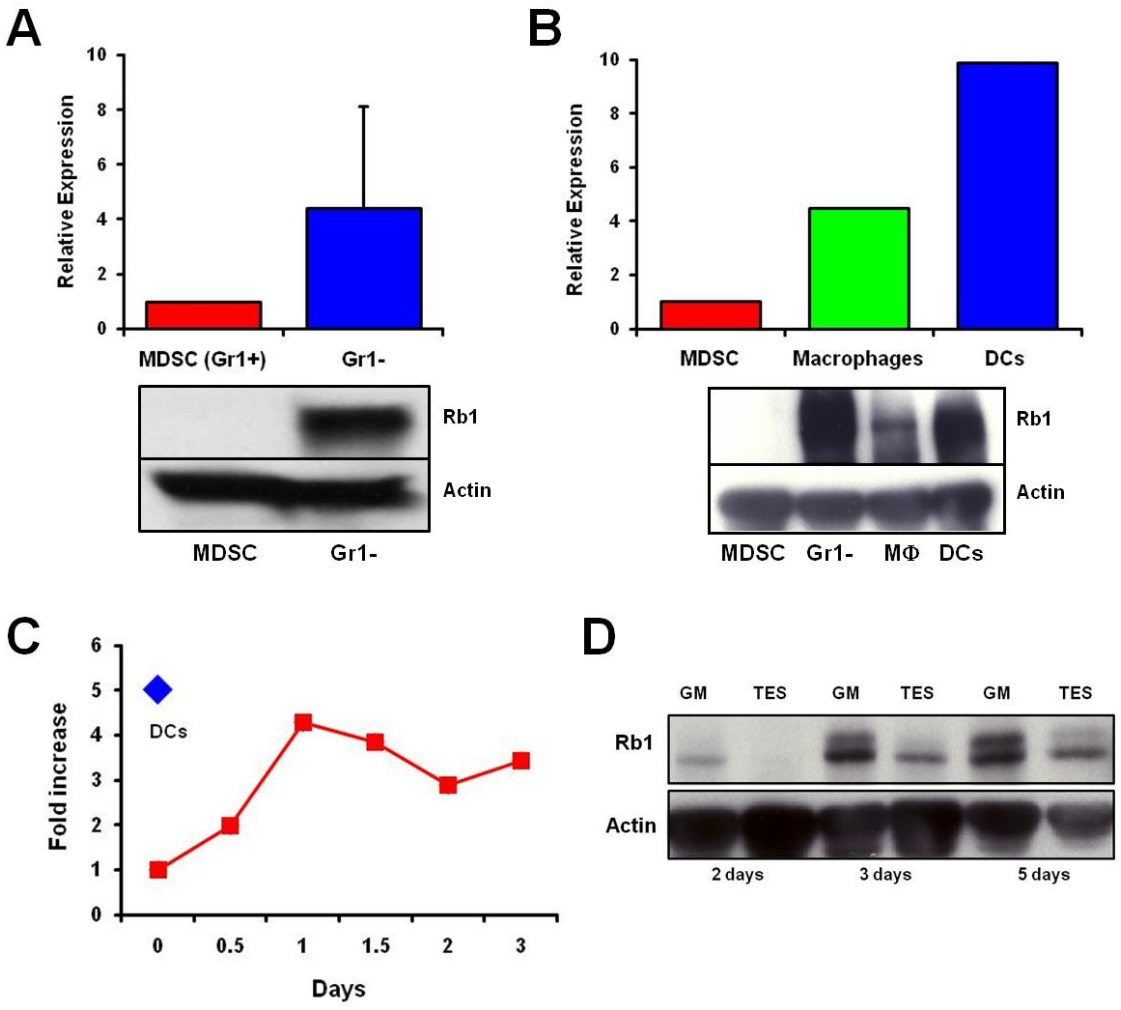

Figure 23. MDSC express low levels of Rb1 mRNA and non-detectable levels of Rb1 protein, which increases over time in culture.
(A) Rb1 mRNA and protein expression levels in splenic MDSC obtained by magnetic isolation of $Gr1^+$ cells compared to $Gr1^-$ cells were determined by RT-PCR and Western blot, respectively. Bar graph represents Rb1 mRNA relative expression calculated by normalizing Rb1 quantitative values against the endogenous control, 18S. (B) Same as in A, instead comparing MDSC Rb1 expression levels to MΦ obtained from the peritoneum after casein-induced mobilization, and DCs obtained from whole BM cells differentiated with GM-CSF and IL-4 for 5 days. (C) Rb1 mRNA expression levels in magnetically isolated MDSC cultured over time with GM-CSF and compared to that in DCs. (D) Rb1 and β-Actin protein level in magnetically isolated MDSC cultured for 5 days with GM-CSF with or without TES.

MDSC Subsets Express Different Levels of Rb1 mRNA and Protein When Freshly Isolated and When Placed in Culture Over Time. As mentioned, MDSC can be divided further into two distinct groups; M-MDSC, which are mononuclear $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells and G-MDSC, which are polymorphonuclear $CD11b^{+}Ly6G^{+}$ $Ly6C^{lo}$ cells. Consistent with their phenotypic differences, M-MDSC and G-MDSC also differ in the expression of Rb1 mRNA and protein. M-MDSC expressed very high levels of Rb1 mRNA and protein while G-MDSC expressed very low levels of mRNA and undetectable protein levels, even when loading five times more protein (Figure 24). Interestingly, the expression of Rb1 by M-MDSC from SPL and BM of tumor-bearing and from cells of the same phenotype in naïve BM is very similar. The lack of Rb1 expression is also shared by G-MDSC from of the same various sources. E2F is a downstream target of Rb1, its protein expression down-regulated by Rb1 activity. Consistently, E2F protein expression correlated inversely with Rb1 expression, with high expression in G-MDSC and undetectable expression in M-MDSC (Figure 24A).

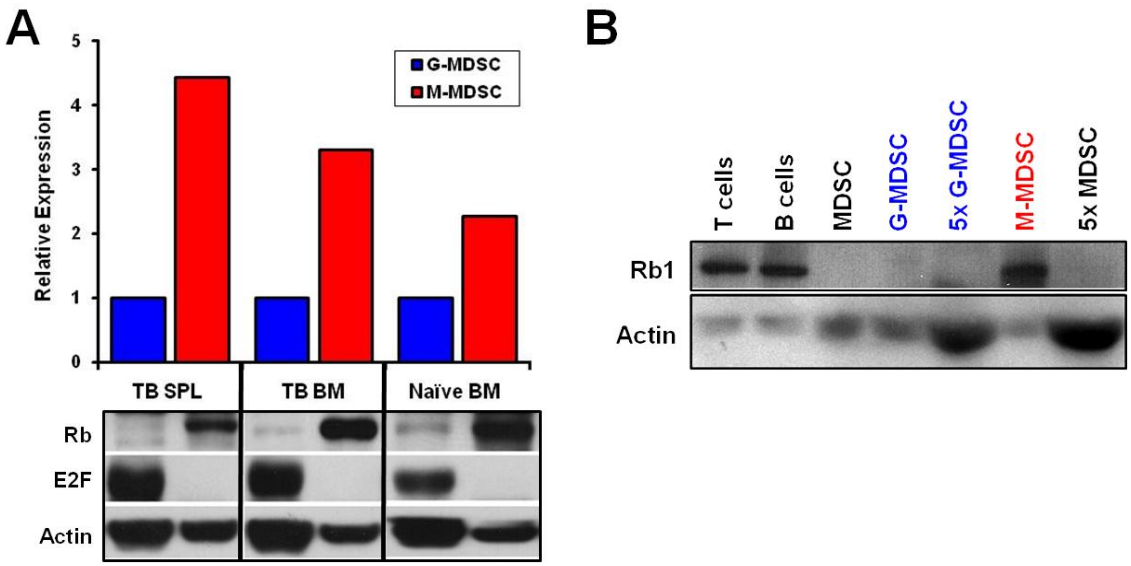

Figure 24. Freshly isolated M-MDSC express high levels of Rb1 while G-MDSC express low levels of Rb1.
(A) G-MDSC ($CD11b^{+}Ly6G^{+}Ly6C^{lo}$) and M-MDSC ($CD11b^{+}Ly6G^{-}Ly6C^{hi}$) were sorted from SPL and BM of tumor-bearing mice and from BM of naïve mice. RNA and protein were extracted from each sample to perform RT-PCR and Western blot analysis, respectively. Bar graph represents Rb1 mRNA expression normalized against the expression of endogenous control, 18s. Western blot analysis depicting Rb1, phosphorylated Rb1 (pRb), E2F and β-Actin expression in the corresponding samples. (B) Western blot analysis of Rb1 and β-Actin expression in MDSC, G-MDSC and M-MDSC sorted from SPL of tumor-bearing mice compared to T cells and B cells sorted from SPL of naïve mice. For MDSC and G-MDSC, five times more protein was also loaded depicted as 5x MDSC and 5x G-MDSC.

It has also been shown that M-MDSC survive and proliferate as seen in a 5 day culture with GM-CSF; while G-MDSC do not, with only 20% survival observed in a 3 day culture with GM-CSF[556]. Thus, we examined the change in Rb1 expression in M-MDSC and G-MDSC over a 3 day culture. When placed in culture supplemented with GM-CSF, the change in Rb1 expression level in M-MDSC and G-MDSC was very distinct (Figure 25A). In M-MDSC, Rb1 mRNA expression did not change significantly over time with GM-CSF regardless of the presence of TES (Figure 25B). Conversely, G-MDSC

cultured with GM-CSF displayed a rapid increase in Rb1 mRNA expression, reaching its peak by day 2. This was delayed by the presence of TES (Figure 25C). When analyzing Rb1 protein levels in G-MDSC, a difference could easily be distinguished between freshly isolated G-MDSC and G-MDSC in culture for 2 days (Figure 25D) but no difference was visible between G-MDSC in culture for 2 days with GM-CSF only and G-MDSC cultured for 2 days with GM-CSF plus TES. This can be due to the lack of sensitivity in the Western blot assay or that there indeed is no difference in protein expression. Of particular interest, is the intense band representing Rb1 protein expression in neutrophils obtained from the peritoneum after casein-induced mobilization thus representing active neutrophils (Figure 25D).

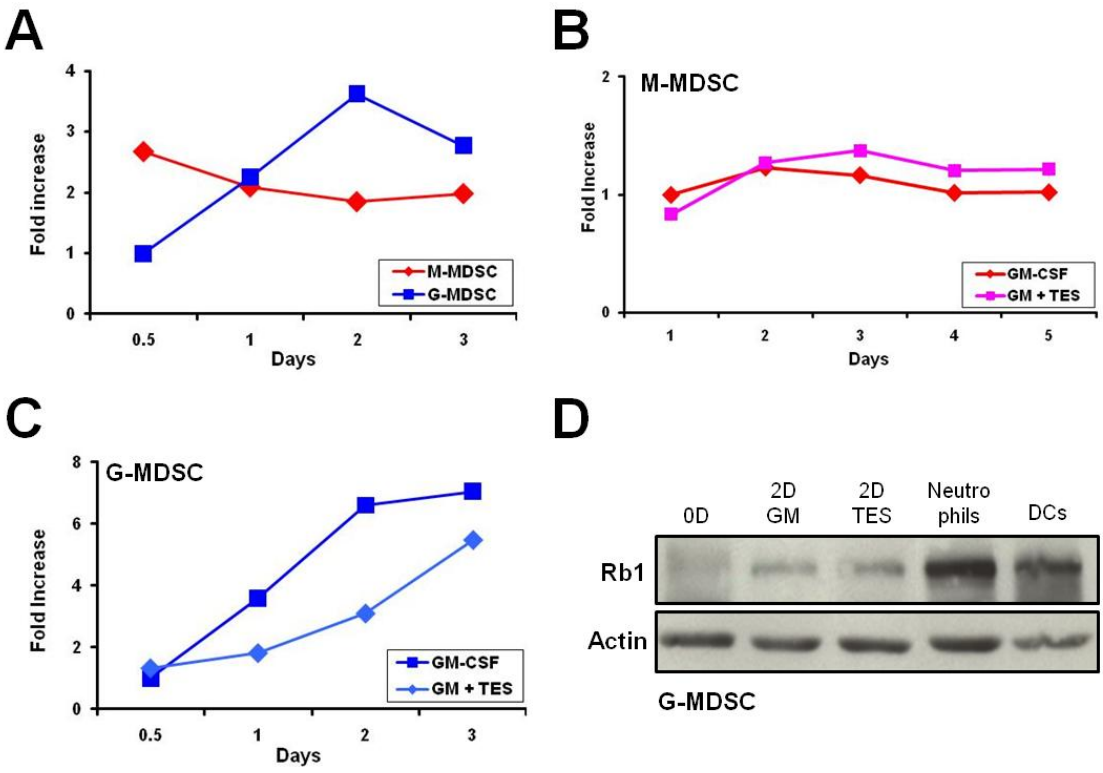

Figure 25. Rb1 mRNA and protein levels increase in G-MDSC but not in M-MDSC *in vitro* over time.

(A) RT-PCR was performed on RNA extracted from sorted M-MDSC and G-MDSC cultured with GM-CSF on the indicated days for 3 days. (B) RT-PCR was performed on RNA extracted on the indicated days from sorted M-MDSC that were cultured with GM-CSF with or without TES for 5 days. (C) RT-PCR was performed on RNA extracted on the indicated days from sorted G-MDSC that were cultured with GM-CSF with or without TES for 3 days. (D) Western blot analysis of Rb1 and β-Actin protein expression in freshly isolated G-MDSC, G-MDSC cultured for 2 days in GM-CSF only, G-MDSC cultured for 2 days in GM-CSF plus TES, neutrophils isolated from the peritoneum after casein-induced mobilization and DCs obtained from whole BM cells differentiated with GM-CSF and IL-4 for 5 days.

Rb1 Expression is Regulated by Histone Acetylation. Protein expression can be regulated by many means employed at the transcriptional, translational and post translational level. To regulate transcription, for example, modifications such as histone acetylation results in more relaxed chromatin associated with gene activation, while DNA methylation results in more compact, inactive chromatin associated with gene inactivation. Acetylation of histones is primarily regulated by the competing activity of HATs, histone acetyltransferase, and HDACs, histone deacetyltransferases. DNA methylation is mediated by DNMT, DNA methyltransferases[558]. To study the role of histone acetylation and methylation in regulating gene expression, HDAC inhibitors (HDACi) such as trichostatin (TSA), valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA) and MS275; and DNMT inhibitors (DNMTi) such as 5-aza-2'-deoxycytidine (AZD) are commonly used. The effects of TSA, an HDACi and AZD, a DNMTi on Rb1 mRNA expression were analyzed in BM cells enriched for hematopoietic progenitor cells (HPC). HPC were cultured for two days with GM-CSF plus TES and then treated with TSA or AZD for 12 hours before harvesting for RNA extraction. Surprisingly, TSA treatment resulted in a dose-dependent increase in Rb1 mRNA expression while AZD treatment had no effect (Figure 26A). Furthermore, magnetically isolated G-MDSC cultured in GM-CSF plus TES treated overnight with SAHA, and more so with VPA,

exhibited an enhanced increase in Rb1 mRNA expression that lasted at least for 2 days compared to untreated G-MDSC (Figure 26B). This data suggest that Rb1 expression is primarily regulated by changes in histone acetylation and not by DNA methylation. To further confirm this, G-MDSC were treated overnight with and without MS275, a class I HDAC-selective inhibitor, to analyze the change in acetylation level on the Rb1 promoter using the CHIP assay and Acetyl-Histone H3 specific antibody. When cultured overnight in GM-CSF plus TES, G-MDSC exhibit an increase in acetylation of the Rb1 promoter region which correlates with the observed increase in Rb1 mRNA levels. When MS-275 is added to this culture condition, the increase in acetylation at the Rb1 promoter region is enhanced compared to untreated cells (Figure 26C).

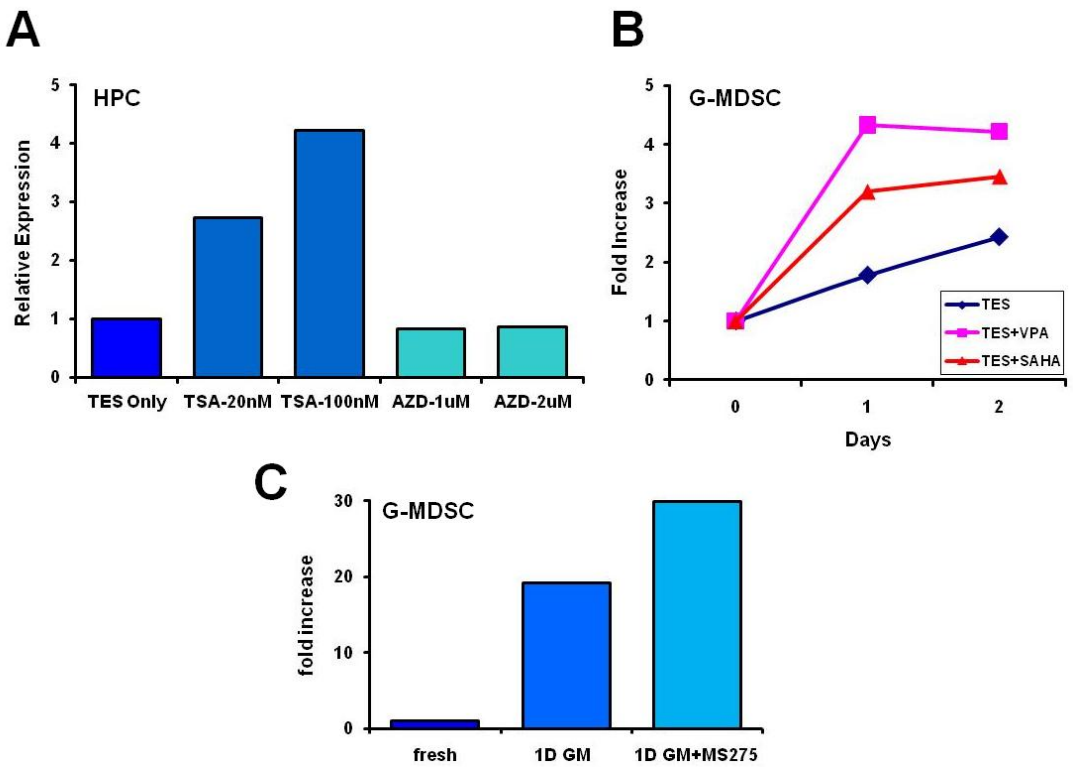

Figure 26. Effect of HDACi and DNA methylation inhibitors on Rb1 mRNA expression.
(A) Bar graph representing Rb1 mRNA relative expression in BM cells that were magnetically enriched for HPC by lineage depletion, cultured for two days with GM-CSF

plus TES, then treated with the indicated amounts of TSA and AZD before harvesting RNA. (B) Line graph depicting change in Rb1 mRNA expression over time in G-MDSC cultured with GM-CSF plus TES and treated overnight with VPA or SAHA. (C) Bar graph representing relative acetylation levels at the Rb1 promoter analyzed after overnight treatment with and without MS275, an HDACi, compared to freshly isolated G-MDSC.

Rb1 Expression Regulates MDSC Subset Accumulation and Differentiation.

M-MDSC and G-MDSC differ not only in their phenotype, morphology, suppressive mechanism and Rb1 expression, as shown here; they also differ in their proliferative and differentiation capacity. M-MDSC, when in culture with GM-CSF, are capable of differentiating into DCs, MΦ and importantly into G-MDSC. Furthermore, in the presence of GM-CSF plus TES, there was a much higher frequency of G-MDSC that differentiate from M-MDSC than that seen with GM-CSF alone (Figure 27). When M-MDSC were transferred into a tumor-bearing mouse, the majority of M-MDSC differentiated into G-MDSC, thus suggesting that G-MDSC in tumor-bearing mice are mainly sourced by M-MDSC[556].

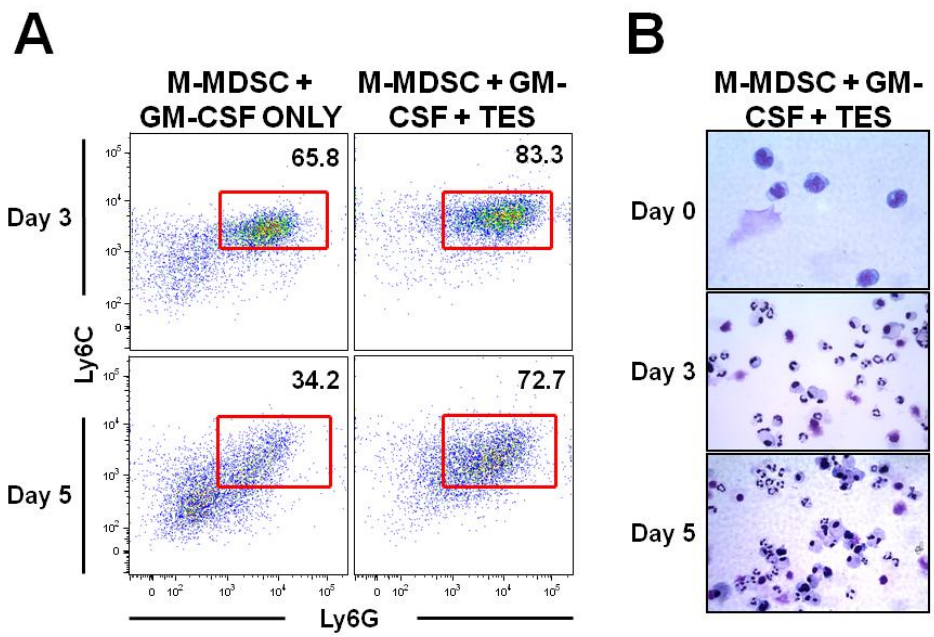

Figure 27. M-MDSC differentiate preferentially to G-MDSC in the presence of GM-CSF plus TES compared to GM-CSF alone.
Sorted M-MDSC from tumor-bearing mice were cultured with GM-CSF with or without TES for the indicated amount of time and then analyzed. (A) Flow plots depicting cells expressing Ly6C vs. Ly6G, first gated on viable $CD11b^+$ cells. (B) Wright-Giemsa staining showing the presence of polymorphonuclear cells derived from M-MDSC in culture for the indicated amount of time.

Next, we wanted to explore if changes in Rb1 expression serves as the switch that drives this preferential differentiation which may also be promoted by the tumor microenvironment. Otherwise, Rb1 expression is simply a consequence and unconnected to the process. Because HDACi treatment increased Rb1 mRNA expression in HPCs and in G-MDSC, M-MDSC were treated with VPA to see if this would affect their preferential differentiation to G-MDSC and even perhaps push them to differentiate into DCs or MΦ. When treated with VPA in the presence of GM-CSF plus TES, M-MDSC differentiation to G-MDSC was nearly abolished as well as to

differentiation to DCs and MΦ. Interestingly, when analyzing proliferation with BrdU incorporation, VPA caused a massive increase in proliferation in M-MDSC (Figure 28).

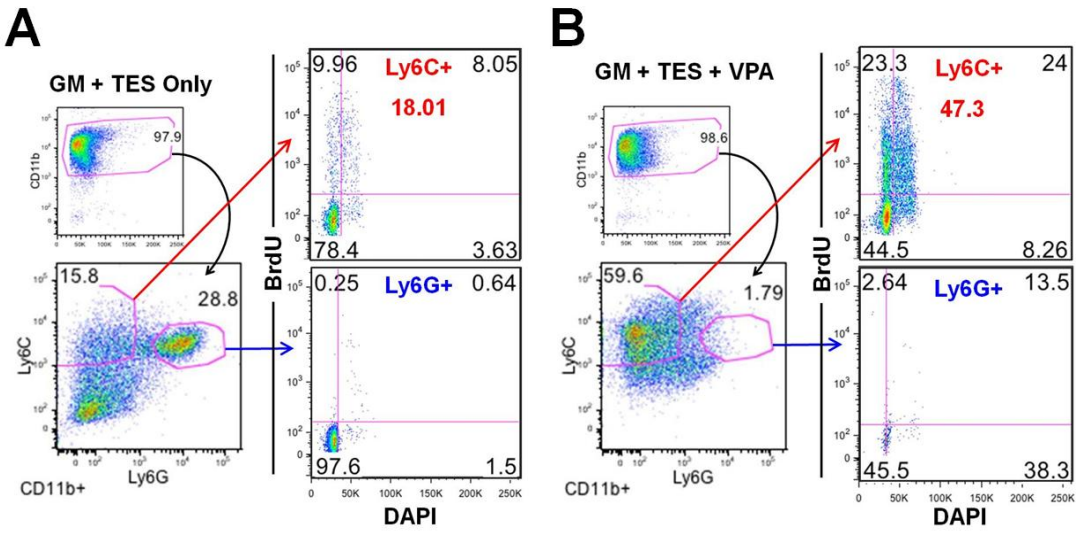

Figure 28. HDACi treatment prevents M-MDSC from differentiating into G-MDSC. (A) Splenic Ly6C$^+$ from EL-4 tumor-bearing mice were sorted and cultured for three days supplemented with GM-CSF plus TES to allow for differentiation to G-MDSC and other myeloid lineages. BrdU was added during the last four hours in culture to analyze BrdU incorporation by flow in remaining M-MDSC and differentiated G-MDSC in culture. (B) Same as in A, except that VPA was added after resting cells overnight and removed after 18hrs of treatment.

Other studies have shown that Rb1-deficiency induced in adulthood results in a myeloproliferative-like disease with myeloid hyperplasia in the BM and increased extramedullary hematopoiesis with a significant expansion of myeloid cells, CD11b$^+$Gr1$^+$ in the SPL[471]. In tumor mouse models, a similar expansion of MDSC occurs with increased myeloid cell cellularity in the BM and in the SPL[241]. Thus, the inducible Rb1-deficiency model was further analyzed to see if the expansion of myeloid cells was primarily monocytic or granulocytic in nature. After induction of Rb1 deficiency in MxCre Rb1$^{flox/flox}$, an increased accumulation of CD11b$^+$Ly6G$^+$Ly6C^{lo} in the SPL both in

frequency and in absolute number of cells was observed (Figure 29), suggesting that lack of Rb1 expression indeed promotes the preferential expansion of myeloid cells with a granulocytic phenotype.

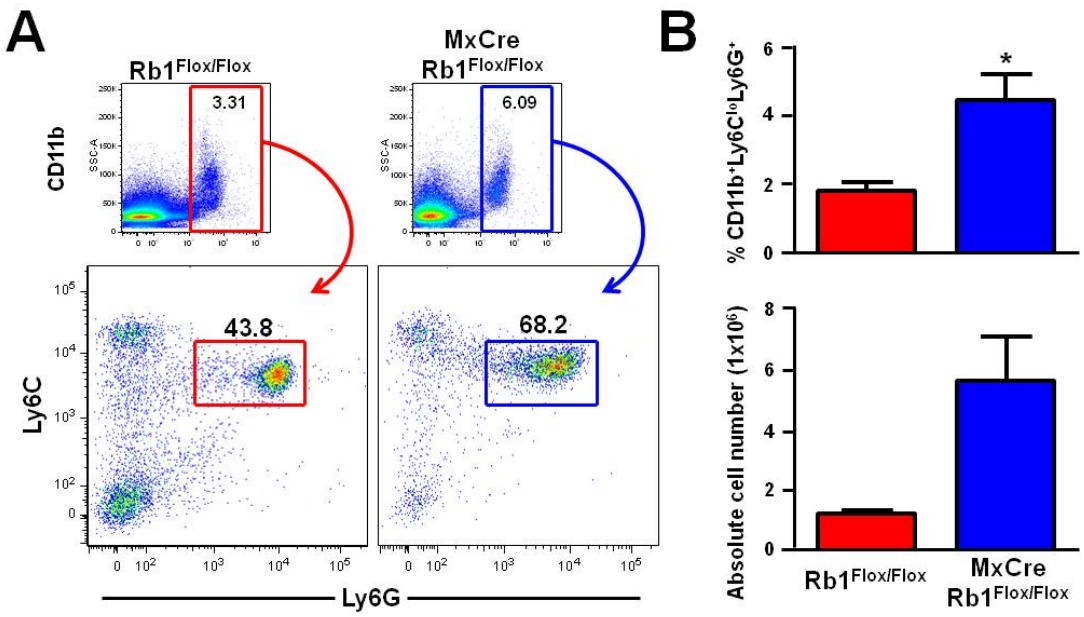

Figure 29. Induction of Rb1 deficiency promotes an expansion of splenic CD11b^{+}Ly6G^{+}Ly6C^{lo} cells.
(A) Representative Ly6C vs. Ly6G staining after gating on viable CD11b^{+} cells in SPL from Rb1$^{flox/flox}$ and MxCreRb1$^{flox/flox}$ mice after poly(I/C) administration. (B) Percentage frequency and total absolute number of CD11b^{+}Ly6G^{+}Ly6C^{lo} cells in the SPL of the indicated genotype. For MxCreRb1$^{flox/flox}$: n = 4 and Rb1$^{flox/flox}$: n = 4. *P < 0.05.

Discussion

In this study, the role of Rb1 in the accumulation and differentiation of MDSC subsets was examined. Freshly isolated splenic MDSC from tumor-bearing mice, without discriminating the G-MDSC and M-MDSC subsets, exhibited very low or

undetectable Rb1 expression levels at the mRNA and protein level, respectively, compared to more differentiated cells, such as DCs and MΦ. Interestingly, when discriminating between M-MDSC and G-MDSC, different levels of Rb1 expression at both the mRNA and protein level were seen. M-MDSC expressed high levels while G-MDSC expressed undetectable levels. As shown by Youn *et al*, in most of the tumor models observed, particularly in the tumor model used throughout this study, EL-4 thymoma, MDSC were primarily composed of G-MDSC, while M-MDSC only made up about 10-15% of MDSC[241]. Additionally, when using Gr1 antibody and magnetic isolation, the majority of $Gr1^+$ cells isolated are those that express high levels of Gr1 which are mainly G-MDSC. This may explain why when examining MDSC as a whole, they seemed to express low or undetectable levels of Rb1 since the majority of isolated cells were G-MDSC.

MDSC subsets differ in their morphology, phenotype, preferred mode of suppression and, as shown here, expression of Rb1 mRNA and protein when freshly isolated, as well as when measured over time in culture. In culture with GM-CSF, M-MDSC Rb1 expression levels stayed relatively the same while increasing in G-MDSC over time, which was slightly hindered in the presence of TES at the mRNA level. Although very different, M-MDSC and G-MDSC do share an important relationship. M-MDSC have been shown to preferentially differentiate into G-MDSC in the presence of tumor-derived factors[556]. We hypothesize that M-MDSC in tumor-bearing mice are promoted to differentiate preferentially into G-MDSC, which is mediated by the tumor microenvironment. Recent studies performed by Youn *et al* have shown that when placed in culture with GM-CSF, G-MDSC are capable of differentiation further, acquiring characteristics common to neutrophils, such as increased expression of a lysosomal enzyme, decreased expression of specific surface markers and increased phagocytic

activity[556]. To add to this list, G-MDSC also acquired Rb1 protein expression. Thus, it seems that in tumor-bearing mice, G-MDSC do not further differentiate to neutrophils and instead remain as pathologically activated precursors, capable of immune suppression. Rb1 expression seems to play a role in this differentiation, but up to now with the results presented here, it can only be said that Rb1 expression correlates with differentiation stage, where it is high in M-MDSC, low in G-MDSC and high in neutrophils, as well as any other differentiated cell such as DCs, MΦ, T cells and B cells.

One way we attempted to investigate this further was by altering Rb1 expression levels in MDSC subsets. Notably, the increase of Rb1 expression observed over time in G-MDSC correlated with increased histone acetylation at the Rb1 promoter. This prompted us to examine the control of Rb1 expression at the transcriptional level by using HDACi and DNA methylation inhibitors. Initially, HPC were used since they have been shown to be capable of differentiating into MDSC in culture[559] and thus represent potential precursors of MDSC *in vivo*. When treated with HDACi in culture, HPCs exhibited an increase in Rb1 mRNA expression in a dose-dependent manner. This was not observed when using the DNMTi. Furthermore, when treating G-MDSC with HDACi, the increase in Rb1 expression was enhanced with increased acetylation at the Rb1 promoter region. Specifically the HDACi used in this case was MS-275, a class 1 specific HDACi. This suggests that in MDSC subsets, Rb1 expression is controlled by histone modifications mediated by class 1 HDACs.

To take it a step further, the differentiation capacity of M-MDSC to G-MDSC was analyzed in the presence of HDACi. Interestingly, treatment of M-MDSC with HDACi abrogated their ability to differentiate into G-MDSC, even in the presence of TES. We have shown here that HDACi treatment causes increased Rb1 expression and thus may cause Rb1 expression to maintain itself in M-MDSC, preventing them from further

differentiating. In other words, HDACi treatment presumably prevented the downregulation of Rb1 expression required to drive further differentiation of M-MDSC to G-MDSC by sustaining or enhancing histone acetylation at the Rb1 promoter region. Though, this did not push M-MDSC to differentiate into DCs and MΦ which would probably require more than just sustained Rb1 expression.

Also, HDACs are well known for controlling the transcription of an enormous variety of genes. Thus, it cannot be concluded that the effect on M-MDSC differentiation capacity seen is solely due to the affect of HDACi treatment on Rb1 expression. The alteration of various other genes may have contributed to the observed phenotype. It is also notable that when treated with HDACi, M-MDSC proliferation is enhanced. This is contradictory to other studies which have shown that HDACi treatment instead induces cell cycle arrest in normal and transformed cells and induces terminal cell differentiation[560, 561]. In addition, of all the genes known to be controlled by Rb1, when in complex with HDAC1, Rb1 represses a specific subset of promoters and transcription factors[562]. Perhaps the HDACi treatment also affected the interaction between Rb1 and HDAC1 and subsequently inhibited their selective repressive activity while maintaining Rb1 expression. This may have also contributed to the observed increase in proliferation and lack of differentiation.

Finally, a mouse model of inducible Rb1-deficiency was used to explore the effect of Rb1 in the development and accumulation of myeloid cells. Analysis of the myeloid compartment using the same phenotypic markers used to distinguish G-MDSC and M-MDSC subsets showed that there is a preferential expansion of myeloid cells with a phenotype similar to that of G-MDSC. Further studies are required to elucidate if the accumulation of these G-MDSC-like cells is due to the affect that Rb1-deficiency has on the differentiation capacity of M-MDSC-like cells; or due to other possible reasons, such

as the affect that Rb1-deficiency has on the proliferation and survival of G-MDSC-like cells.

Although not fully demonstrated, this data does allow one to speculate that a tumor-derived factor or factors, or a specific aspect of tumor progression promotes this preferential G-MDSC expansion via down-regulation of Rb1 expression. If so, further experiments would be aimed at elucidating this as well as examining where along the differentiation process from HSCs to G-MDSC does this regulation occur. Furthermore, it remains possible that Rb1 expression serves as a switch that promotes the differentiation of MDSC subsets from M-MDSC to G-MDSC to neutrophils where the transition from M-MDSC to G-MDSC is promoted and the transition from G-MDSC to neutrophils is impeded by tumor-derived factors. Regardless, these results do suggest that Rb1 plays a role in the accumulation and differentiation of cells of the myeloid compartment such as MDSC and particularly G-MDSC in cancer. Furthermore, if Rb1 indeed plays a role in MDSC accumulation in cancer by dictating their differentiation, this can have clinical implication. As mentioned before, the expansion of MDSC and their potent suppression of anti-tumor responses contribute significantly to tumor progression. Thus, if MDSC differentiation is controlled by Rb1, manipulation of Rb1 expression in the myeloid compartment by pharmacological means *in vivo* may in turn prevent MDSC accumulation and thereby suppressive hold that MDSC have on anti-tumor immune responses. In addition, if a tumor-derived factor(s) is responsible for the down-regulation of Rb1 expression in the myeloid compartment and resulting differentiation block and accumulation of G-MDSC, then this factor(s) can also be targeted.

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