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The Interleukin-12 Family of Cytokines and Receptor Signaling in Graft-versus-Host Disease

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The Interleukin-12 Family of Cytokines and Receptor Signaling in Graft-versus-Host Disease

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Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HCT) is an effective means by which to treat a wide variety of diseases resulting from dysfunctional hematopoiesis due to a potent anti-tumor response known as the graft-versus-tumor (GVT) effect after chemotherapy and/or irradiation. The necessity of donor T cells to mediate GVT is offset by the consequential development of graft-versus-host disease (GVHD); a leading cause of mortality among allo- HCT recipients. The IL-12 family of cytokines signals through Janus Kinase 2 (JAK2) and is comprised of IL-12, IL-23, IL-27, IL-35 and IL-39. IL-12 (p35+p40) and IL-23 (p19+p40) have well documented proinflammatory functions responsible for Th1 differentiation and Th17 stabilization, respectively, and play critical roles in GVHD development. As such, we found that targeting p40 resulted in reduced GVHD. IL-12R and IL-23R also share a β-chain, IL-12Rβ1. While IL-23R is widely implicated in autoimmunity and GVHD, the role of IL-12Rβ1 remains much less defined. We found that donor splenocytes deficient for IL-12Rβ1 or IL-23R had an impaired ability to induce chronic GVHD (cGVHD). We also found a pathogenic role for IL-23R on donor T cells in acute GVHD (aGVHD); strikingly, a similar effect was not seen for IL-12Rβ1. Our studies then focused on determining how pharmacologically targeting IL-12/23p40 can be efficacious in reducing GVHD severity in experimental and clinical settings, yet also to explain why IL-12Rβ1 may be dispensable in aGVHD. The newest member of the IL-12 family, IL-39, is composed of IL-23p19 and EBI3. Given the cognate receptor for IL-39 includes IL-23R and gp130, we hypothesized that IL-39 may play a role in

aGVHD. We detected significantly higher IL-39 in serum of mice with aGVHD compared to recipients of bone marrow alone (BMA) and cGVHD, providing a potential explanation for how IL-23R signaling could bypass IL-12β1. We also evaluated how JAK2 impacted GVHD. We found that T cell deficiency of JAK2 or neutralization via pacritinib significantly reduced mortality from GVHD. In summary, targeting IL-12 family signaling is an effective strategy to reduce GVHD. Further, future studies should focus on whether targeting IL-23/IL-39p19 is as effective as targeting IL-12/IL-23p40 in the clinic due to IL-39.

I.   INTRODUCTION

I.1. Allogeneic Hematopoietic Stem Cell Transplantation

The use of the atomic bomb in World War II not only revolutionized warfare but also the methods to treat bone marrow aplasia caused by radiation damage. In 1950, it was found that syngeneic bone marrow transplantation (BMT) could effectively compensate for bone marrow dysfunction resulting from irradiation using murine models[1]. By 1956, this concept of replacing dysfunctional bone marrow was applied to murine models of leukemia and consequential aplasia following irradiation. This study involved two groups of mice: one that received a syngeneic (genetically identical) BMT and the other that received an allogeneic (genetically disparate) BMT. While aplasia was effectively treated in both groups, all mice that received a syngeneic transplant succumbed to leukemia relapse. However, the group of mice that received an allogeneic BMT (allo-BMT) did not experience any sign of disease relapse, but instead died from a "wasting disease", which would later manifest into what is currently known as Graft-versus-Host disease (GVHD)[2]. The aforementioned experiments led to two major discoveries which catalyzed the birth of immunology: the identification and typing of human leukocyte antigens (HLA), as well as the ability of a genetically mismatched immune system to eradicate tumor cells[3–5]. In 1990, the Nobel Prize was awarded to E. Donnall Thomas for his unrelenting pursuit and discoveries regarding allo-BMT as a therapy for cancer.

Today, allogeneic hematopoietic cell transplantation (allo-HCT) is performed with curative intent for high-risk blood cancers and bone marrow failure syndromes. The efficacy of allo-HCT lies in the ability of the donor graft to mediate a potent anti-tumor response in transplant recipients, known as the graft-versus-tumor (GVT) effect, coupled with the benefit derived from pre-transplant conditioning[6,7]. This GVT effect is primarily derived from donor T cells but can also be attributed to natural killer (NK) cells and B cells[8]. However, B cell responses against tumors are largely driven by T-B cell interactions, and B cell reconstitution is delayed after allo-HCT[9]. With regards to NK cells, generation occurs early after transplant, and have been demonstrated to possess GVT capability[10,11]. While this treatment holds much promise, poor functionality in haplo-identical recipients early after transplant as well as graft failure due to large number of stem cells that must be infused, especially in the case of T cell depleted (TCD) transplants, remain obstacles for clinical success[12].

The success of allo-HCT is compromised by the development of GVHD, a complication mediated by mature donor T cells present in the graft that respond against normal host tissue. GVHD can occur in 2 phases: acute GVHD (aGVHD) and chronic GVHD (cGVHD), both of which are significant causes of morbidity and mortality after allo-HCT. The incidence of acute GVHD (aGVHD) has been significantly reduced over the past decade. Transplant-related mortality has declined with the implementation of reduced intensity conditioning (RIC) regimens, new GVHD prophylaxis strategies, and the development of molecular methods aiding in early detection of viral and fungal infections in concert with modern anti-

infectious agents[13–15]. However, aGVHD still affects 20% to 70% of allo-HCT patients[16]. Current clinical regimens for GVHD patients are primarily based on nonspecific immunosuppressants for prophylaxis and treatment, such as calcineurin inhibitors or glucocorticosteroids, respectively[17]. These broadly-acting agents fail to induce immune tolerance, increase susceptibility to opportunistic infections, and compromise GVT activity[18]. Research in the field is focused on reducing GVHD without compromising the GVT effect. The current consensus on the initiation of GVHD pathophysiology can be divided into three primary phases:

1) *Host tissue injury caused by conditioning regimens* leads to the release of proinflammatory cytokines. Tissue damage from pre- transplant conditioning regimens results in a prolonged (up to 12 weeks post allo-HCT) increase of various cytokines; these include interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 21 (IL-21), interleukin 23 (IL-23), transforming growth factor β (TGFβ) and tumor necrosis factor α (TNFα)[19–21]. These cytokines are primarily produced by activated dendritic cells (DCs) in response to tissue damage and microbe exposure, in concert with release of damage associated molecular patterns (DAMPs), including high mobility group protein B1 (HMGB-1) and adenosine triphosphate (ATP), as well as pathogen associated molecular patterns (PAMPs), which include lipopolysaccharide (LPS) and peptidoglycan. Both DAMPs and PAMPs can activate APCs, such as DCs and macrophages.

2) *Donor T cell activation* by activated APCs leads to differentiation into effector T cells, such as T helper type 1 (Th1) and T helper type 17 (Th17), both of which are pathogenic and associated with GVHD severity and mortality[22].

3) *Effector T cell migration and target tissue destruction* by activated donor T cells results in the initiation of GVHD[22,23].

A myriad of cytokines, chemokines, receptors, and transcription factors are associated with T cell activation and associated inflammation, hence playing a central role in the development of GVHD. Classically, Th1 cells are believed to play a critical role in the induction of GVHD; although our group and others have demonstrated that Th17 cells also contribute[15]. By targeting Th1 and Th17 specific transcription factors, T-box transcription factor TBX21 (T-bet) and Retinoic acid-related orphan receptor gamma (RORγt), respectively, it was observed that both Th1 and Th17 subsets contribute to GVHD development; yet either lineage alone is sufficient to induce GVHD[25,26]. Thus, both lineages must to be blocked in order to control GVHD. Efficacy of targeting these T cell differentiation pathways at the cytokine level are under investigation in clinical trials. Strategies for protecting/promoting prompt repair of target tissues may also reduce GVHD severity.

I.2. Acute GVHD (aGVHD)

Acute GVHD is manifested by a strong inflammatory component resulting from robust donor T cell activation and expansion. Prior to transplant, conditioning regimens involving chemotherapy and/or irradiation cause damage to host epithelial tissues and subsequent release of danger signals such as chemokines and cytokines. The inflammatory milieu is then amplified by an activated innate immune response, consisting of APCs, natural killer cells (NK cells), neutrophils, and macrophages[23]. Donor CD4 and CD8 T cell recognition of major or minor histocompatibility antigens, directly or indirectly, by host and donor APCs in conjunction with activation of the innate immune response creates a "cytokine storm" consisting of such components as interferon gamma (IFNγ), TNFα, IL-6, IL-12 and IL-23, among others[18,27]. The aforementioned combination of inflammatory factors culminates in T cell infiltration and subsequent destruction of host tissues, namely the skin, lung, liver and gastrointestinal tract (GI tract)[27–30].

I.3. Chronic GVHD (cGVHD)

Chronic GVHD is widely systemic and can affect essentially any of the major organ systems[18,31]. While largely undefined, the origin of cGVHD pathogenesis has been linked to thymic damage caused by conditioning, resulting in aberrant selection and subsequent release of allo/autoreactive T cells[32]. Older patients receiving RIC have also been observed with cGVHD, which is potentially due to reduced thymic reserve/ function[33,34]. The activation of these T cells results in cytokine production and consequential activation of macrophages and fibroblasts. Chronically stimulated donor T cells interact with bone marrow-derived B cells and produce

additional factors contributing to fibroblast proliferation and activation[32,35]. In particular, T follicular helper (Tfh) cells interact with B cells via CD40L-CD40 to promote B cell proliferation, differentiation, and antibody isotype switching[36]. These Tfh-B cell interactions subsequently lead to germinal center formation in which antibody diversification and affinity maturation occur, ultimately leading to an adaptive immune response[32,35,36]. The resultant autoantibody production and tissue fibrosis lead to end organ damage[37].

I.4. The IL-12 Family of Cytokines and Receptors

The IL-12 family of cytokines can direct the donor immune response to execute a range of proinflammatory and immunosuppressive functions that are relevant in GVHD (Fig. 1). They are primarily secreted by cells of myeloid origin in response to inflammatory stimuli, such as microbial products or fungal infections[38]. While part of the type 1 hematopoietin family of cytokines, IL-12 family members are unique in that each member is comprised of two different subunits, or heterodimers, in which either the α or β subunit is shared among the others[39]. The α-subunits include p19 (IL-23/IL-39), IL-27p28 (IL-27), and p35 (IL12/IL-35). The β- subunits include p40 (IL-12/IL-23) and Epstein-Barr virus-induced gene 3 (EBI3) (IL-27/IL-35/IL-39)[40]. Further, each cytokine signals through a distinct heterodimeric receptor that is associated with its cognate subunits: IL-12R (IL-12Rβ2/IL-12Rβ1), IL-23R (IL-23Rα/IL-12Rβ1), IL-27R (IL-27Rα/gp130), IL-39R (IL-23Rα/gp130), and IL-35R (IL-12Rβ2/gp130)[39,40]. The functionality of each respective cytokine and receptor combination ranges from proinflammatory to

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immune suppressive in a host of pathological and physiological conditions. Yet, similar subunits and receptors involved in proinflammatory functions can also form suppressive complexes, as in the case of IL-12Rβ2, involved in IL-12 and IL-35 signaling. Therefore, deciphering the contributions of each cytokine/ receptor subunit combination is critical to understanding the immune response as a whole; the context of allo-HCT is no exception.
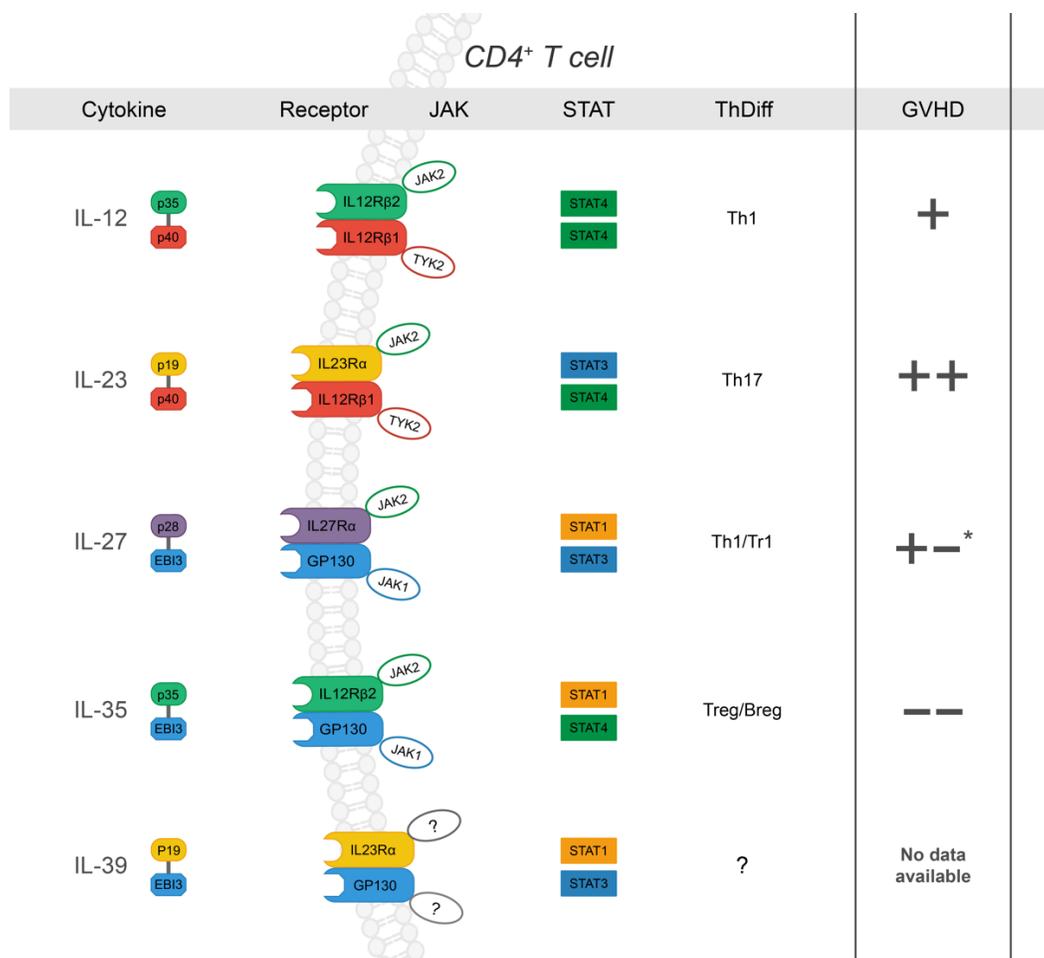
Figure 1. The IL-12 family: cytokines, receptors, JAK-STAT signaling, influence on CD4+ T cell differentiation and subsequent effect on GVHD severity after allo-HCT *('+' and '–' denote increase or decrease in GVHD severity, respectively. Listed from top to bottom)* IL-12 is a heterodimer composed of p35 and p40[41]. Upon ligation, IL-12 signals through IL-12Rβ1 and IL-12Rβ2, which form the receptor complex for IL-12 (IL-12R), subsequently leading to JAK2-STAT4 signal transduction and a positive feedback loop for Th1 differentiation[42–45]. While its role in Th1 differentiation and IFNγ production has been shown to drive GVHD, there are conflicting reports

concerning the specific requirement of IL-12[46,47]; p35 can also associate with EBI3 to form IL-35, and p40 is the shared subunit with IL-23 (+). IL-23 is a heterodimer composed of p19 and p40[48]. IL-23Rα associates with JAK2 to induce primarily STAT3 phosphorylation but also STAT4 to a lesser degree[26,49–51]. IL-23 signaling results in stabilization cues for Th17 cells and has also been implicated exacerbation of intestinal GVHD (++)[52–56]. IL-27, composed of p28 and EBI3, ligation to IL-27Rα/ gp130 promotes IL-10 production by Tr1 cells via STAT1 at early time points post-BMT and plays a role in hindering GVHD- induced inflammation[57]. However, IL-27 also inhibits Treg generation and may promote Th1 differentiation and function (+/-)[58,59]. IL-35 is composed of p35 and EBI3. IL-35 can signal through any combination of IL-12R**β**2 and gp130 was recently described as a potent immunoregulatory cytokine secreted by both T and B regulatory cells[39,60,61]. IL-35 has been reported to suppress GVHD development. IL-39 is the most recent addition to the family and is composed of p19 and EBI3, which signal through STAT3 and STAT1. There are no reports of its function in the context of allo-HCT.
*reported to promote GVHD through increased Th1 and decreased Treg differentiation; yet also dampen GVHD severity via Tr1 during induction phase

I.4.a. IL-12/IL-12R

IL-12 consists of p35 and p40, and acts primarily on NK cells and T cells[33,41]. IL-12Rβ1 binds to IL-12Rβ2 to form the receptor for IL-12 (IL-12R)[41,62]. Upon ligation, IL-12Rβ1 binds to Tyrosine kinase 2 (Tyk2) while IL-12Rβ2 binds to Janus Kinase 2 (JAK2). Tyk2 and JAK2 then phosphorylate tyrosine residues primarily on signal transducer and activator of transcription 4 (STAT4). Ultimately, the STAT4 complex trans locates to the nucleus and binds to the IFNγ promoter; Jun oncogene (c-Jun) is also recruited to the IFNγ promoter via STAT4[42,43,63], potentiating IFNγ transcription and Th1 differentiation. In a STAT4 - dependent manner, IL-12 also promotes expression of Interferon regulatory factor 1 (IRF1) and 4 (IRF4), transcription factors required for Th1 differentiation [64,65]. Notwithstanding the contribution of IL-12 to Th1 differentiation, IL-12/IL-12R also promotes T-cell proliferation and adhesion during activation. It has been reported that IL-12 contributes to expression of Interleukin 2 receptor α (IL-2Rα) by recruiting STAT4 and c-Jun to the promoter of IL-2R, thereby enhancing T cell proliferation[66,67]. IL-12 -induced STAT4 activation also culminates in P-selectin

ligand formation, which augments T cell adhesion during differentiation[68–71].

Furthermore, activation of IL-12/IL-12R signaling induces both positive and

negative feedback queues which can either strengthen or reduce IL-12 signaling,

respectively. For instance, STAT4 activation fosters transcription of IL-12Rβ2 and

Interleukin 18 receptor 1 (IL-18R1), which cooperate to amplify IL-12 signaling

and Th1 cell differentiation. While IL-12R signaling can promote proliferation via

STAT5-JAK2 interactions, evidence exists that STAT5A can suppress IL-12 -

induced Th1 cell differentiation through the induction of Suppressor of cytokine

signaling 3 (SOCS3)[50]. However, this report demonstrated that SOCS3 activity

inhibits IL-12 signaling by binding to the STAT4 docking site of the IL-12Rβ2

subunit[72,73]. Hence, IL-12 is predominately associated with Th1 differentiation,

yet may simultaneously hinder this effect through mobilization of STAT5A

depending on the context of disease or environment. IL-12 promotes the

differentiation of primed CD4$^+$ T cells into Th1 cells, which express *Tbet*, produce

IFNγ, and play a critical role in driving GVHD[25,62]. On the other hand, IL-12

negatively regulates T helper type 2 (Th2) transcription factors and associated

cytokine production[74]. As such, IL-12Rβ2 expression is absent on Th2 cells but

upregulated in Th1; an increase in Th2 differentiation is associated with reduced

acute GVHD yet can exacerbate chronic GVHD[75,76]. In addition, CD40-CD40L

interactions between T cells and APCs can fuel IL-12 production by APCs, which

amplify innate immune cell responses through IFNγ production[77]. With regards to

the IL-12 cytokine itself, the pool of available data is somewhat contradictory in

the context of aGVHD. IL-12 has been reported to drive GVHD due to its

stimulatory effect on Th1 cells[78,79]. IL-12 serum levels in aGVHD patients are increased compared to healthy controls, yet no correlation between higher grade GVHD (II-IV) and IL-12 has been observed[80] (Table 1). Conversely, exogenous IL-12 administration was suggested to be protective in GVHD via an IFNγ-dependent mechanism[81]. Previous studies observed that a single injection of IL-12 at the time of allo-HCT stifles GVHD in myeloablative-conditioned recipients[46,47,81]. The protective or pathogenic role of IL-12 seemingly relies on the dose and timing IL-12 injection, and irradiation type for the recipient conditioning regimen in murine BMT models[82]. In NK cells, IL-12 induces cytotoxic events through STAT4 and subsequent activation of the Perforin 1 (perforin) gene promoter[83]. A recent report a describes IL-12/IL-18 activated donor NK cells mitigate GVHD but enhance GVT activity[84]. Apart from advocating Th1 responses, IL-12 plays a critical role in T follicular helper cell (Tfh) differentiation and function through STAT4 and Tbet[85,86]. Consistent with the crucial role of Tfh cells in cGVHD pathogenesis, administration of anti-p40 mAb in recipient mice significantly reduced Tfh generation and scleroderma manifestations of cGVHD after allo-HCT[57]. Thus, targeting one or more of the IL-12 cytokine/receptor subunits represents a promising therapeutic strategy to reduce cGVHD.

I.4.b. IL-23/IL-23R

IL-23 consists of p19 and p40. The IL-23R is a heterodimer comprised of IL-12Rβ1 and IL-23Rα. IL-23R associates with JAK2 and, in a ligand-dependent manner,

with STAT3. IL-23- induced activation of STAT3 leads to direct binding of phosphorylated STAT3 to IL-17A and IL-17F promoters. STAT3 up-regulates the expression of RORγt, the master transcription factor of Th17, which is critical for the expression of two members of the Interleukin-17 family, IL-17A and IL-17F[87–89]. SOCS3 inhibits JAK2 activity, hence decreasing IL-17A and IL-17F expression[87]. Hence, IL-23 signaling regulates Th17 cells. IL-23 plays an important role in expanding and maintaining the Th17 cell population, a T cell subset involved in homeostatic antimicrobial immune responses as well as in the propagation of many autoimmune diseases[90]. IL-23 is an indispensable factor for promoting pathogenicity of Th17 cells, yet is not required for initial differentiation[91–94]. IL-23 has been shown to control Th17 responses through regulating T cell metabolism. TCR stimulation induces GLUT-1 surface expression and subsequent lactate production, promoting glucose uptake[95,96]. T cells under Th17 polarizing conditions undergo a HIF1-α- dependent metabolic switch to glycolysis, and data indicates that IL-23 might contribute to this effect via PKM2 and HIF1-α[97]. Notably, allogeneic T cells were shown to depend on glycolysis for effector function during GVHD development, yet a connection to IL-23 and glycolysis has not been demonstrated. HIF1-α induction has also been associated with IL-23 production in dendritic cells; this link between HIF1-α and PKM2 has been previously established in cancer cells[98–100]. Therefore, PKM2 may possess more than one function in addition to its role in glycolysis manifested by transcriptional activation as a protein kinase[101]. Taken together, IL-23 signaling through PKM2/STAT3 may directly contribute to the metabolism of Th17 cells and, in concert with IL-6, could

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represent an essential factor for lineage commitment[102]. Glucocorticoid-induced protein kinase 1 (SGK1) is critical for IL-23R expression through deactivating murine Foxo1, which directly represses IL-23R expression[103]. SGK1 is essential for the induction of pathogenic Th17 cells and implicates environmental factors, such as a high-salt diet, as triggers to Th17 development and subsequent tissue inflammation[104]. Lastly, while Blimp-1 IL-23-dependent Blimp-1 enhances Th17 pathogenic factors such as GM-CSF and IFNγ, and co-localizes with RORγt and STAT-3 at *Il23r, Il17a*, and *Csf2* enhancer sites[105]. Studies show IL-23 signaling contributes to the pathogenesis of various autoimmune diseases. In mice, it was demonstrated that bacteria-driven innate colitis is associated with an increased production of IL-17A and IFNγ in the colon. Stimulation of intestinal leukocytes with IL-23 induced the production of IL-17 and IFNγ exclusively by innate lymphoid cells expressing IL-23R, which were demonstrated to accumulate in the inflamed colon. These results identified a previously unrecognized IL-23-responsive innate lymphoid population that mediates intestinal immune pathology and may therefore represent a target in inflammatory bowel disease[106–108]. Intestinal IL-23-responsive innate cells are also a feature of T cell-dependent models of colitis, which resembles many of the features seen in intestinal GVHD with respect to T cell infiltration resulting in inflammation and gut injury. The transcription factor RORγt controls IL-23R expression, as it was shown that Rag/Rorc-null mice failed to develop innate colitis which is dependent on IL-23[106]. In addition, expression of IL-23 and IL-23R was increased in the tissues of patients with psoriasis[109]. Injection

of a neutralizing monoclonal antibody to IL-23p19 in a xenotransplant mouse model showed IL-23-dependent inhibition of psoriasis[109].

I.4.c. IL-39/IL-39R

IL-39 has been proposed to consist of p19 and EBI3, and is the most recent addition to the family[110]. Wang et al. published the first report describing the function of an additional heterodimer that involves p19 complexed with a subunit other than p40. IL-39 is secreted by activated B cells and was demonstrated to be significantly elevated in lupus models compared to other IL-12 members using MRL/lpr mice[110]. The receptor for IL-39 was determined to be formed by dimerization of IL-23R and gp130 and signal through STAT1 and 3. While associated with neutrophil differentiation and expansion, the proinflammatory effects of IL-39 have yet to be fully defined. In a different report by Ramnath et al, IL-39 was shown to be secreted by keratinocytes and contribute to wound healing[111]. While the function of IL-39 may be context dependent, these disparate reports indicate that IL-39 may also act on a broad range of cell types. Hence, further clarification regarding the general role of IL-39 in immunity is required in order to determine its effect in GVHD.

	aGVHD Grade		
	0-1	**2-4**	**References**
IL-12	↑	↑	53
IL-23	↑	↑↑	88
IL-27	↓	↓	123
IL-35	↓	↓↓	126

Table 1: Expression levels of IL-12 family cytokines in aGVHD patients. Representative table of IL-12, IL-23, IL-27 and IL-35 levels detected in the serum of patients with aGVHD. Upward arrows indicate increases compared to healthy donors, while downward arrows indicate decreases.

II. Targeting p40 to reduce acute and chronic GVHD

II.1. Abstract

Graft-versus-host disease (GVHD), in both its acute (aGVHD) and chronic (cGVHD) forms, remains a major obstacle impeding successful allogeneic hematopoietic stem cell transplantation (allo-HSCT). T cells, in particular pathogenic Th1 and Th17 subsets, are a driving force for the induction of GVHD. IL-12 and IL-23 cytokines share a common p40 subunit and play a critical role in driving Th1 differentiation and in stabilizing the Th17 phenotype, respectively. In our current study, we hypothesized that p40 is an essential cytokine in the development of GVHD. By using p40-deficient mice, we found that both donor- and host-derived p40 contribute to the development of aGVHD. Neutralization of p40 with an anti-p40 mAb inhibited Th1- and Th17- polarization *in vitro*. Furthermore, anti-p40 treatment reduced aGVHD severity while preserving the graft-versus-leukemia (GVL) activity. Alleviation of aGVHD was associated with an increase of Th2-differentiation and a decrease of Th1 and Th17 effector T cells in the GVHD target organs. In addition, anti-p40 treatment attenuated the severity of sclerodermatous cGVHD. These results provide a strong rationale that blockade of p40 may represent a promising therapeutic strategy in the prevention and treatment of acute and chronic GVHD while sparing GVL effect following allo-HSCT.

II.2. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only curative option for many hematopoietic neoplastic diseases. However, its efficacy is limited by the occurrence of graft-versus-host disease (GVHD), which remains the major cause of morbidity and mortality in the recipients of allo-HSCT[112,113]. Generally, GVHD attributes its detrimental effects to an immunological attack on recipient parenchymal organs by donor pathogenic cells transplanted in the graft[114]. According to distinct pathophysiology and clinical manifestations, GVHD can be divided into two forms: acute GVHD (aGVHD) and chronic GVHD (cGVHD)[112]. Donor T cell-mediated injuries in the liver, skin and gut are the main characteristics of aGVHD[113,114]. cGVHD manifests itself with more extensive organ involvement and many autoimmune and fibrotic features caused by alloreactive T and B cells[115]. In the clinic, many patients have an overlap syndrome in which features of aGVHD and cGVHD appear together[115,116]. Thus, finding shared therapeutic targets of aGVHD and cGVHD could be a highly efficient method to control GVHD and increase the overall success of allo-HSCT.

T-helper (Th), CD4 cells can differentiate into several distinct subsets, such as: Th1, Th2, Th17, and T regulatory cells (Tregs), depending on the signals encountered. Among these, Th1 and Th17 are the major pathogenic subsets that induce aGVHD and cGVHD[74,117–119]. Paradoxically, exacerbated aGVHD was observed in mice receiving IFNγ−/− or IL-17A−/− T cells[120,121]. This phenomenon occurs because IFNγ−/− T cells produce more IL-17A, whereas IL-17A−/− T cells

produce more IFNγ. These results indicate that Th1 and Th17 lineages are mutually inhibitory, and that each alone is sufficient to induce GVHD. Furthermore, our recent work clearly demonstrates that it is necessary to block both Th1 and Th17 lineages in order to control the development of aGVHD[25].

Cytokines produced by antigen presenting cells (APCs) are critical factors in T-cell activation and differentiation, among which IL-12 and IL-23 play important roles in driving Th1 development and stabilizing the Th17 phenotype, respectively[39]. IL-12 and IL-23 belong to the IL-12 family of cytokines and function as master regulators for innate and adaptive immunity[122]. Both IL-12 and IL-23 are heterodimeric cytokines, which have a shared p40 subunit, paired with either an α chain p35 or p19, respectively[39]. Ustekinumab is an anti-p40 mAb specific for amino acids 1–88 that specifically blocks the interaction of p40 with the IL-12Rβ1 chain in the IL-12 and IL-23 receptors in human[123]. This mAb is approved by the U.S. Food and Drug Administration (FDA) for the treatment of moderate-to-severe psoriasis and shows impressive efficacy in the clinic[123,124].

Increased p40 mRNA has been found in GVHD target organs, which is relevant to the localized production of IFNγ after allogeneic bone marrow transplantation (allo-BMT)[125]. In the current study, we hypothesized that the blockade of p40 could alleviate GVHD by regulating the differentiation and function of Th1 and Th17 cells after (allo-BMT). Here, we provide evidence that both donor- and host-derived p40 contribute to the development of aGVHD. Overall, neutralizing p40 efficaciously

reduces Th1 and Th17 polarization and alleviates the severity of aGVHD and cGVHD while preserving the graft-versus-leukemia (GVL) activity.

II.3. Materials and methods

II.3.a. Mice

C57BL/6 (B6; H-2b), B6.Ly5.1 (H-2b), BALB/c (H-2d), FVB (H-2q) mice were purchased from the National Cancer Institute/National Institute of Health. C3.SW-H2b/SnJ (H-2b), B10.D2 (H-2d), and p40−/− mice on B6 background mice were purchased from the Jackson Laboratory. All animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at Medical University of South Carolina (MUSC, Charleston, SC). Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at MUSC.

II.3.b. Cell staining and flow cytometric analysis

Mononuclear cell isolation from recipient spleen and liver was carried out as previously described[25]. Standard flow cytometric surface staining was used. For intracellular cytokine staining, cells were stimulated with 50ng/ml PMA (Sigma-Aldrich), 500ng/ml ionomycin (Sigma-Aldrich) and 0.7 µl/ml Golgi Plug (BD Biosciences) incubated at 37 °C for 4 hours before staining as recommended by the manufacturer. The flow antibodies used were as follows: CD4-V450 (clone RM4-5, BD), CD8-APC-Cy7 (clone 53-6.7, BD), B220-PE (clone RA3-6B2, eBioscience), H2Kb-APC (clone AF6-88.5.5.3, eBioscience), H2Kq-Alexa Fluor

647 (clone KH114, Biolegend), CD45.1-FITC (clone A20, BD), IFNγ- Percp-Cy5.5 (clone XMG1.2, eBioscience), IL-4-PE (clone 11B11, BD), IL-5-PE (clone TRFK5, eBioscience), IL-17-PE-Cy7 (clone TC11-18H10.1, Biolegend), CXCR3-Biotin (clone CXCR3-173, eBioscience), PD-1-PE (clone J43, eBioscience), CXCR5-PE-Cy7 (clone SPRCL5, eBioscience), Ly9.1-Biotin (clone 30C7, BD), Streptavidin-PE-Cy7 (BD), and Streptavidin-APC-Cy7 (BD). Stained cells were analyzed using Diva software, LSR II (BD Biosciences, San Jose, CA) and FlowJo (TreeStar, Ashland, OR).

II.3.c. In vitro generation of Th1 and Th17 cells

Splenocytes were isolated from normal B6 mice and were CD8/CD25-depleted using negative selection. CD8/CD25-depleted splenocytes were cultured with IL-2 (2ng/ml) or anti-CD3 (1μg/ml) plus IL-12 (10ng/ml), IFNγ (1000U/ml) and anti-IL-4 (10μg/ml) for Th1 generation. For Th17-generation, cells were stimulated with anti-CD3 (1μg/ml) in the presence of IL-6 (10ng/ml), TGF-β (5ng/ml), anti-IFNγ (10μg/ml) and anti-IL-4 (10μg/ ml). Cells were cultured in a 24-well plate in the presence of isotype or anti-p40 mAb at the concentrations indicated for 3 days, and then tested for the expression of intracellular IFNγ and IL-17. Supernatant from the cell culture was harvested, diluted 2 times, and measured IFNγ production using ELISA.

II.3.d. GVHD models

T cells were purified from spleen and lymph node cells by negative selection using magnetic beads as previously described[25]. T-cell purity was typically more than 95%. T-cell- depleted bone marrow (TCD-BM) was prepared by complement lysis as previously described and transferred at 5×10^6/mouse[25]. B6→BALB/c (1 × 106 T cells) and FVB→B6 (2×10^6 T cells) MHC-mismatched BMT models were used for aGVHD experiments. B10.D2 →BALB/c (5×10^6 splenocytes) MHC-matched, minor histocompatibility antigen (MiHA)-mismatched BMT model was used for cGVHD experiments. Recipient mice (8–10 weeks of age) were conditioned with total body irradiation (TBI) administered at 700cGy for BALB/c < 23g or 750cGy for those ≥ 23g of initial body weight and 950cGy for B6 mice < 23g or 1000cGy for those ≥ 23g using an X- RAD 320 X-ray Irradiator (Precision X-ray Inc., North Branford, CT). Within 24 hours after TBI, recipient mice were transplanted with TCD-BM alone or together with purified T cells or splenocytes. The recipient mice in aGVHD experiments were monitored for body weight and survival twice weekly. The recipient mice in cGVHD experiments were monitored for survival and clinical signs such as weight loss, posture, activity, fur texture, and skin integrity weekly[126].

II.3.e. Leukemia/lymphoma models and Bioluminescent imaging (BLI)

B6→BALB/c MHC-mismatched and C3.SW-H2b/SnJ→B6 MHC-matched, MiHA-mismatched BMT models were used to examine the GVL effects of donor T cells. B6→BALB/c (1 × 10^6 T cells) recipients were injected with 2×10^3 luciferase-transduced A20 B cell lymphoma cells, whereas C3.SW-H2b/SnJ→B6 (3 × 10^6 CD44− T cells) recipients with 1×10^5 C1498 luciferase-transduced acute myeloid

leukemia cells at the time of BMT. The recipient mice were monitored for body weight and survival twice weekly, and clinical score and tumor growth weekly. Mice were injected i.p. with luciferin (150mg/kg) and then anesthetized with isoflurane gas using Xenogen XGI Gas Anesthesia System. The BLI was performed by using the IVIS system (Xenogen). The imaging data were analyzed using Living Image Software (Xenogen). The mortality caused by tumor relapse or GVHD was distinguished by BLI signal indicative of tumor relapse and weight loss indicative of GVHD.

II.3.f. Antibody treatment in vivo

Anti-p40 mAb (CNTO3913) and its isotype control (CNTO6601) were kindly provided by Janssen Research & Development, LLC, Spring House, PA, USA. These mAbs were injected i.p. at 25mg/kg body weight on day 0 and then 12.5 mg/kg twice weekly for 4 weeks after BMT.

II.3.g. Statistics

The percentages of body weight change, percentages of cell types, absolute numbers of cells, and clinical scores in WT vs. KO, or isotype vs. anti-p40 treatment were compared using 2-tailed student t test. The Log-rank test was used for survival analysis.

II.4. Results

II.4.a. Donor- and host-derived p40 contribute to aGVHD severity. To

examine the role of p40 produced by donor cells in mediating aGVHD, we

performed an allo-BMT using p40-deficient (p40−/−) mice on B6 background as

donors and tested the effects of p40 deficiency on donor BM and T cells in the

development of aGVHD. The BALB/c recipients of p40−/− BM and T cells had

significantly improved survival compared to those that received WT cells ($p =$

0.046) (Figure 1A), yet weight loss was similar ($p = 0.184$) (Figure 1B) between

the two groups. Consistent with alleviation of aGVHD, the recipients of the

p40−/− graft had improved donor CD4 T- and B-cell reconstitution compared to

those recipients of WT graft ($p = 0.04$ and 0.04, respectively) (Figure 1C).

Furthermore, the function of T and B cells in the recipients of p40−/− graft was

significantly improved compared to those in the recipients of WT graft ($p = 0.03$

and 0.001, respectively) (Figure 1D). These results indicate that donor-derived

p40 contributes to the development of aGVHD after allogeneic BMT. Because

p40 can be produced by either donor or host APCs and host APCs are critical to

inducing aGVHD[127,128], we assessed the role of host-derived p40 on the

development of aGVHD. Host-derived p40 had little or no effect on donor BM

engraftment, because WT and p40−/− recipients infused with BM alone had

comparable outcomes (Figures 2A and 2B) and similar CD4, CD8 T- and B-cell

reconstitution 80 days post BMT ($p =0.33$, 0.78, and 0.32, respectively) (Figures

2C and 2D). However, p40−/− recipients transferred with donor allogeneic T cells

had significantly improved survival ($p = 0.015$) (Figure 2A) and increased donor

B-cell reconstitution ($p = 0.02$) (Figures 2E and 2F). These data suggest that

host- derived p40 also significantly contributes to the development of aGVHD.
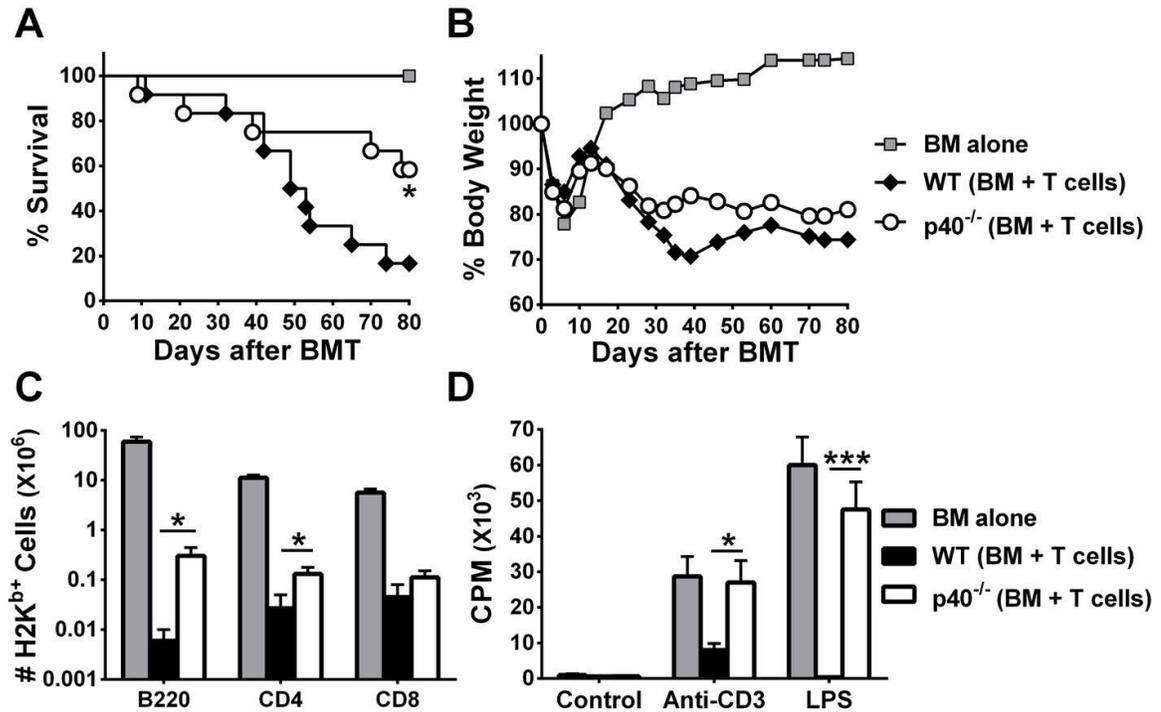
Figure 1. Role of donor-derived p40 in aGVHD
BALB/c mice were lethally irradiated at 700–750 cGy and transplanted with WT graft (5 × 106 BM and 5 × 106 T cells) or p40−/− graft from mice on B6 background. Recipient mice were monitored for survival (A) and body weight changes (B) over time. Data were pooled from 2 replicate experiments with total 10–12 mice per group. (C) Spleens were collected from each survived recipient 80 days after BMT, and stained for expression of CD4, CD8, B220 and H2Kb (donor marker). Absolute numbers of CD4+, CD8+ or B220+ donor cells were calculated and presented in a per spleen basis. (D) T- and B-cell function was measured by stimulating spleen cells with anti-CD3 or LPS for 3 days. Proliferation was assessed using [3H]-TdR incorporation assay. Data shown as Mean ± 1 SD. *$p < 0.05$, ***$p < 0.001$.
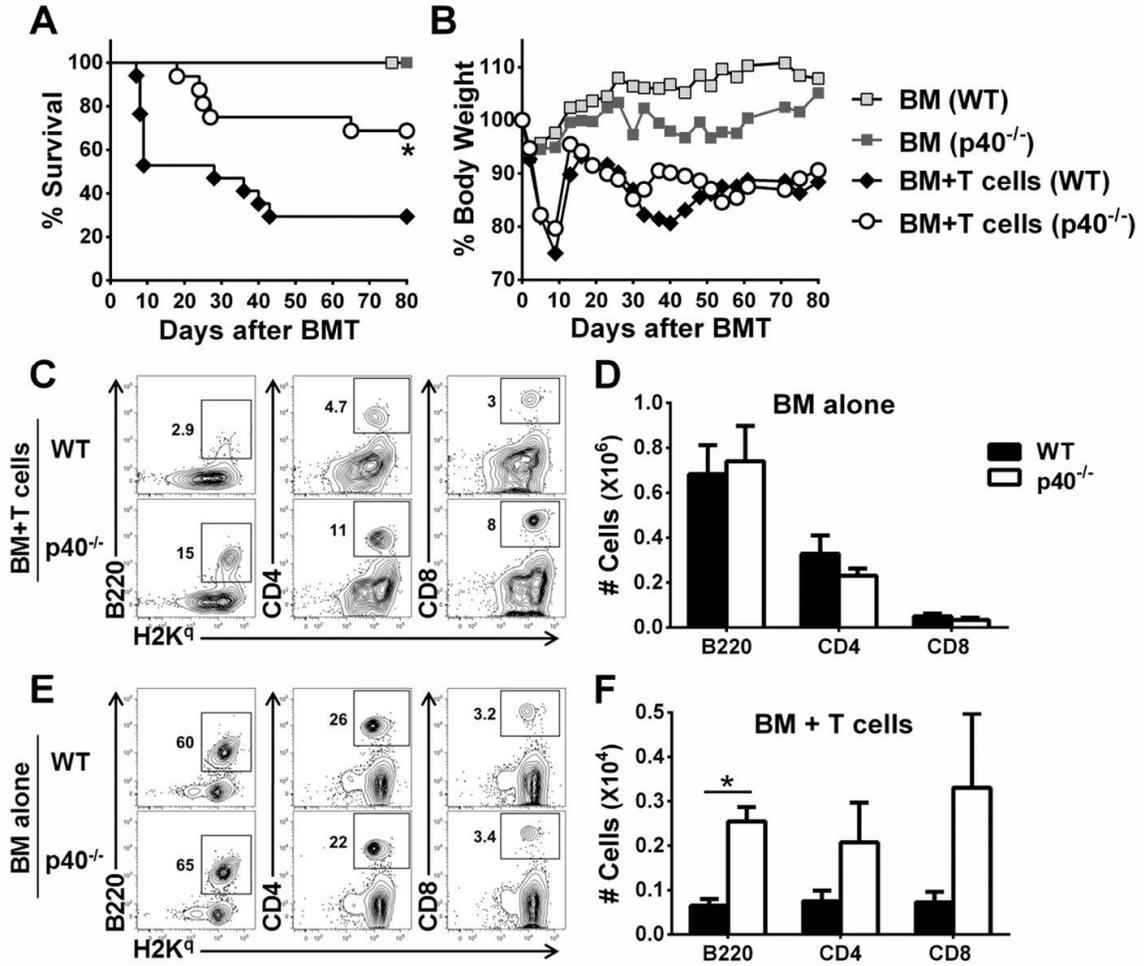
Figure 2. Role of host-derived p40 in aGVHD

WT or p40−/− B6 mice were lethally irradiated at 950–1000 cGy. These recipients were then transplanted with 5 × 106/mouse TCD-BM alone or with 2 × 106/mouse of total T cells isolated from FVB donor mice. Recipient mice were monitored for survival (A) and body weight changes (B) over time. Data were pooled from 3 replicate experiments with total 16 mice per group. Upon completion of the experiment on day 80, spleens were collected from surviving recipients for cell counting and FACS analysis. Percentages or absolute numbers of donor-derived (H2Kq+) CD4, CD8 T cells and B cells were shown in BM alone recipients (C–D) and BM plus T cell groups (E–F). The data present 3–5 mice in each group from one of 3 replicate experiments. *$p < 0.05$.

II.4.b. Anti-p40 mAb inhibits the activity of IL-12 and IL-23 in T-cell

polarization *in vitro*. Using gene deletion (p40−/− mice), the data presented

above indicate that IL-12/23p40 may be a valid therapeutic target for the control

of aGVHD. To translate these findings to a more practical, therapeutic approach,

we then focused our attention on systemically neutralizing IL-12 and/or IL-23 p40

using an anti-p40 mAb. We first tested whether anti-p40 mAb could block Th1

and Th17 polarization *in vitro* by antagonizing the activity of IL-12 and IL-23.

Indeed, anti-p40 mAb inhibited IFNγ production by T cells that were stimulated

with IL-12 plus IL-2 or anti-CD3 under Th1 polarizing conditions in a dose-

dependent manner ($p = 0.007$ and 0.02, respectively) (Figure 3A). Anti-p40

treatment also inhibited intracellular expression of IFNγ and IL-17 in T cells

stimulated by IL-12 (Th1 condition) and IL-23 (Th17 condition), respectively

(Figure 3B and 3C). These data indicate that anti-p40 mAb is efficacious in

suppressing Th1 and Th17 polarization *in vitro*.
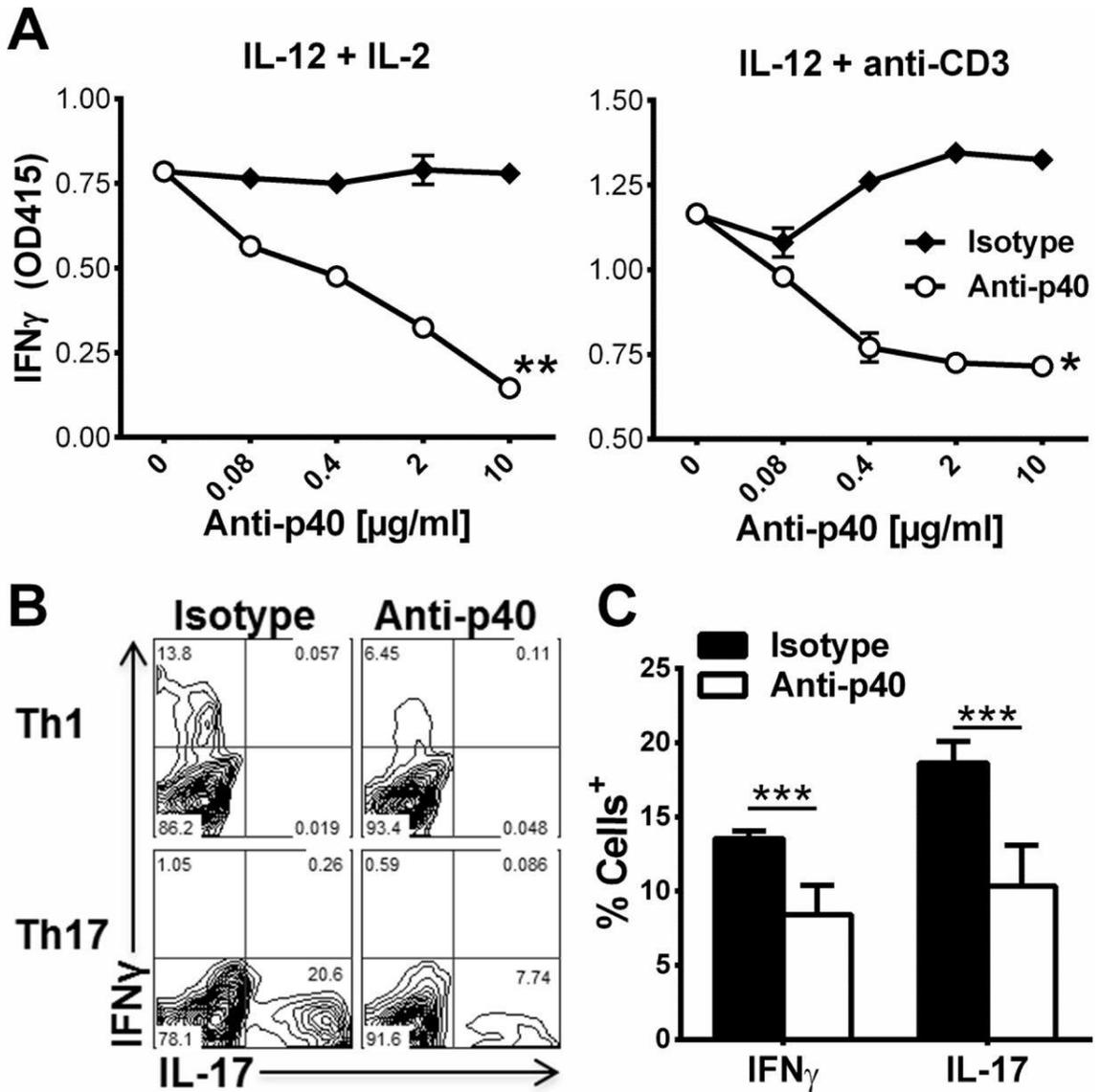
Figure 3. Effect of anti-p40 mAb on T-cell polarization *in vitro*
(A) CD8/CD25- depleted splenocytes were cultured with IL-2 or anti-CD3 plus IL-12 in the presence of isotype or anti-p40 mAb at the concentrations indicated for 3 days (details were described in the Material and Methods). Culture supernatants were harvested and IFNγ was measured by ELISA. The levels of IFNγ were reflected by average OD415 in duplicate wells from one of two replicate experiments. (B) CD8/CD25-depleted splenocytes were cultured in Th1 conditions or Th17 conditions for 3 days. Cultured cells were harvested and stained for the expression of surface CD4 and intracellular IFNγ and IL-17 on gated CD4+ cells (C–D). The data present 5 replicate wells in each group from one of two replicate experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

II.4.c. Neutralizing p40 alleviates aGVHD. Since anti-p40 mAb significantly

reduced Th1 and Th17 polarization *in vitro*, we further hypothesized that

neutralizing p40 would attenuate aGVHD after allo-BMT. By using the B6 →

BALB/c BMT model, we found that systemically giving anti-p40 mAb significantly

improved recipient outcomes as reflected by significantly better survival and less

body weight loss compared to WT controls (p = 0.004 and 0.001, respectively)

(Figures 4A and 4B). These data demonstrate that systemic administration of

anti-p40 mAb to neutralize p40 is an effective way to attenuate aGVHD severity

after allo-BMT.
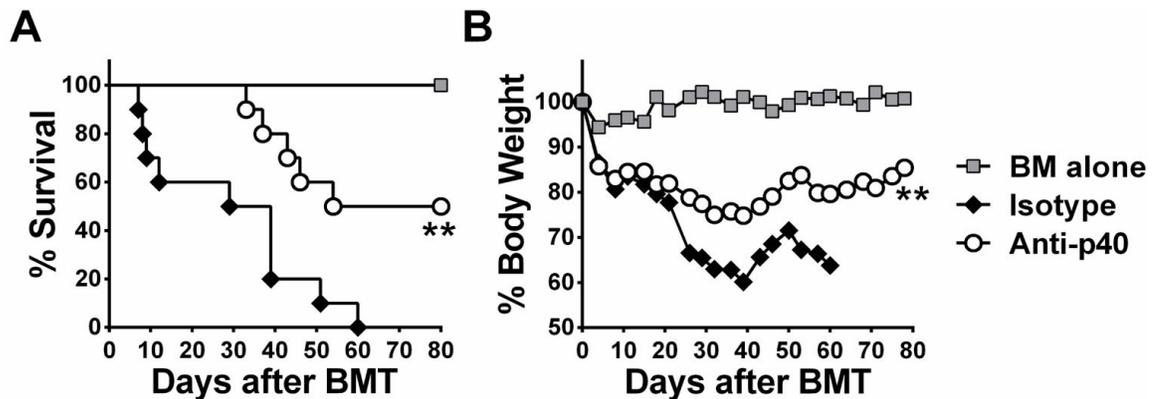
Figure 4. Effect of neutralizing p40 on aGVHD development
BALB/c mice were lethally irradiated at 700cGy and transplanted with 5 × 106/mouse TCD- BM alone or together with total T cells at 1 × 106/mouse from WT B6 mice. Recipient mice were injected i.p. with isotype or anti-p40 mAb at 25 mg/kg body weight on day 0 and then 12.5 mg/kg twice weekly for 4 weeks. Recipient survival (A) and body weight changes (B) were monitored over time. Data were pooled from 2 repeated experiments with 10 mice in each group. **p < 0.01.

To further understand the mechanism by which neutralizing p40 reduces aGVHD severity in vivo, we investigated how anti-p40 treatment affects donor T-cell activation, differentiation and migration after allo-BMT. Treatment with anti-p40 mAb had no impact on IFNγ- or IL-17-producing T cells, but significantly increased IL-4/5-producing CD4 T cells in recipient spleens at 14 days post-BMT (p = 0.028) (Figures 5A and 5C). However, anti-p40 treatment significantly reduced IFNγ-producing CD4 and CD8 T cells in the recipient liver, a major target organ of aGVHD (p = 0.012 and 0.043, respectively) (Figures 5B and 5D). In addition, neutralization of p40 also significantly reduced the number of IL-17-producing CD8 T cells in the recipient livers (p = 0.047) (Figure 5D). Anti-p40 treatment had no impact on Treg differentiation between the two groups (data not shown). Thus, in murine models, neutralizing p40 promoted Th2-differentiation while reducing IFNγ and IL-17 production in GVHD target organs after allo-BMT.

Because donor T-cell migration to target organs is an essential step for the development of aGVHD[129], we further tested the migratory ability of donor T cells during p40 neutralization. As shown in Figure 5E, there were significantly fewer CD4 and CD8 donor T cells in recipient liver 14 days after anti-p40 treatment (p = 0.03 and 0.016, respectively). Given CXCR3 is a key chemokine receptor modulating T cell migration to the liver, we measured CXCR3 expression on donor T cells and found that anti-p40 treatment significantly decreased CXCR3 expression on donor CD4, but not CD8, T cells (p = 0.004 and 0.933, respectively) (Figure 5F). These data suggest that anti-p40 treatment inhibits donor T-cell migration into the liver partially through down-regulation of CXCR3

expression. A trend of reduced pathology scores in the GVHD target organs, such as liver and gut, was observed in the recipients receiving anti-p40 mAb, although the difference did not reach statistical significance at 14 day post-BMT (data not shown).

II.4.d. Neutralizing p40 maintained T-cell-mediated GVL activity after allo-BMT. Preserving the GVL effect is of paramount importance when BMT is used as immunotherapy for hematologic malignances. Hence, we next asked whether neutralizing p40 would maintain GVL activity while alleviating aGVHD. Using B6 → BALB/c BMT model supplemented with A20 B-cell lymphoma cells, the recipients of BM alone plus A20 died within 2 weeks due to tumor relapse, and anti-p40 treatment without T cell infusion had no impact on tumor relapse (Figures 6A–C). The recipients transplanted with additional donor T cells survived significantly longer than those receiving BM alone, illustrated by little or no tumor signal. However, the majority of these recipients treated with the isotype control died from GVHD, as reflected by substantial weight loss and lack of BLI signal. We observed that anti-p40 treatment increased the rate and duration of recipient survival, although not significantly, and resulted in a 50% long-term survival of recipients without GVHD or tumor relapse (Figures 6A–C).
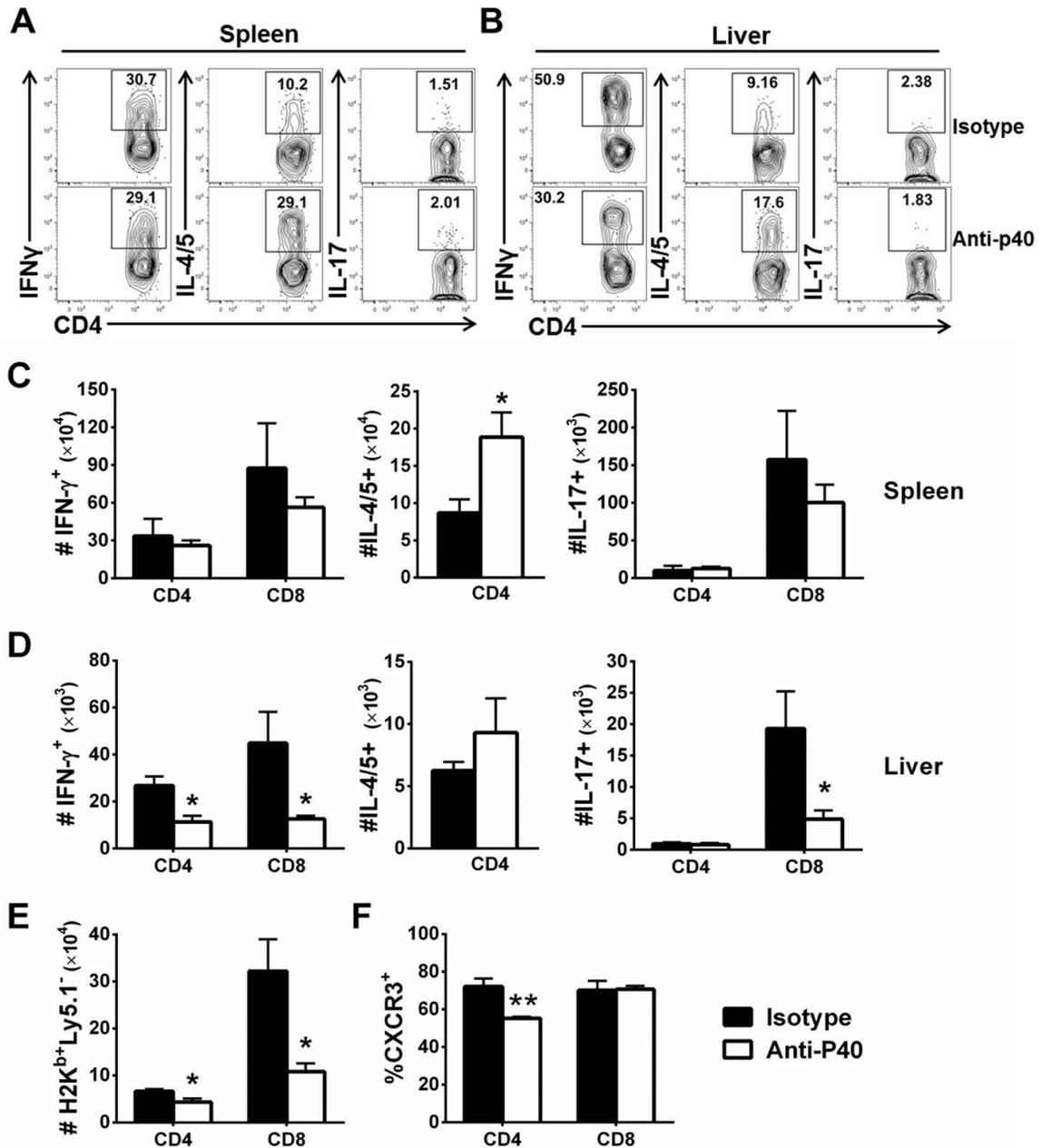
Figure 5. Effect of neutralizing p40 on donor T-cell differentiation and migration
BALB/c mice were lethally irradiated at 700cGy and transplanted with 5 × 106/mouse TCD- BM alone from Ly5.1 B6 mice or together with total T cells at 1 × 106/mouse from Ly5.2 B6 mice. Recipient mice were treated with isotype or anti-p40 mAb as described in the Material and Methods. Fourteen days post-BMT, recipient spleens and livers were collected. Mononuclear cells were isolated for cell counting and FACS staining. (A–B) The expression of intracellular IFNγ, IL-4/5, and IL-17 is shown on gated donor H2kb+Ly5.1−CD4+ cells from the spleen and liver. Absolute numbers of IFNγ, IL-4/5, and IL-17 positive donor CD4 or CD8 T cells were shown in the spleen (C) or liver (D). (E) Absolute numbers of donor pathogenic H2kb+Ly5.1−CD4 or CD8 T cells in recipient livers were shown. (F) Percentage of CXCR3+ cells are shown on gated donor CD4 or CD8 T cells in recipient spleens. The data are pooled from 2 replicate experiments with 10 mice in each group. *$p < 0.05$ and **$p < 0.01$.
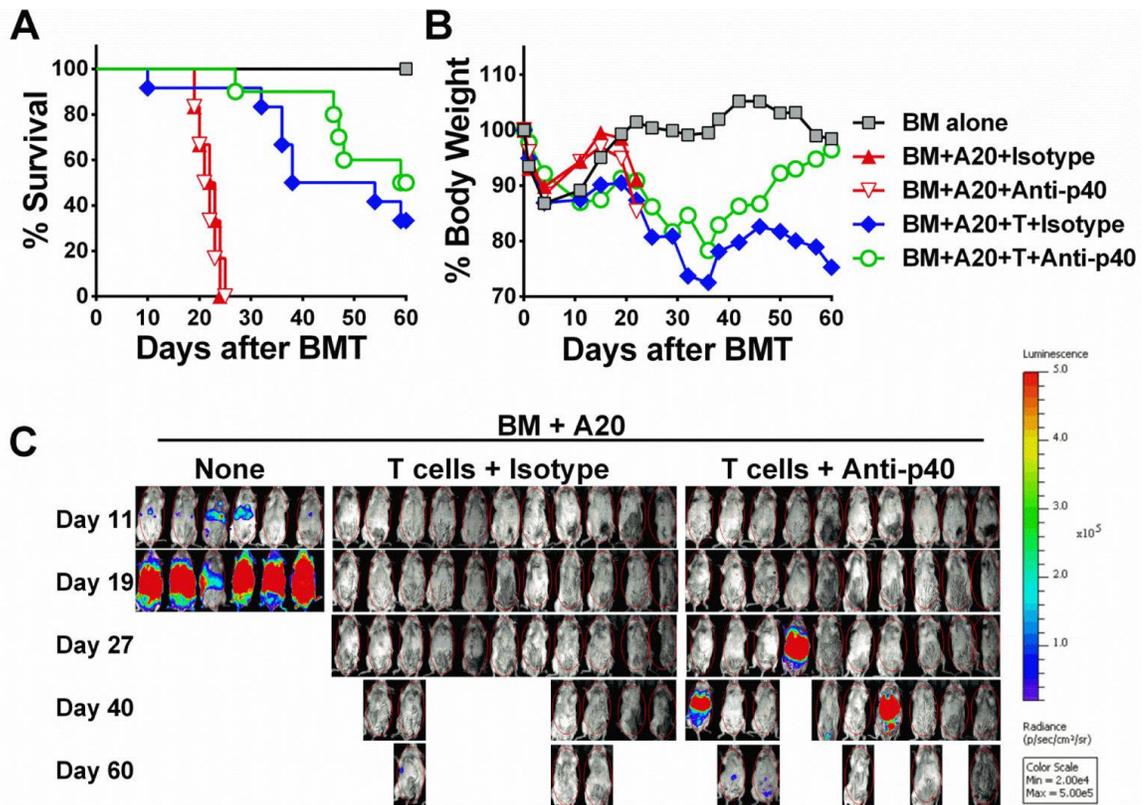
Figure 6. Role of neutralizing p40 on the GVL activity in MHC-mismatched BMT
BALB/c mice were lethally irradiated and transplanted with 5 × 106/mouse TCD-BM alone or plus purified T cells from WT B6 mice at 1 × 106/mouse. Recipient mice were also infused with 2 × 103 luciferase-transduced A20 cells at the day of BMT. Recipient mice were treated with isotype or anti-p40 mAb as described in the Material and Methods. Recipients were monitored for survival (A) and body weight change (B) for 60 days after transplant. Tumor growth was monitored using BLI on the dates indicated (C). The data are pooled from 2 replicate experiments with 10 mice in each group.

Because MHC-matched, MiHA-mismatched BMT models provide additional clinical relevance, we next tested the effects of anti-p40 treatment on GVL activity by using a C3.SW-H2b/SnJ→B6 BMT model with C1498 murine acute myeloid leukemia cells. The recipients of BM alone had leukemic relapse and all mice were dead by day 40 after BMT (Figures 7A–C). The addition of donor T cells prevented leukemia relapse regardless of the treatment, and the mortality of recipients was essentially caused by GVHD. However, anti- p40 treatment

significantly prolonged recipient survival 50 days post-BMT (p = 0.026) (Figure

7A). Taken together, these data indicate that anti-p40 treatment alleviated

aGVHD and largely preserved GVL activity after allogeneic BMT.
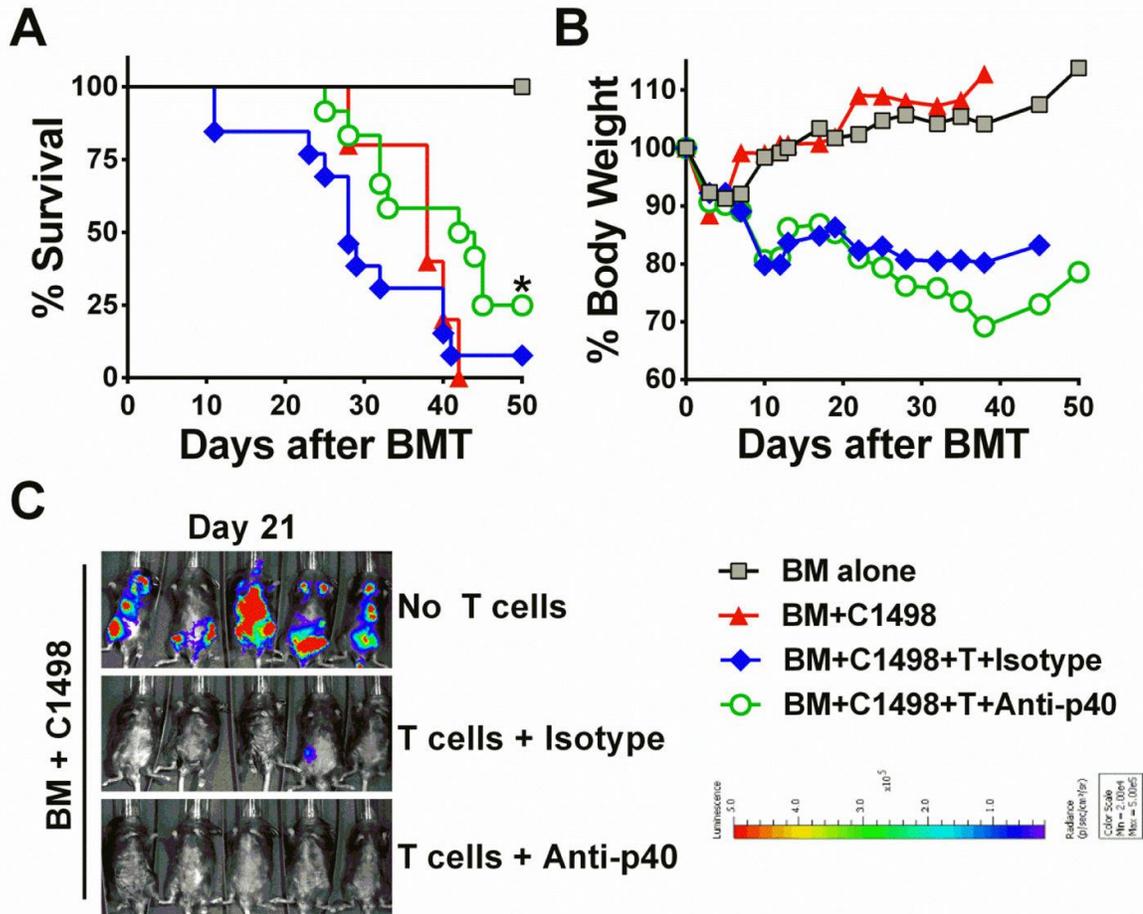
Figure 7. Role of neutralizing p40 on the GVL activity after MHC-matched and MiHA-mismatched BMT
B6 mice were lethally irradiated and transplanted with 5 × 106/mouse TCD-BM alone or with purified CD44− T cells from C3.SW-H2b/SnJ mice at 3 × 106/mouse. Recipient mice were also infused with 1 × 105 luciferase-transduced C1498 cells at the day of BMT. Recipient mice were treated with isotype or anti-p40 mAb as described in the Material and Methods. Recipients were monitored for survival (A) and body weight change (B) for 50 days after transplant. Tumor growth was monitored using BLI on day 21 (C). The data are pooled from 2 replicate experiments with 12 mice in each group. *p < 0.05.

II.4.e. Neutralizing p40 alleviates cGVHD. We next wanted to determine

whether neutralizing p40 could attenuate cGVHD. Using an MHC-matched but

MiHA-mismatched B10.D2 → BALB/c model of sclerodermatous cGVHD, we

found that anti-p40 treatment significantly alleviated the clinical manifestations of

recipients at day 50 post-BMT (p = 0.036) (Figure 8A). In contrast to 30%

mortality seen in the isotype group, all of the recipients survived with anti-p40

treatment (p = 0.06) (Figure 8B). Consistent with the observed alleviation of

cGVHD clinical manifestations, neutralizing p40 significantly increased donor

derived CD4 and CD8 T-cell, but not B-cell, reconstitution in the recipient spleens

60 days after BMT (p = 0.03, 0.036, and 0.143, respectively) (Figures 8C and

8D). In addition, the generation of follicular T helper (Tfh) cells, which are critical

for germinal center (GC) formation and cGVHD induction[130], was inhibited by

blocking p40 (p = 0.014) as measured by co-expression of CXCR5 and PD-1

(Figures 8C and 8D). These data demonstrate that neutralizing p40 reduces the

severity of cGVHD and improves immune reconstitution by constraining Tfh

generation after allo- BMT.
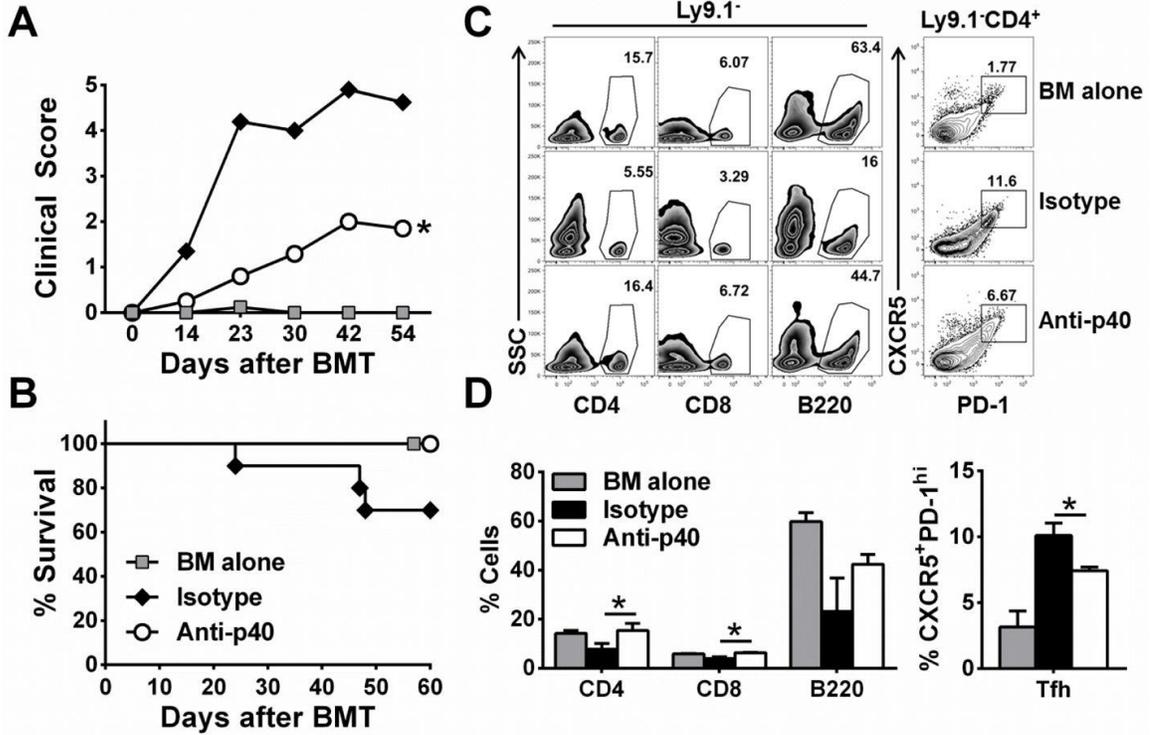
Figure 8. Effect of neutralizing p40 on cGVHD
BALB/c mice were lethally irradiated and reconstituted with 5 × 106/mouse TCD-BM alone or plus 5 × 106/mouse splenocytes from B10.D2 donors. Recipient mice were treated with isotype or anti-p40 mAb as described in the Material and Methods. Recipients were monitored for clinical manifestations (A) and survival (B). Sixty days after transplant, recipient spleens were harvested and subjected to flow staining for the expression of Ly9.1 (recipient marker), CD4, CD8, B220, CXCR5 and PD-1. The representative flow figures (C) and statistic bar graph (D) are shown. The data are pooled from 2 replicate experiments with 10 mice in each group. $*p < 0.05$.

II.5. Discussion

The current study provides data to demonstrate that both donor- and host-derived p40 contributes to the development of aGVHD, and that neutralization of p40 reduces aGVHD severity while preserving GVL activity. Treatment with anti-p40 mAb inhibits Th1 and Th17 polarization in vitro and promotes Th2 differentiation in murine GVHD models. Anti- p40 treatment also inhibits donor T-cell migration into GVHD target organs such as the liver. In addition, anti-p40 treatment improves recipient clinical manifestations of cGVHD.

Host APCs play a critical role in the initiation of aGVHD, while donor APCs also contribute to the activation and differentiation of donor allogeneic T cells[131,132], and thus to the development of GVHD[133]. IL-12 and IL-23 are mainly produced by activated APCs, such as dendritic cells (DCs) and macrophages[39]. Along with modulating T-cell differentiation, IL-12 and IL-23 also act in an autocrine manner to enhance the ability of APCs to present antigen to T cells[122]. Our data illustrated that genetic deficiency of p40 in the donor or host significantly improved recipient survival after allo-BMT (Figures 1 and 2), indicating that both host APC- and donor APC-derived IL-12/23 promotes GVHD development. Consistent with this, we found that neutralization of p40 effectively inhibited Th1 and Th17 polarization in vitro (Figure 3). In contrast, we were unable to demonstrate the effectiveness of p40 neutralization with mAb treatment in blocking Th1- or Th17- differentiation after allo-BMT (Figure 5). The potential explanation would be: 1) the dose of anti-p40 tested may not be sufficient to

block p40 in vivo; 2) other cytokines may compensate for the function of IL-12 and IL-23 in the generation and maintenance of Th1 and Th17 subsets. However, anti-p40 treatment increased Th2-differention and decreased the numbers of Th1 and Th17 cells in recipient liver in the B6 → BALB/c model, which likely contributed to the alleviation of GVHD. Our observation is also consistent with a previous study that showed neutralizing IL-12 by anti-IL-12 Ab could attenuate aGVHD by driving Th2-differentiation in mouse models[78].

T-cell migration into peripheral organs is required for the induction of GVHD. We found that anti-p40 treatment significantly reduced donor T cells present in the liver, a key aGVHD target organ, which likely contributed to the alleviation of aGVHD (Figure 5). It is well accepted that the chemokine systems plays an important role in facilitating donor T cell migration to GVHD target organs. Among these, CXCR3 has been shown to play a pivotal role in Th1 migration[134]. In accordance with these observations, we found that the expression of CXCR3 on donor CD4 T cells was significantly reduced with anti-p40 mAb treatment, possibly due to the requirement of Th1-commitment to promote CXCR3 expression[134,135]. Comparable CXCR3 expression in CD8 T cells was seen regardless of anti-p40 treatment, suggesting that CD8 T-cell trafficking is likely less dependent on CXCR3 expression, which is consistent with a published report that anti-CXCR3 treatment reduced CD4 but not CD8 T-cell infiltration in the central nervous system in response to virus infection[135]. However, there is another explanation that is relevant to the GVHD model used. In B6 to BALB/c model, GVHD is induced in a CD4-dependent manner, so we are more likely to

see differences in CD4 T cells. Whereas in MiHA-mismatched C3.SW- H2b/SnJ to B6 model used in Figure 5, GVHD is driven predominately by CD8 T cells. Thus, it is possible that CXCR3 may differentially be expressed on CD4 vs. CD8 donor T cells in different models, which might be responsible for the distinct effects of anti-p40 on the migration of CD4 vs. CD8 T cells.

Although significantly attenuated aGVHD was seen when blocking p40, the efficacy of preventing aGVHD was not as potent as was observed in our previous study with T-bet and RORγt blockade[25]. The moderate alleviatory effect of anti-p40 treatment on aGVHD could be due to the insufficient treatment dose and compensatory cytokine mechanisms in vivo. In addition, p40 has also been shown to be a minor regulator of inflammatory responses, suggesting that blockade of p40 reduces IL-12 and IL-23 inflammatory cytokines but simultaneously decreases the small regulatory effect of p40. The p40 subunit is promiscuously expressed, while p35 and p19 are only secreted as part of the heterodimer when p40 is also produced by the same cells. As a result, p40 is often secreted in larger quantities than p35 and p19[39,122,136]. In mice, free p40 can form homodimers or present as free monomers, both of which have been shown to be natural inhibitors of IL-12[122,136–138]. This could explain the potent effect of anti-p40 in polarizing conditions in vitro yet only moderate effects in vivo in regard to blockade of Th1/Th17 differentiation. The potentially inhibitory roles of p40 homodimers or monomers may also explain why targeting p19 seems more effective than targeting p40 to block the function of IL-23 in the prevention of GVHD reported by others[52].

However, it is worth noting that homodimers and monomeric p40 have not been found in humans, indicating neutralization of p40 could possibly be more effective in human diseases[122]. Indeed, our colleagues reported that ustekinumab treatment of one patient with advanced, refractory GVHD showed a brief clinical remission accompanied by a decrease in IFNγ production and an increase in IL-4 secretion by whole blood cells stimulated ex vivo with PMA/ionomycin[139]. In addition, ustekinumab has been found to be effective in several Th1/Th17 mediated human diseases, although the shift toward Th2-differentiation has not been observed, such as in psoriasis[123,140–142].

Of clinical importance, we assessed the T cell mediated GVL activity upon neutralization of p40 in two preclinical BMT models. When compared with BM alone, additional T cells mediated robust anti-tumor effect regardless of anti-p40 or isotype treatment (Figures 6 and 7), indicating that anti-p40 treatment largely preserved GVL activity. The preserved GVL effect could be attributed to the maintained total donor T cells (data not shown) and the numbers of IFNγ producing donor T cells (Figure 5C) in recipient spleens after anti-p40 treatment. Furthermore, a previous study has demonstrated that p40 is not required for the CTL function of CD8 T cells in vivo[143]. Taken together, we concluded that inhibition of p40 attenuates GVHD while sparing GVL activity following allo-BMT.

Considering that both Th1 and Th17 subsets contribute to the development of cGVHD, we evaluated the effect of neutralizing p40 on the development of cGVHD. We found that anti- p40 treatment significantly improved the clinical

manifestations and immune reconstitution without GVHD related mortality (Figure 8). IL-12 stimulation is critical for DC-mediated priming of naïve CD4 T-cell into Tfh[144], and subjects deficient for IL-12Rβ1, a receptor for p40, displayed reduced circulating Tfh, memory B cells and impaired GC formation[85]. Consistent with this, we found that Tfh generation was reduced with anti-p40 mAb treatment, which possibly contributed to the reduced severity of cGVHD. The current study agrees with a recent report that anti-p40 treatment attenuates cGVHD in mice[145].

We illustrate that blocking IL-12/23p40 attenuates cGVHD. Paradoxically, a previous study shows exogenous IL-12 administration reduces the severity of cGVHD in a systemic lupus erythematosus (SLE) model[146], in which a large numbers of splenocytes from DBA/2 donors were transferred into non-irradiated B6D2F1 recipients. There are several differences in these two models: 1) The pathophysiology of the SLE cGVHD model is characterized by B-cell hyper-activation, as well as autoantibody and immune complex-mediated glomerulonephritis, in which Th2 cells are the major pathogenic T cells contributing to allo- reactive B-cell activation. The mechanism of reducing cGVHD by administration of IL-12 in that study was likely because that IL-12 reduced Th2-differentiation and further inhibited the pathogenesis of allo-reactive B cells. On the other hand, sclerodermatous cGVHD model is characterized by fibrosis in target organs, which is predominately mediated by activated macrophages and Th1/Th17. In the current study, we demonstrate that blocking IL-12/23p40 reduces sclerodermatous cGVHD, which is consistent with the recent publish by Okamoto, et al.; 2) The pre-conditioning in the two cGVHD

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models are different. The recipients in the SLE model did not receive any irradiation before transplantation, which may affect the contribution of IL-12 in the development of cGVHD; 3) IL-12 has been found to have a paradoxical role in the development of GVHD[82,147]. A single injection of IL-12 on the day of BMT inhibits aGVHD through Fas-mediated reduction of donor T-cell activation and expansion. The dose and timing of IL-12 administration was critical for the protective effect, yet only a low dose of IL-12 was protective and administration 1 hour before BMT was the most efficacious; 4) More importantly, we targeted IL-12 and IL-23 in combination rather than IL-12 alone. In short, the precise mechanisms that account for the discrepancy from the work by Okubo et al. and the current work in the context of neutralization are not known and desirable for further investigation.

In conclusion, our study provides strong evidence that both donor- and host-derived p40 are required for the development of GVHD. Ustekinumab has been approved for the treatment of moderate-to-severe psoriasis, and in a Phase 2b trial, adults with moderate-to-severe Crohn's disease resistant to anti-tumor necrosis factor treatment showed an increased rate of response to ustekinumab[148]. Our current work provides evidence to support evaluation of p40 blockade as a potential therapeutic target for the prevention and/or treatment of GVHD while sparing the GVL effect following allo-HSCT.

III.   IL-23R signaling can bypass IL-12Rβ1 through IL-39

III.1. Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HCT) is an effective means by which to treat a wide variety of diseases resulting from hematological dysfunction. However, the development of graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality post transplantation. The IL-12 family of cytokines is comprised of IL-12, IL-23, IL-27, IL-35, and IL-39. IL-12 family members are unique in that each cytokine and cognate receptor is comprised of heterodimers in which one subunit is shared among the others. IL-12 (p35+p40) and IL-23 (p19+p40) have well documented proinflammatory functions responsible for Th1 differentiation and Th17 stabilization, respectively, and play critical roles in GVHD development. IL-12R and IL-23R share a β-chain (IL-12Rβ1) yet use distinct α-chains to mediate their respective receptor signaling. While both IL-12R and IL-23R are widely implicated in inflammatory disorders, the role of IL-12Rβ1 in this context remains much less defined. We therefore studied the impact of eliminating the common IL-12Rβ1 chain or the unique IL-23R chain in T cells on GVHD using murine models of allogeneic bone marrow transplantation (BMT). We found that donor T cells deficient for IL-12Rβ1 or IL-23R had an abrogated ability to cause cGVHD. In agreement with previous publications, we found a pathogenic role for IL-23R on donor T cells in aGVHD. Strikingly, a similar effect was not seen for IL-12Rβ1. These data suggest that that IL-23R contributes to GVHD pathogenesis via a pathway independent of IL-12Rβ1. To confirm that functional differences existed between T cells deficient for IL-23R or IL-12Rβ1 in GVHD, we

assessed cytokine profiles of these T cells in target organs 14 days post-BMT. We found that GM-CSF production by CD4^{+} T cells was reduced exclusively in T cells deficient for IL-23R in the spleen, liver and gut. The newest member of the IL-12 family, IL-39, has been described to be composed of IL-23p19 and EBI3. Given the cognate receptor for IL-39 includes IL-23R and gp130, we hypothesized that IL-39 may play a role in aGVHD as this would explain why IL-12Rβ1 is dispensable. To validate that p19 and EBI3 can form a heterodimer, we transfected SV40 cells with vectors containing control, IL-23p19, EBI3 or both cDNAs. We detected IL-39 heterodimers only in the supernatant of cells transfected with both IL-23p19 and EBI3 via ELISA. Furthermore, we observed significantly increased levels of IL-39 in allogeneic recipients at day 14 post BMT compared to recipients of BM alone. This may implicate IL-39 in the GVHD development. Taken together, our studies indicate that IL-23R plays an essential role, whereas IL-12Rβ1 is dispensable, for donor T cells to induce aGVHD, yet that both chains contribute to cGVHD. Our proposed model is that in the absence of IL-12Rβ1, IL-39 could transmit IL-23Rα signaling, hypothetically by forming a heterodimer with gp130. This new finding indicates that IL-23R and IL-39 are potential therapeutic targets for controlling aGVHD in the clinic.

III.1. Introduction

Allogeneic hematopoietic cell transplantation (allo-HCT) is an effective means by which to treat a wide variety of diseases resulting from dysfunctional hematopoiesis; ranging from certain immune deficiencies to severe blood diseases and cancers[7,13]. This therapeutic benefit is primarily due to the donor T cells present in the graft, which have the ability to mediate a potent anti-tumor response known as the graft-versus-tumor effect (GVT)[6]. However, donor T cells present in the graft also recognize foreign antigens present on host organ tissue, such as the skin, lung, GI, and liver, which culminates in the development of graft-versus-host disease (GVHD)[23]. The development of GVHD is second only to primary disease as the leading cause of mortality among allo-HCT recipients[16]. GVHD can occur in two phases: acute (aGVHD) and chronic GVHD (cGVHD)[18]. While aGVHD is primarily mediated by mature T cells, cGVHD pathogenesis involves widespread autoantibody production by B cells and fibrosis[22]. In either phase, inflammatory cytokines play a critical role in promoting donor T and B cell responses in GVHD.

Currently, the IL-12 family of cytokines is comprised of IL-12, IL-23, IL-27, IL-35 and, more recently, IL-39[149]. This family of cytokines is unique in that each individual member is heterodimeric, and the individual alpha and beta subunits are shared among other members. The shuffling of alpha and beta subunits can lead to dramatically different signaling cascades, culminating in a range of pro- to anti-inflammatory cellular responses[39]. For example, the alpha cytokine subunit p35 is

shared between IL-12, which promotes T helper 1 (Th1) differentiation, and IL-35, which is a potently immunosuppressive molecule produced by T regulatory cells (Tregs). Similarly, the receptors for IL-12 family members are also heterodimeric and bind to their cognate ligand. Briefly, the p19 subunit binds to IL-23R, p35 binds to IL-12Rβ2, p28 binds to IL-27R (WSX-1), p40 binds to IL-12Rβ1, and EBI3 binds to gp130. Clinically, this property of subunit sharing has been exploited in order to target two pathways at once, the best example being ustekinumab, which targets the shared p40 subunit of IL-12 and IL-23[150]. Recent studies have demonstrated the requirement of IL-23R expression on pathogenic T cells in various autoimmune diseases as well as in aGVHD[56,108]. While IL-12/23p40 is commonly targeted in order to alleviate inflammatory disorders, the contribution of its cognate receptor, IL-12/23Rβ1, in T cell-mediated inflammation has not been well defined.

Our studies found that donor splenocytes deficient for IL-23R or IL-12Rβ1 had an impaired ability to induce cGVHD compared to WT controls. During cGVHD development, both IL-23R and IL-12Rβ1 contributed to thymic damage, as well as follicular T cell (Tfh) propagation and IL-21 production, while IL-23R on donor T cells played a predominate role in promoting germinal center (GC) B cell activation and plasma cell differentiation. Strikingly, a similar effect was not seen for IL-12Rβ1 across aGVHD models. To explain this, we hypothesized that IL-39 could account for these observed disparities in aGVHD. Here, we show that IL-12Rβ1 is partially required for aGVHD, and that donor T cells lacking IL-12Rβ1 function differently than those completely void of IL-23R signaling. We demonstrate that

p19 and EBI3 can form biological heterodimers, and that these heterodimers, herein called IL-39, are detectable in the sera of aGVHD recipients. We found IL-39 levels to be highest at two weeks post BMT in aGVHD, but that IL-39 levels in cGVHD were significantly lower at the same time point. Collectively, we provide evidence that IL-39 levels are increased in aGVHD, but not in cGVHD models, providing a potential explanation for why GVHD progression may be observed in the absence of IL-12Rβ1 in aGVHD. In cGVHD, both IL-23R and IL-12Rβ1 contribute to disease pathogenesis and are viable targets in clinical scenarios. We interpret that donor T cells have a differential requirement for IL-12Rβ1 or IL-23R, which is dependent on the level of histocompatibility antigen mismatch and/or degree of inflammation (e.g. acute vs chronic phase) in GVHD.

III.2. Materials and methods

III.2.a. Mice

C57BL/6 (B6; H-2b), B6.Ly5.1 (H-2b), BALB/b (H2b), BALB/c (H2d), B6D2F1(B6 x DBA2) (H2b/d) mice were purchased from the National Cancer Institute/National Institute of Health. IL-12Rβ1KO mice were purchased from Jackson labs and IL-23R[null] mice were provided by Dr. Benny Yang. All animals were housed at the American Association for Laboratory Animal Care–accredited Animal Resource Center at the Medical University of South Carolina. All mice were treated in adherence to the NIH Guide for the Care and Use of Laboratory Animals and their

respective protocols approved by local Institutional Animal Care and Use Committees.

III.2.b. Murine GVHD models

Recipient mice were lethally irradiated at 700 cGy for BALB/c and 1000-1200 cGy (2 split doses, 3-hour interval) for B6D2F1 recipient mice using an X-RAD 320 irradiator (Precision X-Ray). Within 24 hours of irradiation, Balb/c or B6D2F1 recipient mice were transplanted with 5.0×10^6 T cell depleted bone marrow cells (TCD-BM) from B6 donors with or without T cells ($0.5–1 \times 10^6$/mouse). Recipient survival was monitored throughout the experiment. Body weight loss was monitored twice per week and clinical signs of GVHD were monitored once per week and include posture, skin damage, hair loss, ruffled fur, diarrhea, and decreased activity.

III.2.c. Transfected Cell Lines

SV40 cells were transfected with vectors (Sino Biological) containing control, IL-23p19, EBI3 or both cDNAs according to the manufacturer's instructions (lipofectamine 3000).

III.2.d. Flow cytometry

Mononuclear cells were isolated from mouse recipient spleen or liver. Live/dead fixable yellow from Invitrogen (cat # L34968) was used to distinguish live and dead cells. To measure intracellular cytokines, cells were stimulated for 4–5 h at 37°C

with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (100 ng/mL; Calbiochem, EMD) in the presence of GolgiStop (BD Biosciences). Fix and permeabilization were performed using Cytofix/Cytoperm Plus (BD Biosciences). Stained cells were analyzed using FACSDiva software, LSR II (BD Biosciences, San Jose, CA), and FlowJo (Tree Star, Ashland, OR). The following antibodies were used for cell-surface staining: anti-CD4–V450 (RM4-5), -APC, and -PEcy7 (BD Biosciences), anti–CD8-PEcy5 (53-6.7), -APCcy7 and -AF700 (BD Biosciences,); anti-CD45.1-FITC (A20), - and -APC (BD Biosciences), anti-B220-FITC (RA3-682) and -PE (eBioscience), anti-CD44–APC,PE(eBioscience,) (IM7) anti-CD62L-Pecy5,-FITC(eBioscience) (MEL-14),CD25–FITC (7D4) ,Pecy7 (eBioscience) (PC 61.5) and anti-H2K^b (AF6-88.5.5.3). Intracellular staining was carried out using anti-IFN-γ-PE or Per-cp 5.5 (XMG1.2; BD Biosciences), anti-IL-4-PE (11B11; BD Pharmingen), anti–IL-5–PE (TRFK5; BD Pharmingen), anti-Foxp3-PE (FJK-16s; eBioscience).

III.2.e. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates (Costar) were coated with anti-p19 (5B2; ebioscience) and incubated overnight at 4°C. Plates were washed 4 times with 0.1% Tween 20 in PBS, blocked with 1% BSA for 1hr, then serum samples (1:5 dilution) or supernatant (1:5 dilution) were added to wells and incubated overnight at 4°C. Plates were washed 4 times with 0.1% Tween 20 in PBS and incubated with biotinylated anti-EBI3 (210-306-C55; Rockland) for 1hr at room temperature. Plates were washed again as previously described and incubated for 1hr with streptavidin (18-4100-94;

ebioscience). After washing, TMB substrate (00-4201-56; Invitrogen) was added and incubated for 10-30 minutes at room temperature. Reaction was stopped by adding 1M H3PO4.

III.2.f. Immunoblots/ Immunoprecipitation

SV40 cells were transfected as described. Transfected cells were harvested and lysed in 1% Triton-X lysis buffer for 60 minutes. After lysis, samples were centrifuged and soluble fractions were separated from non-soluble fractions, boiled in laemmli buffer, then probed for gp130 (Cell signaling; 3732S) or IL-23R (RnD; AF1689) using western blot. For cytokine immunoprecipitation, supernatant from transfected cells was probed for p19 (RnD;) and immunoblotted for EBI3 (Rockland).

III.2.g. Statistics

For comparisons of independent murine data, the 2-tailed Student's t-test was used. The Mann-Whitney test was used for comparisons of nonparametric data. ANOVA was used for group comparisons. The log-rank test was used to analyze GVHD survival.

III.3. Results

III.3.a. IL-12Rβ1 and IL-23R are required for cGVHD development

Given that IL-12R and IL-23R share a common receptor subunit, IL-12Rβ1, our initial studies focused on whether this was as important for GVHD pathogenesis as its ligand, IL-12/23p40. To investigate the role individual IL-12Rβ1 or IL-23Rα on T-cell activation and pathogenicity, we utilized IL-12Rβ1 knock-out (KO) and IL-23Rα functional inactive mice (refer as KO hereafter for simplicity)[151]. Immunological phenotypes of either strain of mice showed no significant differences in CD4 or CD8 memory frequency, B cells, or Tregs as compared with WT counterparts. Recent data has demonstrated that blocking p40 alleviates cGVHD[150,152]. cGVHD can affect more than 50% of patients and is the primary long-term cause of morbidity after allo-HCT[31]. Furthermore, both donor T and B cells contribute to cGVHD pathogenesis and both express IL-12Rβ1 and IL-23Rα[35,36,43,108]. Therefore, we first endeavored to define the roles of IL-12Rβ1 and IL-23R on cGVHD using a major MHC-mismatch B6 to BALB/c model. Interestingly, cGVHD development was observed to depend on both IL-12Rβ1 and IL-23R (Figure 1A,B). To confirm this, we also utilized a B6 to B6D2F1 cGVHD model, in which we observed consistent results (Figure 1C,D). Pathological analyses on target organs at multiple time-points supported reductions in cGVHD of the recipients deficient for either receptor unit (Figure 1E).
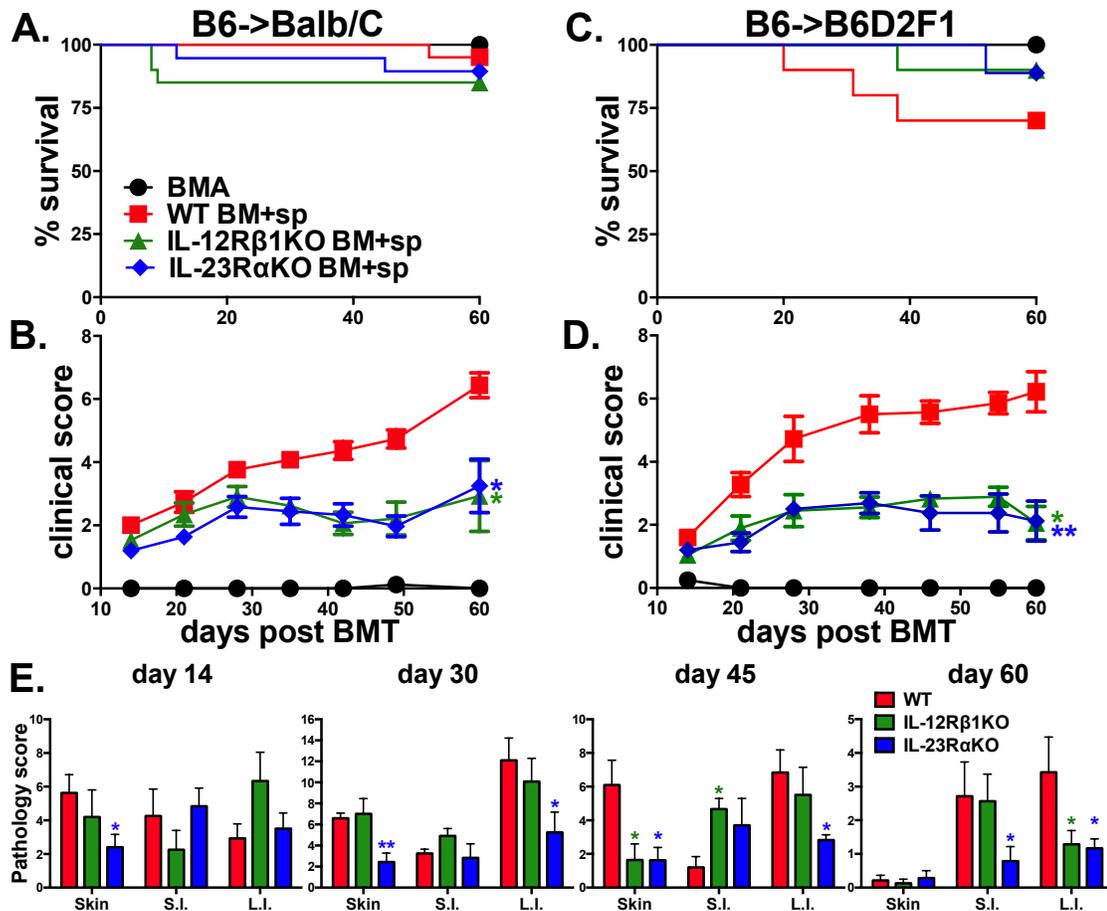
Figure 1. IL-23Rα and IL-12Rβ1 contribute to cGVHD. Lethally irradiated BALB/c (A, B) or B6D2F1 (C, D) mice were transplanted with 5×10^6 TCD-BM alone or plus 0.35×10^6 whole splenocytes (BALB/c) or 2.5×10^6 purified T cells (B6D2F1) from WT B6, IL-12Rβ1KO, or IL-23RαKO mice; donor grafts containing TCD-BM and splenocytes/ T cells were matched based on genetic background. Survival and clinical score of BALB/c (A, B) and B6D2F1 (C, D) are shown. Quantified pathology scores at days indicated are shown from 2 pooled experiments (E). $n_{exp}=4$, $n_{mice/group}=20$. *$p<0.05$, **$p<0.01$

Since cGVHD pathogenesis involves thymic damage, as well as a much broader array of cellular subsets such as B cells and T follicular helper (Tfh) cells, we hypothesized that IL-12Rβ1 may be important for B cell activation or Tfh differentiation. Because BM derived cells also contribute to cGVHD, we initially examined cGVHD development when both BM and splenocytes were the donors

deficient for IL-12Rβ1 or IL-23R. At 60 days post allo-HCT, we evaluated B cell reconstitution and T cell differentiation (Figure 2). Both CD4 and CD8 Tfh (CXCR5+PD1+) differentiation were reduced (Figure 2A,B), while Tfr (CD4+CXCR5+PD1+Foxp3+) differentiation was increased in IL-12Rβ1KO and IL-23RKO recipients (Figure 2C,D). While B cell reconstitution was significantly improved in recipients of IL-12Rβ1 or IL-23R deficient donor grafts (Figure 2E,F), we found that plasma cell differentiation was reduced only in IL-23RKO recipients (Figure 2G,H). However, germinal center B cells were significantly reduced in both cohorts at day 60 versus WT controls (Figure 2I,J). IFNγ production was significantly decreased in both IL-12Rβ1KO and IL-23RKO recipients, while IL-17 was reduced only in IL-23RKO cohorts, and IL-21 was reduced only in IL-12Rβ1KO cohorts (Figure 2K,L). Collectively, both IL-12Rβ1 and IL-23R contribute to cGVHD development.
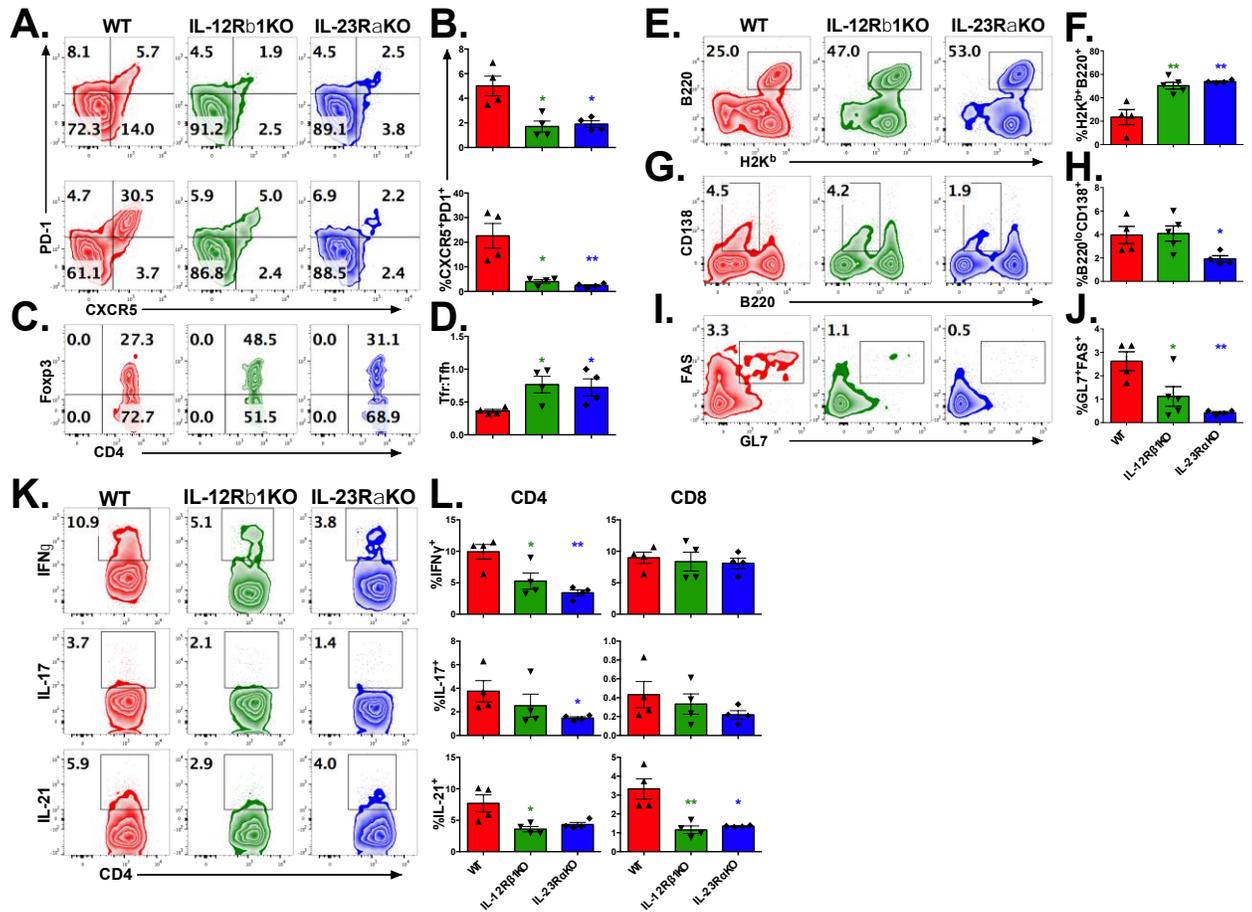
Figure 2. Similar effect of IL-23Rα and IL-12Rβ1 in cGVHD. Lethally irradiated BALB/c mice were transplanted with 5×10^6 TCD-BM alone or plus 0.35×10^6 whole splenocytes from WT B6, IL-12R**β**1KO, or IL-23RαKO mice. At 60 days post BMT, remaining mice were sacrificed, and spleens were taken for analysis by flow cytometry. Representative frequencies of H2K^{b+}CD4$^+$CXCR5$^+$PD1$^+$ (A, top), H2K^{b+}CD8$^+$CXCR5$^+$PD1$^+$ (A, bottom), as well as graphical depictions (B), are shown. A ratio of Foxp3$^+$:Foxp3$^-$ among H2K^{b+}CD4$^+$CXCR5$^+$PD1$^+$Foxp3$^+$ cells (C) and associated graphical data (D) is shown. Representative frequencies of donor B cells (E), plasma B cells (G) and germinal center B cells (I) and associated graphical summaries (F, H, J) are shown. Representative frequencies of donor CD4$^+$ T cell production of IFNγ (K, top), IL-17 (K, middle) and IL-21 (K, bottom), as well as graphical summaries (L) for CD4 (left) and CD8 (right) are shown. n_{exp}=4, $n_{mice/group}$=20. *p<0.05, **p<0.01

III.3.b. IL-12Rβ1 and IL-23R differentially impact cGVHD development

While our data at day 60 post-transplant (Figure 2) was consistent with the notion that both IL-12Rβ1 and IL-23Rα are essential for cGVHD, we observed discrepancies in the function of these receptors on donor splenocytes, for example, in the context of plasma B cell differentiation which was heavily affected by IL-23Rα (Figure 2A,B). We then tested whether using WT BM had any impact on outcomes when transplanted with receptor deficient splenocytes and found that it did not. To determine potential mechanisms for the role of IL-12Rβ1 and/or IL-23Rα during cGVHD development, we utilized CD45.1+ BM to evaluate how these receptors on donor splenocytes affected thymus reconstitution (Figure 3) at days 14, 30, and 45. Thymus reconstitution was notably improved in cohorts receiving IL-23Rα deficient splenocytes at earlier time points, yet comparable effects were observed in cohorts that received IL-12Rβ1KO splenocytes by day 45 (Figure 3A,B). Absolute numbers of double positive (DP) thymocytes were consistently improved (Figure 3C). Notably, in the single positive (CD4+ or CD8+) populations, there were significant reductions in frequencies of non-BM derived donor T cells (Figure 3D-F), suggesting that both IL-12Rβ1 and IL-23Rα were required for thymus infiltration. We also examined the spleen and peripheral lymph nodes (pLN) at days 14, 30, and 45. In the Tfh compartment, CD4+CXCR5+PD1+ T cells were significantly reduced in cohorts that received IL-12Rβ1KO splenocytes, while CD8+CXCR5+PD1+ T cells were significantly reduced in the recipients of either IL-12Rβ1KO or IL-23RαKO graft as compared to those of WT graft (Figure 4). This decrease in CD4+CXCR5+PD1+ correlated with Bcl6 expression, the

transcriptional regulator of Tfh differentiation, in recipients of either IL-12Rβ1KO or IL-23RαKO splenocytes at day 45 (Figure 4A-C). As the main effector cytokine of Tfh cell is IL-21, we measured and found that WT controls produced significantly more IL-21 from CD4+ T cells at days 30 and 45 than either KO cohorts in recipient spleen; similar results were observed on CD8+ cells at day 45 (Figure 4D). Analysis of the pLN revealed reductions in CD4+ IL-21 for IL-23RKO cohorts at days 30 and 45, IL-12Rβ1KO exhibited similar reductions at day 45 (Figure 4E). Interestingly, B cell differentiation and activation was significantly reduced in cohorts that received IL-23RKO splenocytes, as indicated by stagnant GC development, plasma cell differentiation, and CD86 expression at days 30 and 45, which was not observed in IL-12Rβ1KO cohorts (Figure 4 F-I). In general, IL-12Rβ1 or IL-23RKO donor T cells produced lower levels of IFNγ or IL-17, respectively, in both the spleen and pLN. However, IL-4 production was significantly increased in IL-12Rβ1KO T cells at day 30 in the spleen and lymph nodes while the opposite trend was seen for IL-23RKO T cells. Collectively, both IL-12Rβ1 and IL-23R contribute to thymic damage and Tfh differentiation, yet IL-23R may play a predominant role vs IL-12Rβ1 in regulating donor T cell interactions with B cells during cGVHD development.
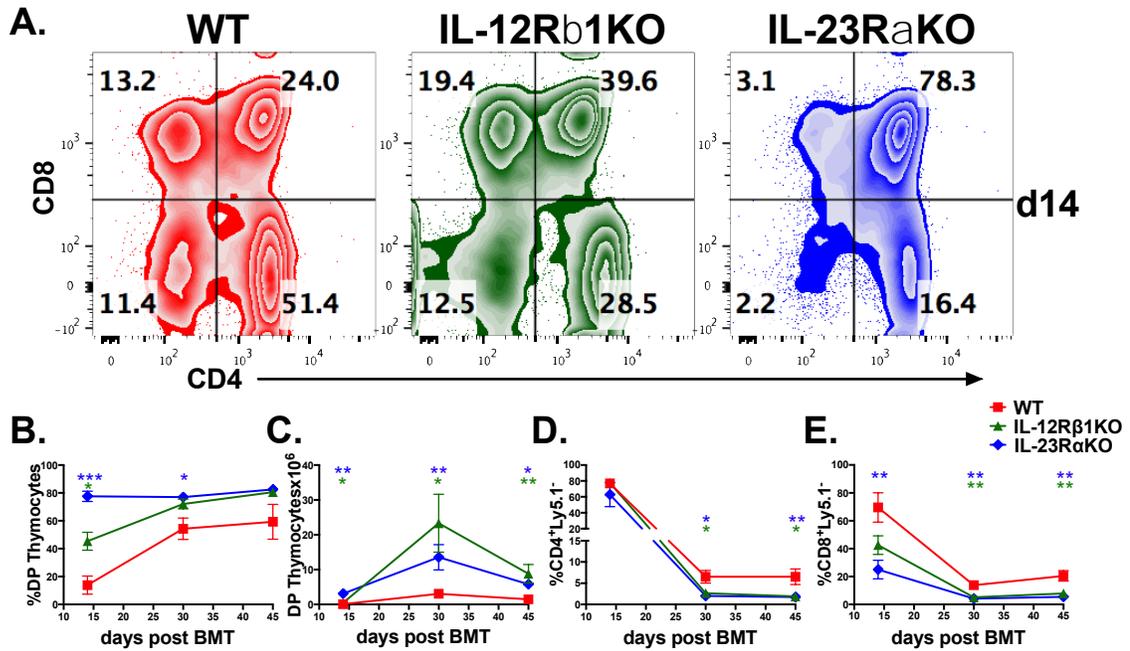
Figure 3. IL-23Rα and IL-12Rβ1 inhibit thymus reconstitution. Lethally irradiated BALB/c mice were transplanted with 5×10^6 WT B6Ly5.1[+] alone or plus 0.35×10^6 whole splenocytes from WT B6, IL-12Rβ1KO, or IL-23RαKO mice. Thymus was collected from mice 14, 30, and 45 days post-BMT. Flow cytometry depictions of double positive (CD4^{+}CD8^{+}) from the thymus at day 14 (A) and graphical representations of double positive (CD4^{+}CD8^{+}) frequencies (B), absolute number of double positive (CD4^{+}CD8^{+}) cells (C), as well as single positive for CD4 (D) or CD8 (E) non-BM derived (Ly5.1^{-}) H2K[b+] cells over time are shown. $n_{exp}=2$, $n_{mice/group}=6-8$. *p<0.05, **p<0.01, ***p<0.001
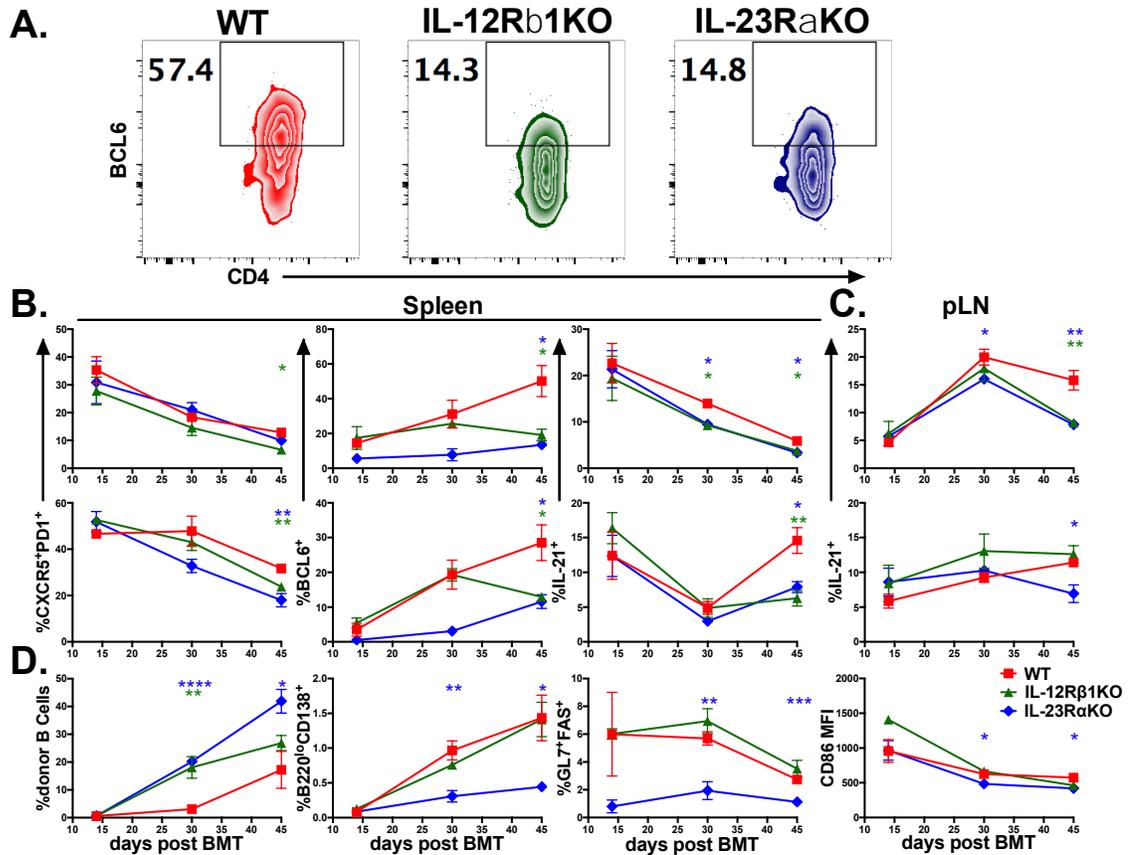
Figure 4. IL-23Rα and IL-12Rβ1 promote Tfh/B cell responses. Lethally irradiated BALB/c mice were transplanted with 5×10^6 WT B6Ly5.1+ alone or plus 0.35×10^6 whole splenocytes from WT B6, IL-12Rβ1KO, or IL-23RαKO mice. Spleen was collected from mice 14, 30, and 45 days post-BMT. Frequency of BCL6 from gated CD4+ cells at day 45 (A) and graphical depictions of CD4+ (B, top) and CD8+ (B, bottom) CXCR5+PD1+, BCL6, and IL-21 in the spleen (left to right) over time are shown. (C) IL-21 production by CD4 (top) and CD8 (bottom) in the peripheral LNs over time are shown. (D) Graphical depictions of frequencies of donor B cells, plasma cells, germinal center B cells and CD86 expression (left to right) over time are shown. *p<0.05, **p<0.01, ***p<0.001

III.3.c. IL-23R signaling can mediate aGVHD in the absence of IL-12Rβ1

We then focused on the role of IL-23R and IL-12Rβ1 in aGVHD. Initial studies to characterize T cell responses were focused on proliferation and cytokine production after being adoptively transferred into allogeneic recipients. We observed similar reductions in IL-23R and IL-12Rβ1 deficient donor T cells compared to WT controls in terms of IFNγ, IL-17 or GM-CSF production. Therefore, we hypothesized that IL-23R and IL-12Rβ1 would contribute to aGVHD. To test this, we used 3 murine models of allo-HCT: B6 to BALB/b (miHA mismatch), B6 to B6D2F1 (haploidentical mismatch), and B6 to BALB/c (complete mismatch). In the miHA model, we observed improvements in survival among recipients of IL-23R and IL-12Rβ1 deficient donor T cells compared to WT controls, although clinical scores were significantly reduced only in recipients of IL-23RKO T cells. Experiments using haploidentical recipients also yielded improvements in survival versus WT, yet recipients of IL-23RKO T cells also had significantly better survival than recipients of IL-12Rβ1KO T cells (Figure 5C). However, in complete mismatch models, outcomes in recipients of IL-12Rβ1KO T cells showed no improvements compared to WT controls, while recipients of IL-23RKO T cells also had significantly improved survival and clinical scores (Figure 5E,F). In fact, no significant differences were observed when donor T cells lacked IL-12Rβ2 or IL-12Rβ1 compared to WT controls in the B6 to BALB/c model. This data indicates a dominant role for IL-23R in minor-, haplo-, and major-mismatch GVHD models, and the differences correlated with degree of MHC-mismatch (Figure 5 A-F).
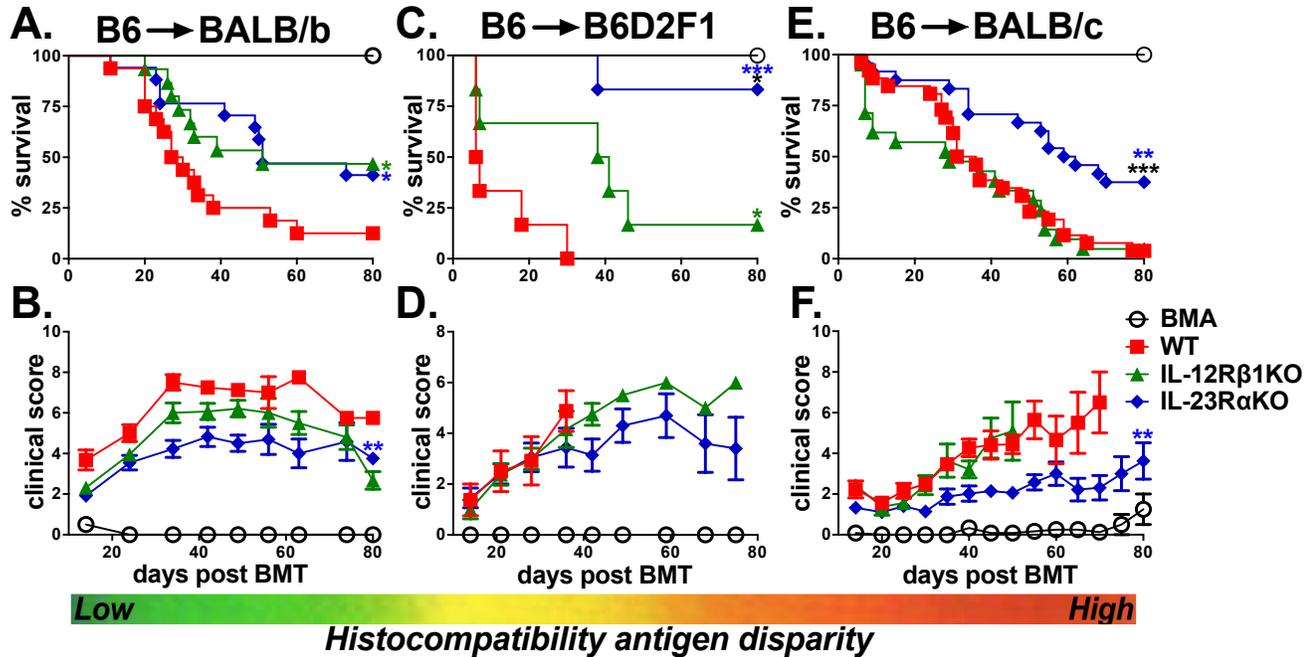
Figure 5. Effect of IL-12Rβ1 and IL-23Rα in aGVHD. Lethally irradiated BALB/b (A, B), B6D2F1 (C, D) or BALB/c (E,F) mice were transplanted with 5×10^6 TCD-BM alone or plus 1×10^6 (BALB/c), 3×10^6 CD25$^-$ (B6D2F1), or 3×10^6 (BALB/b) purified T cells from WT B6, IL-12Rβ1KO, or IL-23RαKO mice. Survival (A, C, E) and clinical score (B, D, F) of BALB/b (left), B6D2F1 (middle), and BALB/c recipients (right) are shown. Data shown is pooled from 2-4 replicate experiments with a total of 15-30 mice per group. *p<0.05, **p<0.01, ***p<0.001

To further evaluate the role of IL-23R in GVHD development, we blocked IL-23R using a commercially available antibody and found that the treatment with anti-IL-23R significantly improved recipient survival and clinical scores compared to with vehicle control. Together, these data show that IL-23R signaling is required for donor T cells to induce aGVHD, and that this requirement is positively correlated with the degree of histocompatibility disparity among donor and recipients. Furthermore, these data indicate that IL-23R signaling may be able to bypass IL-12Rβ1 in certain scenarios.

III.3.d. IL-23R and IL-12Rβ1 are functionally different on donor T cells during aGVHD

We then sought to determine how IL-12Rβ1 and IL-23R differentially impacted the donor T cell response in the context of aGVHD. We utilized the B6 to BALB/c model, as we had observed the most significant differences in survival and clinical score (Figure 5 A,B). Hence, we assessed cytokine profiles of T cells in target organs 14 days post-BMT. While production of IFNγ and IL-17 of donor T cells in recipient spleen was similarly decreased in both cohorts, GM-CSF production by CD4+ T cells was reduced exclusively in T cells deficient for IL-23R (Figure 6). Further, a significant reduction of IFNγ and GM-CSF in target organs, such as the liver (Figure 6A,B) and gut (Figure 6C), was only observed in T cells deficient for IL-23R. We also observed stark increases in GM-CSF production in IL-12Rβ1, but not IL-23R, deficient T cells in the spleen (Figure 6E). Albeit, this data suggests that IL-23R signaling promotes GM-CSF production by donor T cells which occurs independently of IL-12Rβ1.
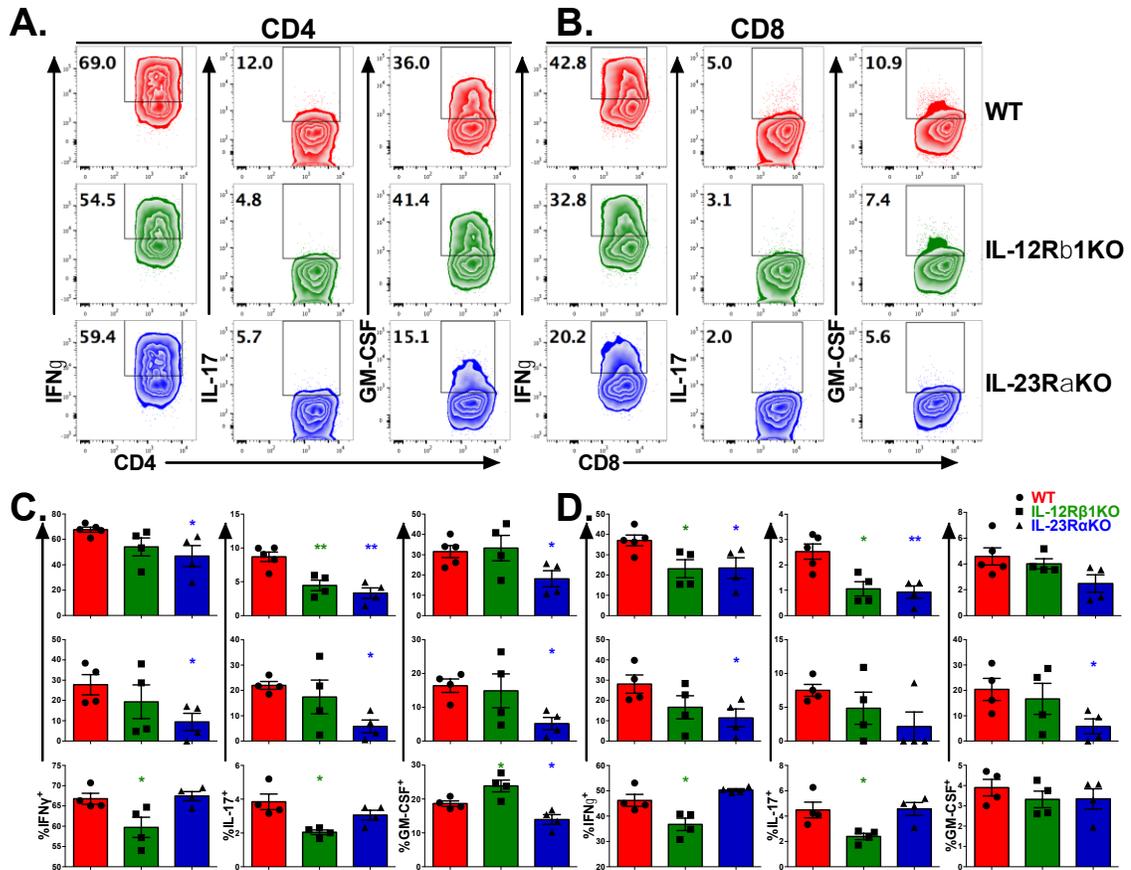
Figure 6: IL-23Rα regulates GM-CSF production in aGVHD. Lethally irradiated BALB/c mice were transplanted with 5×10^6 TCD-BM alone or plus 0.75×10^6 purified T cells from WT B6, IL-12R**β**1KO, or IL-23RαKO mice. Lymphocytes were isolated from the liver (A, B), gut (C) and spleen (D) of mice 14 days post-BMT. (A) Frequencies of donor CD4+ and CD8+ IFN**γ**, IL-17 and GM-CSF (left to right) as well as graphical representations (B) from the liver, gut (C), and spleen (D) are shown *p<0.05, **p<0.01, ***p<0.001

III.3.e. p19 and EBI3 heterodimers are detectable in aGVHD

The newest member of the IL-12 family, IL-39, was recently shown to contribute to SLE pathogenesis; this cytokine has been described to be composed of IL-23p19 and EBI3[110]. Given the cognate receptor for IL-39 includes IL-23Rα and gp130, we hypothesized that IL-39 may play a role in aGVHD as this would explain why IL-12Rβ1 is dispensable in aGVHD models. To validate that p19 and EBI3 can form

a heterodimer, we transfected SV40 cells with vectors containing control, IL-23p19, EBI3 or both cDNAs. Antibody specificity for both p19 and EBI3 correlated with transfected subunits in the supernatant of SV40 cells (Figure 7A,B). We detected IL-39 heterodimers only in the supernatant of cells transfected with both IL-23p19 and EBI3 via ELISA (Figure 7C). Heterodimer formation was confirmed with immunoprecipitation experiments (Figure 7D). Given that differences between IL-12Rβ1 and IL-23R seemed to correlate with the degree of histocompatibility antigen disparity in aGVHD models, together with the fact that both receptors played a role in cGVHD, we hypothesized that IL-39 would be increased in aGVHD compared to cGVHD. To test this, we used ELISA to measure serum IL-39 at various time points in aGVHD and cGVHD (Figure 7E). We observed significantly increased levels of IL-39 in allogeneic recipients at day 14 post BMT in aGVHD compared to the recipients of BM alone. This trend of higher IL-39 in aGVHD continued at later time points, but mortality in the aGVHD setting prevented our ability to adequately evaluate significance. In contrast, we did find elevated IL-39 levels in sera from the recipients with cGVHD as compared to those from the recipients of BM alone in any time-point tested (data not shown). Taken together, the data implicates IL-39 in aGVHD development and could serve as one explanation for why IL-12Rβ1 is dispensable in certain scenarios.
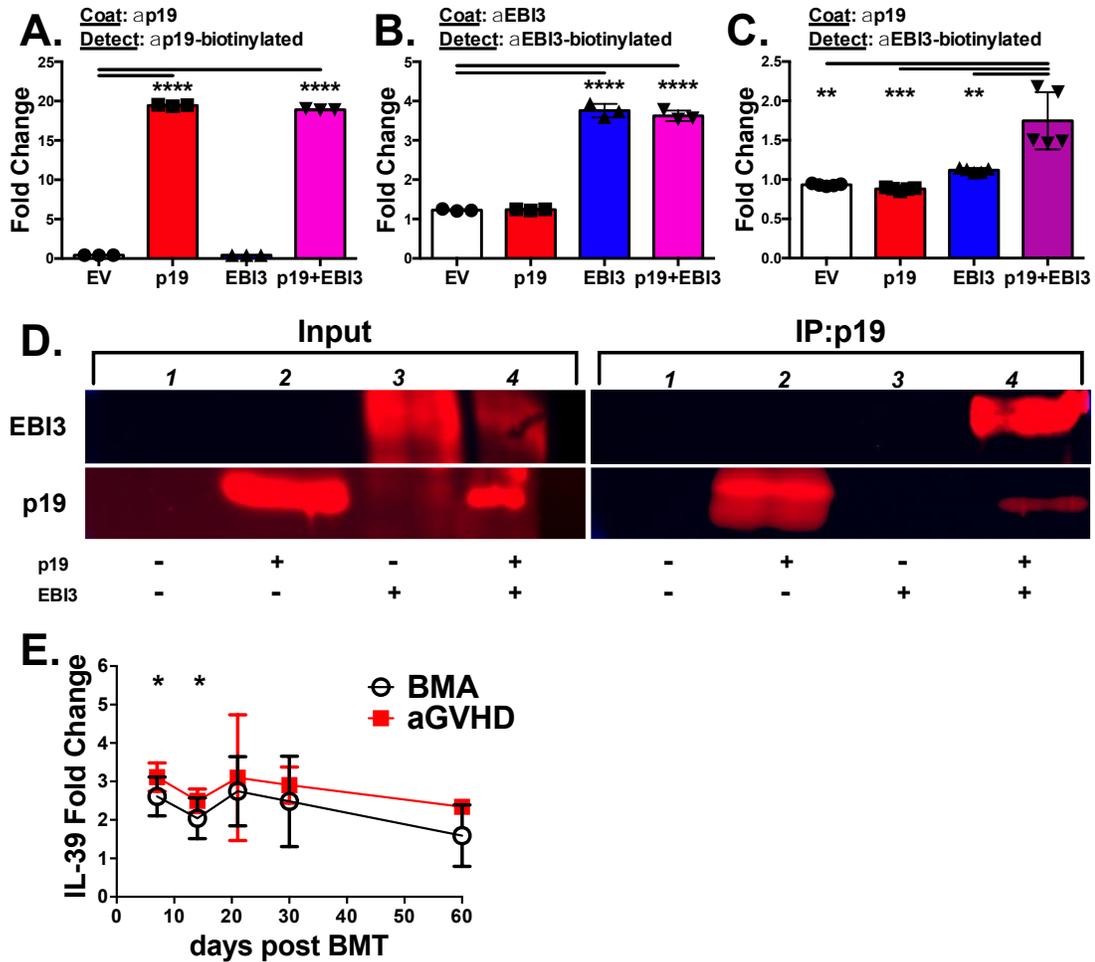
Figure 7: p19 and EBI3 form heterodimers that are detectable in aGVHD. SV40 cells were transfected with vectors containing control, IL-23p19, EBI3 or both cDNAs. After 2 days, supernatant was harvested and ELISA was performed for (A) p19, (B) EBI3 or (C) p19/EBI3 heterodimers. (D) Supernatant from transfected SV40 cells was TCA precipitated (input) or immunoprecipitated with anti-p19 and probed by western blot for EBI3 (top) and p19 (bottom). Recipient Balb/C mice were lethally irradiated and transplanted with 5×10^6 TCD-BM alone or plus 0.75×10^6 purified T cells (acute GVHD) or 0.35×10^6 whole splenocytes (chronic GVHD) from B6 mice. ELISA for IL-39 was performed as previously described using serum taken at days 7 (aGVHD only), 14, 35, or 60 days post BMT (E). $n_{exp}=4$, $n_{mice/group}=5$-20. $^{**}p<0.01$, $^{***}p<0.001$

III.4. Discussion

The current study indicates that both IL-23R and IL-12Rβ1 play a role in cGVHD, whereas IL-12Rβ1 is dispensable for donor T cells to induce aGVHD; potentially due to IL-39, a novel cytokine in the allo-HCT field. The contribution of p40 to cGVHD has been shown. However, to our knowledge, these are the first studies evaluating the effect of IL-23R and IL-12Rβ1 in cGVHD.

In cGVHD, thymic damage by donor T cells was abrogated in the absence of either receptor chain, leading to improvements in thymus reconstitution, especially in recipients of IL-23RKO splenocytes at early time points. IL-23 has been shown to promote RORγt-dependent apoptosis in DP thymocytes, which may explain the accumulation of DP thymocytes in recipients of IL-23R and IL-12Rβ1 deficient splenocytes[153]. Interestingly, studies in lupus models found that IL-23p19KO mice developed less SLE than WT controls via influencing thymic selection, indicating that IL-23 may promote pathogenic T cell development in the thymus during cGVHD[154].

Additionally, IL-23R and IL-12Rβ1 both had a significant impact on Tfh differentiation during cGVHD development in our models, which culminated in similar reductions in BCL6 expression and IL-21 production by CD4+ T cells at day 45 post- BMT in the spleen and lymph nodes. However, recent reports by others in the field suggest that CXCR5 and PD1 alone may not be sufficient to distinguish Tfh cells[35]. Albeit, we did find that the master transcription factor of Tfh cells, BCL6,

had a correlated reduction with CXCR5+PD1+ cells in the spleen. Importantly, IL-12 and IL-23 have both been shown to contribute to Tfh development and function in humans[155]. Lastly, B cell differentiation and activation was profoundly affected by IL-23R expression on donor splenocytes, but not by IL-12Rβ1, indicating a potential requirement for IL-23R signaling to induce inflammatory stimulation of B cells in cGVHD. Studies using lupus models have indicated that IL-23 signaling is critical for SLE development not only by promoting pathogenic T cell responses but also by enhancing B cell responses and autoantibody production[156]. Consistent with this, we found significantly lower frequencies of plasma cells in cohorts that received IL-23R deficient grafts. Germinal center formation is known to be preceded by Bcl-6 expression in CD4+ T cells. We found that, although not significantly different at earlier time points, IL-23R deficient donor T cells had a notable trend of less Bcl6 expression over time, which could possibly explain the dramatic reductions in GC formation compared to WT controls.

We found a pathogenic role for IL-23R on donor T cells in aGVHD, yet a similar effect was not seen for IL-12Rβ1. At baseline, these data suggest that that IL-23R may contribute to GVHD pathogenesis via a pathway independent of IL-12Rβ1. We found that GM-CSF production by CD4+ T cells was reduced exclusively in T cells deficient for IL-23R. Further, a significant reduction of IFNγ and GM-CSF in target organs, such as the liver and gut, was only observed in T cells deficient for IL-23R. This indicates that IL-23R may play a direct role in promoting GM-CSF production by donor T cells. As GM-CSF was recently shown to license DC and

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macrophage production of proinflammatory mediators such as IL-1β, this may be a contributing factor to differences observed between cohorts receiving IL-12Rβ1KO or IL-23RKO T cells. Recent publications support the notion that GM-CSF is critical, especially in the gut, for GVHD development[157]. However, these studies on GM-CSF focus primarily on aGVHD (versus cGVHD), hence we did not check GM-CSF in our cGVHD models. Thus, we cannot rule out a role for GM-CSF in cGVHD; as both receptor chains were important for cGVHD, the impact of GM-CSF may be less vital in our studies. More research is required on this point. Given the cognate receptor for IL-39 includes IL-23Rα and gp130, we hypothesized that IL-39 may play a role in aGVHD as this would explain why IL-12Rβ1 is dispensable. We detected IL-39 heterodimers only in the supernatant of cells transfected with both IL-23p19 and EBI3 via ELISA and we observed significantly increased levels of IL-39 in allogeneic recipients at day 14 post BMT compared to cGVHD mice or recipients of BM alone. While our finding is novel in the field, many questions still remain regarding the role of IL-39 in GVHD. IL-39 was originally identified in lupus models and found to be produced by B cells[110]. However, aGVHD is primarily T cell mediated, leaving a large pool of cells that may potentially produce IL-39. Studies to identify these cells are ongoing. Importantly, the translational impact of IL-39 has yet to be validated as it has not been observed in humans. Hence, the most critical question we are focused on is detecting this cytokine in patient samples. Our proposed model is that in the absence of IL-12Rβ1, IL-39 could transmit IL-23R signaling by forming a

heterodimer with gp130. This new finding indicates that IL-23R and IL-39 may serve as potential therapeutic targets for controlling aGVHD in the clinic.

Summary Figure
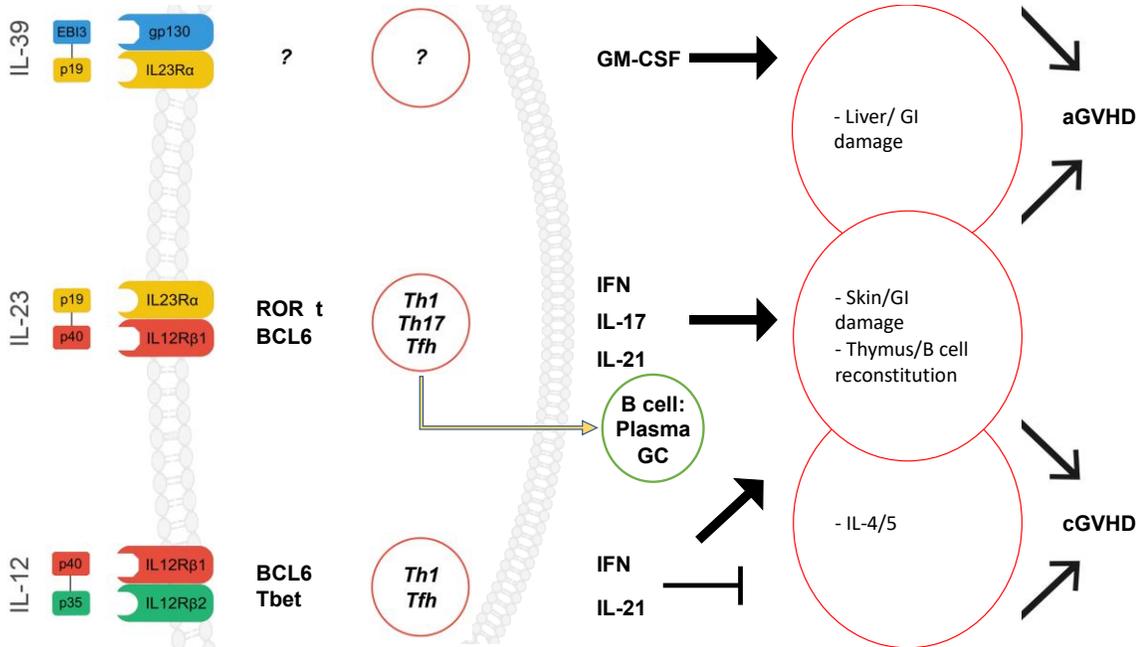
IV.   Targeting JAK2 reduces GVHD and xenograft rejection through regulation of T cell differentiation

IV.1. Abstract

Janus kinase 2 (JAK2) signal transduction is a critical mediator of the immune response. JAK2 is implicated in the onset of graft-versus-host disease (GVHD), which is a significant cause of transplant-related mortality after allogeneic hematopoietic cell transplantation (allo- HCT). Transfer of JAK2$^{-/-}$ donor T cells to allogeneic recipients leads to attenuated GVHD yet maintains graft-versus-leukemia. Th1 differentiation among JAK2$^{-/-}$ T cells is significantly decreased compared with wild-type controls. Conversely, iTreg and Th2 polarization is significantly increased among JAK2$^{-/-}$ T cells. Pacritinib is a multikinase inhibitor with potent activity against JAK2. Pacritinib significantly reduces GVHD and xenogeneic skin graft rejection in distinct rodent models and maintains donor antitumor immunity. Moreover, pacritinib spares iTregs and polarizes Th2 responses as observed among JAK2$^{-/-}$ T cells. Collectively, these data clearly identify JAK2 as a therapeutic target to control donor alloreactivity and promote iTreg responses after allo-HCT or solid organ transplantation. As such, a phase I/II acute GVHD prevention trial combining pacritinib with standard immune suppression after allo-HCT is actively being investigated (https://clinicaltrials.gov/ct2/show/NCT02891603).

IV.2. Introduction

Janus kinase 2 (JAK2) signal transduction is implicated in human autoimmune syndromes[158,159] and graft-versus-host disease[26,160,161]. IL-6, IL-12, and IL-23 mediate inflammation and activate T cells via JAK2[25,160,161]. IL-6 receptor blockade has demonstrated efficacy in a phase II GVHD prevention trial[162], but does not fully impair pathogenic Th1/Th17 responses[163]. IL-12 and IL-23 promote Th1 and Th17 differentiation, respectively, via JAK2[159]. Neutralizing the shared p40 subunit of these cytokines prevents GVHD in rodents[152] and may have merit in treating patients with steroid refractory GVHD[139].

JAK2 inhibition is an alternative approach to suppress IL-6 and p40 receptor signal transduction and induce durable tolerance to alloantigens[160]. JAK2 inhibitors are clinically efficacious in myelofibrosis, a disease often driven by constitutive JAK2 activation[164]. The existing evidence regarding JAK2 as a therapeutic target for acute GVHD is primarily supported by observations using ruxolitinib, an equimolar inhibitor of JAK1 and JAK2[161,165–168]. Ruxolitinib has demonstrated efficacy in treating steroid-refractory GVHD, yet is immune suppressive[161,165]. In part, JAK1 mediates the biologic effects of common gamma chain cytokines, including IL-2 and IL-15[160]. Ruxolitinib suppresses host-reactive T cells in mice[161,166–168] and humans[161,165]. Although not observed in murine transplant studies, ruxolitinib reduces the quantity of human Tregs[169] and NK cells[170,171]. Therefore, targeting JAK2 has the potential to prevent GVHD without conceding JAK1-mediated functions provided by donor lymphocytes.

We report that genetic deletion or pharmacologic inhibition of JAK2 significantly reduces GVHD lethality and spares the GVL effect. Moreover, we demonstrate that JAK2 blockade significantly delays skin graft rejection. We have shown that JAK2 blockade abrogates human Th1 and Th17 responses using TG101348[26,172]. TG101348 is now regarded as a tool compound, as its use was associated with Wernicke encephalopathy caused by off-target inhibition of thiamine uptake[173]. Therefore, we investigated the use of pacritinib, a JAK2 inhibitor that does not impair thiamine metabolism[174]. Distinct from ruxolitinib, pacritinib spares JAK1 activity required by anti-tumor cytotoxic T lymphocytes and Tregs[175]. Here, we identify that eliminating JAK2 signal transduction significantly enhances Th2 and Treg differentiation while dramatically reducing Th1 responses. Thus, we prove that JAK2 inhibition significantly suppresses donor T cells across species without untoward effects on Tregs or GVL responses.

IV.3. Materials and Methods

IV.3.a. Mice

C57BL/6 (B6; H-2b), B6.Ly5.1 (H-2b), BALB/b (H2b), BALB/c (H2d), and (B6 x DBA2)F1 (H2b/d) mice were purchased from the National Cancer Institute/National Institute of Health. Mice with a conditional KO of JAK2 on the T cell lineage were generated by breeding JAK2fl/fl (provided by Dr. Wagner at University of Nebraska) with CD4-Cre (purchased from Taconic). NOD-scid gamma-deficient (NSG) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). With exception to the NSG mice who were housed at the Moffitt Cancer Center, all other animals were housed at the American Association for Laboratory Animal Care–accredited Animal Resource Center at Medical University of South Carolina. All mice were treated in adherence to the NIH Guide for the Care and Use of Laboratory Animals and their respective protocols approved by local Institutional Animal Care and Use Committees.

IV.3.b. Murine GVHD and Bioluminescent Imaging (BLI)

Lethally irradiated BALB/c (MHC-mismatched) or BALB/b (MHC-matched, minor histocompatibility antigen-mismatched) mice received 5×10^6 TCD-BM alone or plus 1×10^6 (BALB/c) or 3×10^6 (BALB/b) T cells from WT B6 or JAK2 KO mice. Recipient body weight and survival was assessed twice weekly. GVHD pathology scores were assessed in a blinded fashion by an independent pathologist. Where indicated, B6.Ly5.1+ TCD-BM and B6.Ly5.2+ WT and JAK2

KO T cells were used. For GVL experiments, BALB/c recipients received TCD-BM alone, TCD-BM with 2×10^3 luciferase-transduced A20, or TCD-BM plus WT B6 or JAK2 KO T cells and luciferase-transduced A20. (B6 x DBA2)F1 mice received TCD-BM alone, TCD-BM with 5×10^3 luciferase-transduced P815 tumor cells, or 3×10^6 WT B6 T cells and luciferase-transduced P815 tumor cells. BLI was performed as previously described[25]. As indicated, pacritinib or its vehicle control were administered at 25-100mg/kg by oral gavage daily from day 0 for 3 weeks after BMT. Where indicated, ruxolitinib (a JAK1/2 inhibitor control) was given at 30mg/kg twice a day by oral gavage.

IV.3.c. Xenograft model

NSG mice (male or female, 6-24 weeks old) received a $1cm^2$ split thickness human skin graft under anesthesia. Eligible patients undergoing mastectomy were consented and skin was collected in accordance with an IRB-approved protocol at Moffitt Cancer Center (MCC 17634). The bandage and sutures were removed after 7-10 days. Thirty days later, recipient mice then received 5×10^6 fresh, human PBMCs (OneBlood) i.p. using a random donor allogeneic to the skin graft[172,176]. Each transplant experiment used a unique donor pair of skin and PBMCs. Pacritinib 100mg/kg or vehicle was given by oral gavage twice a day from day 0 until day +14. The skin grafts were followed closely for signs of rejection, such as ulceration, necrosis, or scabbing. Skin grafts that were >75% non-viable were considered rejected. Pathologic skin rejection grading was performed according to criteria set forth by Bejarano and colleagues[177]. Tissue

samples were prepared, stained (Ventana Medical Systems, Tucson, AZ), and imaged (Vista, CA, USA) as previously described[26].

IV.3.d. Statistics

For comparisons of independent murine data, the 2-tailed Student's t-test was used. For comparisons of dependent human data, the 2-tailed paired t-test was used. The Mann-Whitney test was used for comparisons of nonparametric data. ANOVA was used for group comparisons. The log-rank test was used to analyze GVHD and skin graft survival.

IV.4. Results

IV.4.a. JAK2 signaling promotes Th1 differentiation, but inhibits Th2- and Treg-responses by allo-activation in vitro

To examine the effect of JAK2 in T cells, we employed mice in which JAK2 is conditionally deleted in T cells(JAK2flox/flox x CD4- Cre). Phenotypically, the frequency of splenic CD4+ T cells among JAK2-/- mice was decreased compared to that of naive B6 mice (Supplemental Figure 1 A, D), though proportions of CD8+ T cells and CD4+ Tregs were similar (Supplemental Figure 1 A, B, D, E). The composition of T cell subsets within the naïve and central memory compartments were also similar between JAK2-/- and WT mice (Supplemental Figure 1 C, F,G); with only a subtle increase in CD8+ effector memory T cells in JAK2-/- mice (Supplemental Figure 1 G). In response to alloantigen stimulation, proliferating (CFSElow) JAK2-/- CD4+ and CD8+ T cells produced significantly less IFNγ, yet

higher levels of IL-4/5 and IL-10 compared to WT T cells (Supplemental Figure 2A, B). These data indicate that JAK2 signaling is critical for Th1 differentiation, and opposes Th2 and Treg development after alloantigen stimulation in vitro.

JAK2 contributes to T cell-mediated GVHD, but is dispensable for the GVL effect

To study how JAK2 affects donor T cell responses to alloantigen in vivo, we first evaluated GVHD severity after transfer of JAK2-/- or WT T cells to major MHC-mismatched recipients. JAK2-/- T cells had a significantly reduced ability to induce lethal GVHD compared to WT T cells (Figure 1A, B). Furthermore, a consistent reduction in GVHD severity was e observed in a minor MHC-mismatch model of BMT (Figure 1C, D). Impaired immune reconstitution is associated with GVHD[178]. We therefore examined donor–derived T and B cell reconstitution in surviving BALB/c recipients 80 days post BMT. In the thymus, the percentage and absolute number of double positive thymocytes (CD4+CD8+) were comparable to cohorts receiving TCD-BM only (Supplemental Figure 3A and B). In the spleen, the absolute numbers of CD4+ T cells and B cells in recipients of JAK2-/- T cells were reduced compared to those of TCD-BM alone (Supplemental Figure 3B); the frequencies of donor-derived CD4+ and CD8+ T cells, and of B cells were comparable (Supplemental Figure 3A). These data indicate that JAK2 signaling in T cells facilitates recipient thymic damage typically associated with GVHD(22), yet may enhance peripheral T and B cell counts after allo-HCT.

In studies examining the effect of JAK2 on GVL activity, recipients of T cells from both WT and JAK2-/- donors exhibited significantly less tumor mortality compared to those that did not receive T cells , supporting the notion that JAK2 is partially dispensable for T cell mediated GVL activity (Figure 1E-G). The tumor mortality among recipients of JAK2-/- T cells was not significantly different (p=0.15, Log-rank test) compared to those of WT T cells (Figure 1E-G). These experiments demonstrate that JAK2 signaling in donor T cells contributes to GVHD but is partially dispensable for the GVL effect.
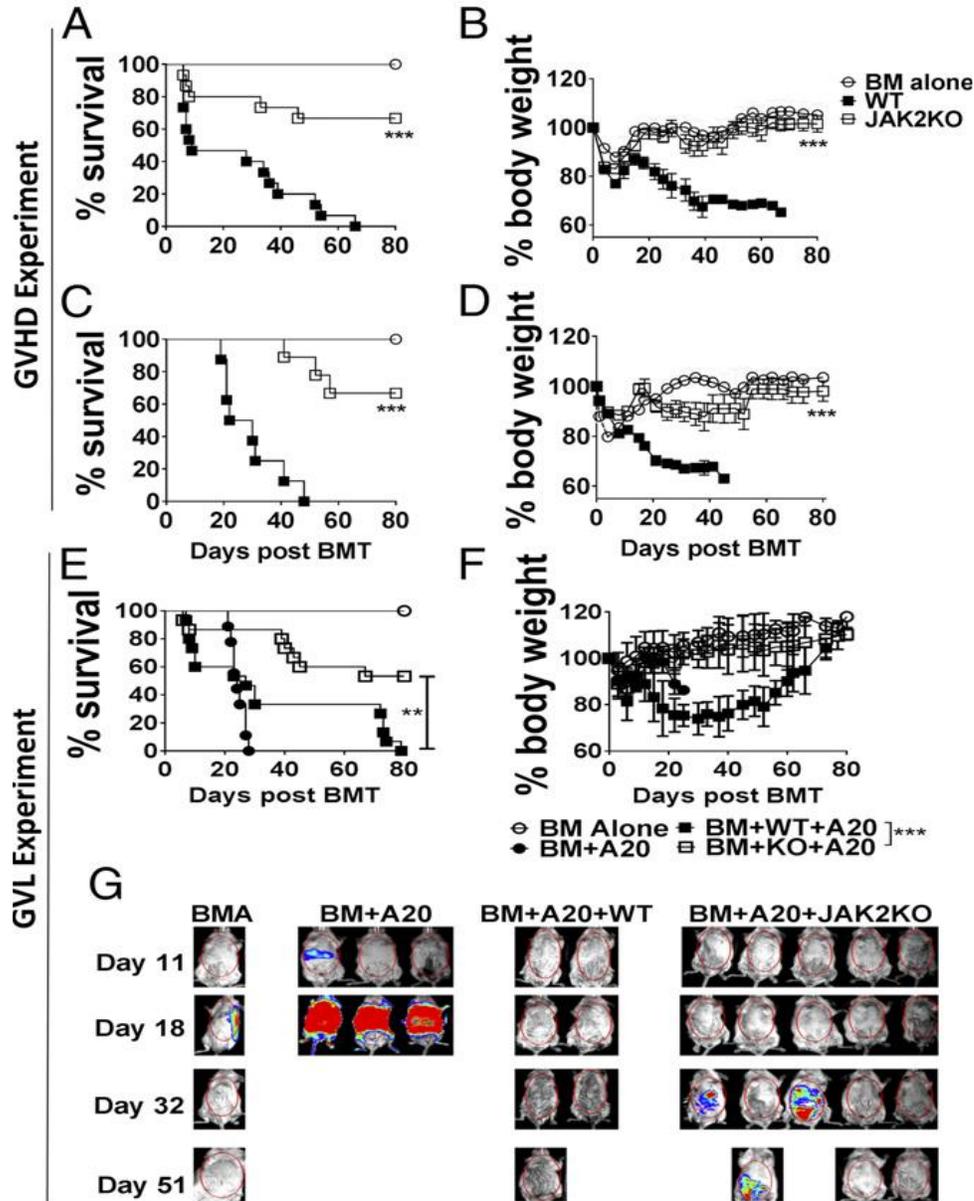
Figure 1. JAK2 contributes to T cell-mediated GVHD, but is dispensable for GVL. Lethally irradiated BALB/c (A and B) or BALB/b (C and D) mice were trans- planted with 5 × 106 TCD-BM alone or plus 1 × 106 (BALB/c) or 3 × 106 (BALB/b) purified T cells from WT B6 or JAK2 KO mice. Survival and body weight loss of BALB/c (A and B) and BALB/b recipients (C and D) are shown. Data shown are pooled from two to three replicate experiments with a total of 6–15 mice per group. Lethally irradiated BALB/c mice were transplanted with 5 × 106 TCD-BM alone or plus 2 × 103 luc-A20 cells and either 1 × 106 purified T cells from WT or JAK2 KO mice plus 2 × 103 luc-A20 cells. Recipient survival (E), body weight loss (F), and tumor burden (G) are shown. Percentage survival and tumor mortality data shown were pooled from three replicate experiments with a total of 6–15 mice per group. Representative BLI images were taken from one of three replicate experiments. **P < 0.01; ***P < 0.001.

IV.4.b. JAK2 inhibits Th2 and Treg polarization in vivo

To determine how JAK2 contributes to T cell differentiation, we analyzed the cytokine production profile of WT and JAK2-/- T cells in allogeneic recipients 14 days post BMT., JAK2-/- T cells had significantly less IFNγ+ Th1 differentiation in the spleen (Figure 2A-C). Conversely, JAK2-/- T cells had significantly enhanced IL4/5+ Th2 and Foxp3+ Treg polarization (Figure 2A-C). JAK2-/- CD8+ T cells also produced less IFNγ compared to WT controls (Figure 2A-C). Though limited to a small positive population of cells, JAK2-/- T cells exhibited increased IL-17+ Th17 differentiation (Figure 2A-C). Similar results were observed in the liver (Supplemental Figure 4).
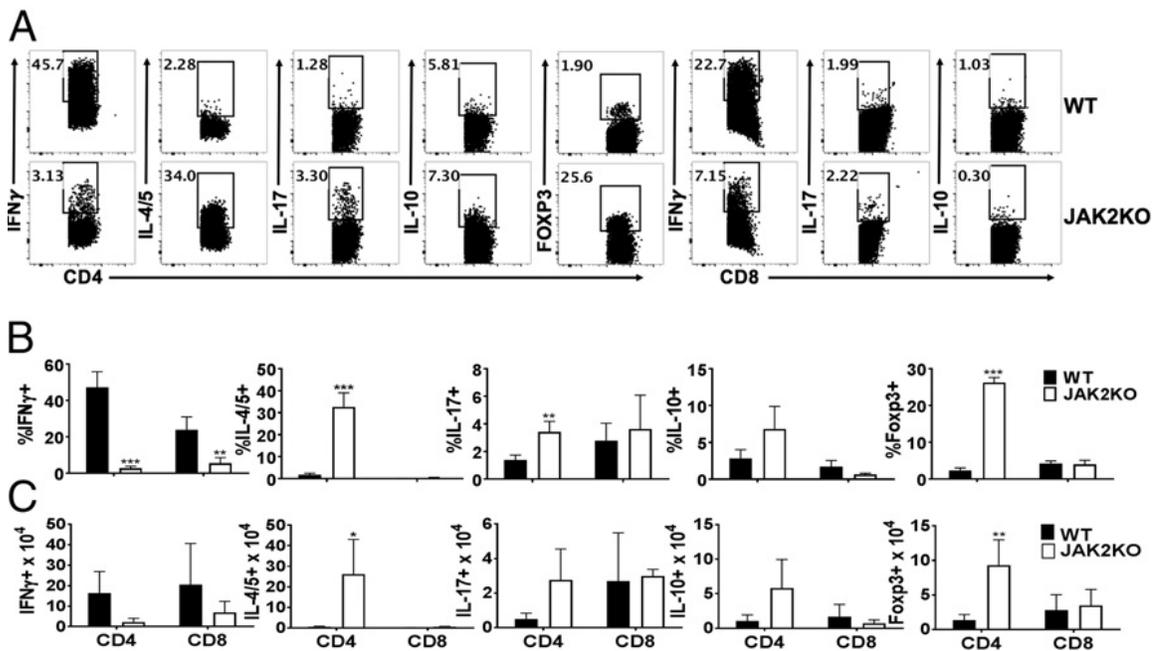
Figure. 2. Donor T cells deficient for JAK2 are prone to Th2 and Treg polarization in vivo. Lethally irradiated BALB/c mice were transplanted with 5 × 106 Ly5.1+ TCD-BM alone or plus 1 × 106 purified T cells (Ly5.2+) from WT B6 or JAK2 KO mice. Recipient splenic mononuclear cells were isolated for immunopheno- typing on day +14 post-BMT. Data depict one repre- sentative mouse per group for IFNγ+, IL-4/5+, IL-17+, Foxp3+ (Tregs), or IL-10+ among gated H2Kb+Ly5.1-CD4+ or CD8+ cells (A). Average percentages +SD (B) or absolute numbers (C) of splenic T cell subsets are shown from one of three replicate experiments. Splenic cells from 9 to 11 mice per group were ana- lyzed in total. *P < 0.05; **P < 0.01; ***P < 0.001.

IV.4.c. JAK2 contributes to the migratory capacity of donor T cells

We then investigated the impact of JAK2 signaling on donor T cell migration. Expression of chemokine receptor, CXCR3, and the integrin, α4β7, were significantly decreased among CD4+ JAK2-/- T cells in the spleen of recipients (Figure 3A,B). This reduction in CXCR3 expression is indicative of reduced trafficking among Th1 cells[179], while decreased α4β7 demonstrates impaired migration to intestinal tissues[28,180]. However, the expression of CCR6l, a key Th17 chemokine receptor, was similar among cohorts (Figure 3A,B). These data show that JAK2 activation promotes T cell homing potential to GVHD target organs. Consistent with the observed decrease in α4β7 expression, recipients of JAK2-/- T cells had significantly less tissue damage in the small and large intestine (Figure 3C, D); a similar decrease in target organ damage was also observed in the skin and liver of JAK2-/- T cell recipients (Figure 3C, D). Although these results do not directly assess T cell trafficking, the data suggests JAK2 contributes to the expression of necessary chemokines and integrins required for lymphocyte migration.
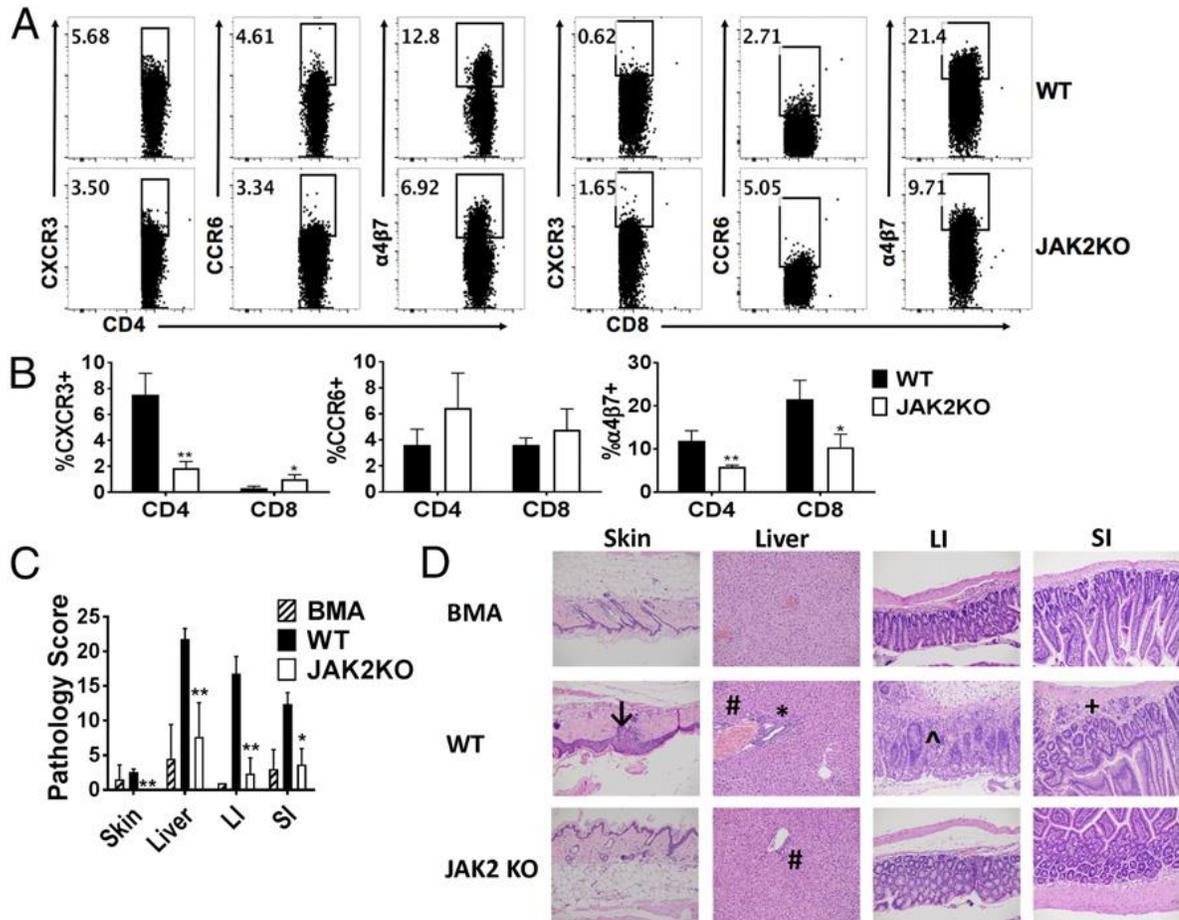
Figure. 3. JAK2 contributes to the migratory capacity of donor T cells. Lethally irradiated BALB/c mice were transplanted with 5×10^6 Ly5.1+ TCD-BM alone or plus 1×10^6 purified T cells (Ly5.2+) from WT B6 or JAK2 KO mice. Recipient splenic mononuclear cells were isolated for T cell migration surface markers on day +14 post-BMT. Flow plots show expression of CXCR3, CCR6, or α4β7 (A) among gated H2Kb+Ly5.1- CD4+ or CD8+ cells. (B) The mean frequency of T cell CXCR3, CCR6, or α4β7 expression ±SD for one of three replicate experiments is shown. A total of 9–11 mice per group were analyzed. (C) Quantified GVHD tissue damage scores ±SD from one representative experiment are shown (BMA: 6 mice; WT: 14 mice; JAK2 KO: 15 mice). (D) Representative H&E sections of GVHD target organs from each cohort showing vacuolar changes in the skin (↓); endothelialitis (#) and mononuclear infiltrates in the liver (*); crypt regeneration in the large intestine (LI, ^); and lamina propria inflammation in the small bowel (SI, +). *P < 0.05; **P < 0.01. (Magnification: 200×.)

IV.4.d. Pharmacologic inhibition of JAK2 with pacritinib reduces GVHD and spares GVL

To translate the observations made using JAK2-/- T cells, host WT BALB/c mice received B6 allografts and were treated with pacritinib, a JAK2 inhibitor, or vehicle for 3 weeks. Pacritinib significantly reduced acute GVHD mortality among recipient BALB/c mice (Figure 4A,B). At 4 days' post BMT, similar to results observed in JAK2-/- T cells, pacritinib significantly reduced CD4+ T cell production of IFNγ and proliferation of Th1 cells (Figure 4C). Notably, pacritinib treatment did not significantly impact JAK2-/- T cells in these studies, indicating the potential off target effects of pacritinib in this context were minimal. While ruxolitinib (JAK1/2 inhibitor) impaired murine CTL activity against tumor in vitro (Supplemental Figure 5), neither pacritinib nor ruxolitinib interfered with the GVL effect in vivo (Supplemental Figure 6A-C). Additionally, ruxolitinib was associated with greater immune suppression against GVHD (p<.05, Supplemental Figure 6A-C), supporting that dual JAK1/2 inhibition induces broad donor T cell inactivation compared to pacritinib. Pacritinib had no direct effect on P815 cells (Supplemental Figure 6D-F).
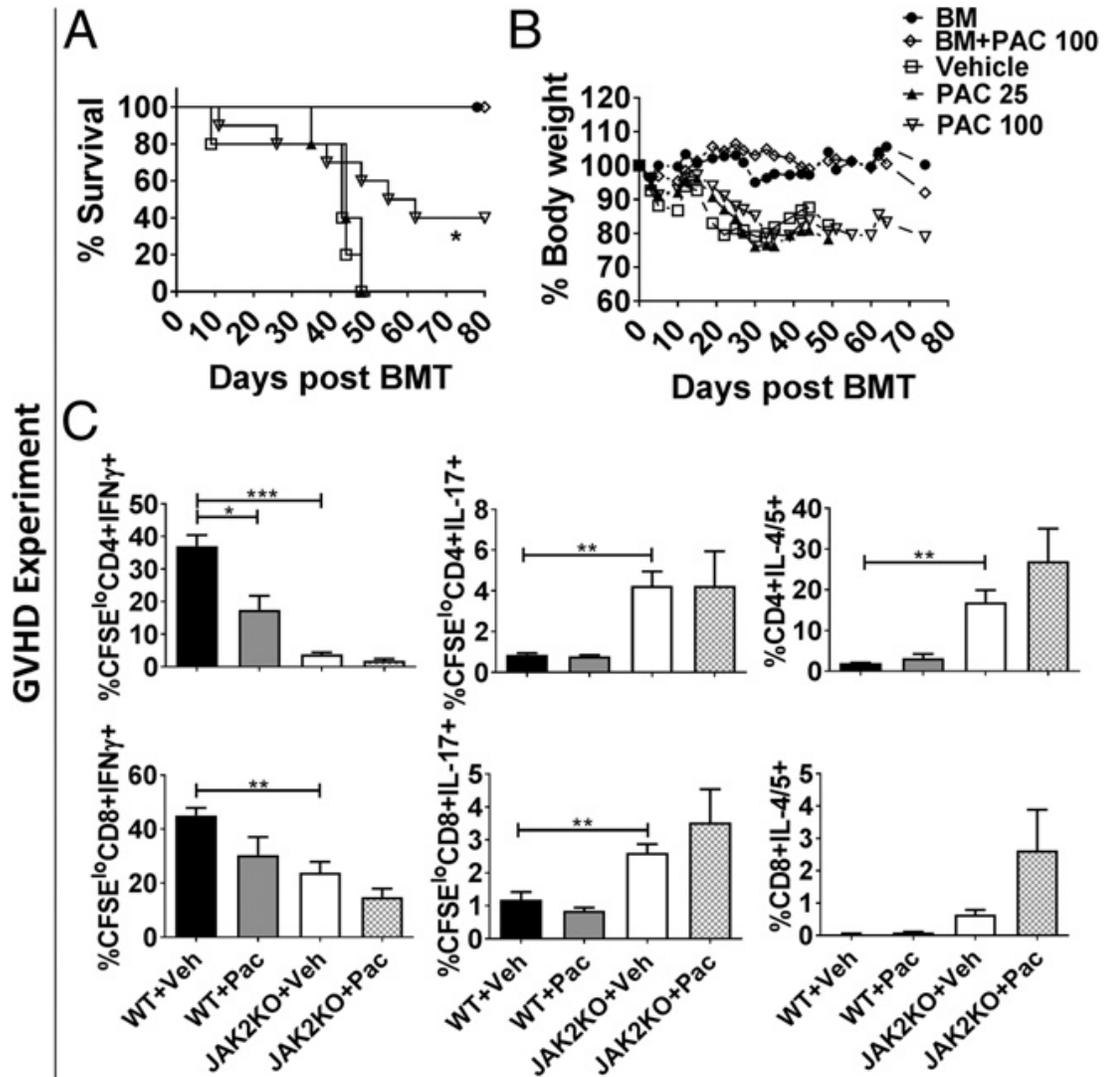
Figure 4. Pharmacological inhibition of JAK2 with pacritinib reduces GVHD and spares GVL. Lethally irradiated BALB/c mice were transplanted with 5×10^6 TCD- BM alone or plus 1×10^6 T cells/mouse from WT B6 donors. Pacritinib 100 mg/kg or methylcellulose vehicle was given by oral gavage daily for 3 wk starting on day 0 of BMT. Recipient survival (A) and body weight loss (B) are shown. In separate experiments, lethally irradiated BALB/c mice were transplanted with carboxy- fluorescein succinimidyl ester (CFSE)-labeled T cells from either WT or JAK2 KO donors and treated with pacritinib or vehicle. Average percentages +SD of CFSE- diluted CD4+ and CD8+ T cells positive for IFNγ, IL-4/5, and IL-17 for are shown (C). A total of 12 mice/group were used across the three experiments (A–C). *P < 0.05; **P < 0.01; ***P < 0.001. Pac, pacritinib; Veh, vehicle.

IV.4.e. Pacritinib polarizes a Th2 response among DC-allostimulated, human T cells

We went on to verify the immune suppressive effects of pacritinib in a human system. Using cytokine- or DC-stimulated human T cells, pacritinib inhibited JAK2-dependent phosphorylation of STAT3 and significantly suppressed alloreactive T cell proliferation (Supplemental Figure 7A, B). IL-2-mediated STAT5 signal transduction, required by Treg and CTL alike, was largely preserved among T cells exposed to pacritinib (Supplemental Figure 7A). JAK2 inhibition of human T cells directed robust Th2 polarization and significantly decreased Th1 and Th17 differentiation (Figure 5A-C). Pacritinib also suppressed lymphocyte production of IL-6, IL-17A, IL-17F, and TNF-alpha[181] (Supplemental Figure 7C). As demonstrated in rodents[168], both pacritinib and ruxolitinib reduced IFNγ-mediated signaling in human T cells (Supplemental Figure 8).
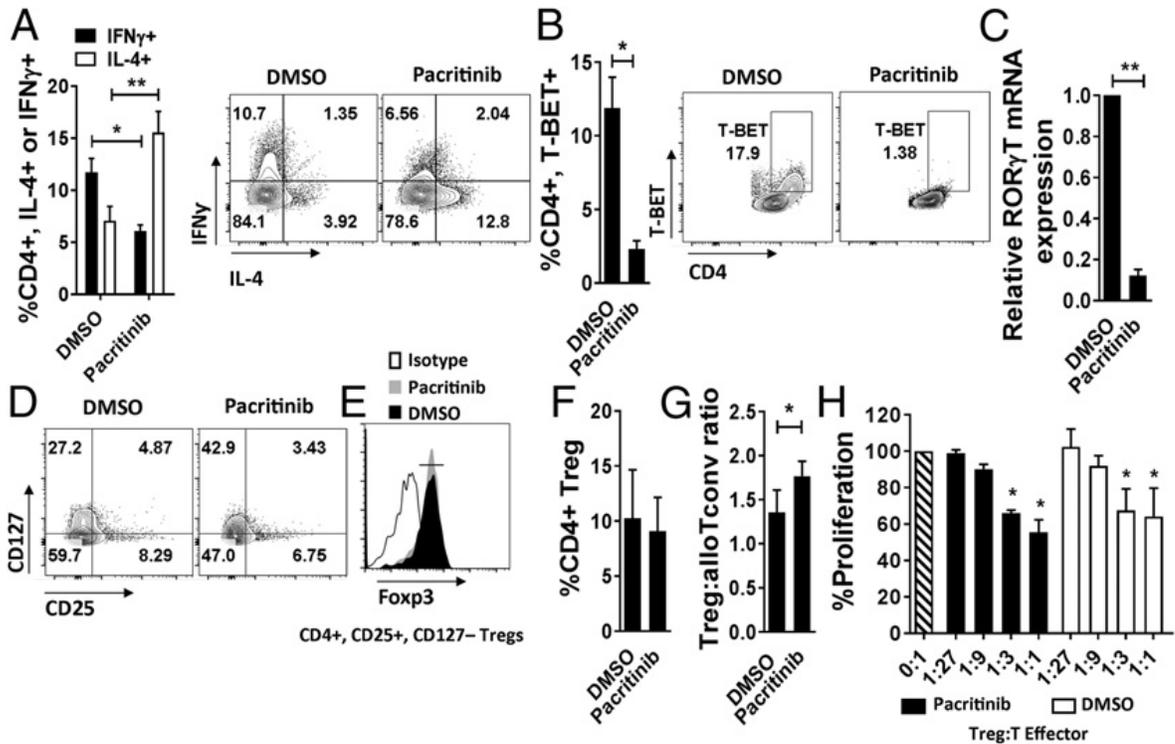
Fig. 5. Pacritinib polarizes a Th2 response by human T cells after allogeneic stimulation in vitro and permits the differentiation of suppressive iTreg. Data show the effect of pacritinib (2.5 μM) on (A) Th1 (IFNγ) versus Th2 (IL-4), (B) T-BET expression (replicate means ± SEM, n = 4 experiments), and (C) RORγT expression ±SD among CD4+ T cells after DC allostimulation (one of two experiments is shown, performed in triplicate). (D and E) iTregs were generated from Treg-depleted, DC-allostimulated CD4+ T cells in the presence of pacritinib (2.5 μM) or vehicle control. Contour plots show iTregs and corresponding Foxp3 expression. (F and G) Graphs show the mean frequency of iTreg and ratio of iTreg to alloreactive Tconv (CD4+, CD25+, CD127+) ±SD after DC allostimulation. n = 6 experiments. (H) iTreg-suppressive potency is demonstrated against self-T cell responders stimulated by allogeneic DCs without additional drugs. One of two experiments are shown, each performed in triplicate. *P < 0.05; **P < 0.01.

IV.4.f. Pacritinib permits the differentiation of suppressive human induced Treg

CD4+ T cells were purified, depleted of natural Tregs as previously described (>99% non-Treg), and stimulated with allogeneic, cytokine-matured DCs for 5 days with pacritinib or DMSO added once on day 0. Induced Treg (iTreg) differentiation was similar following DC-allostimulation, regardless of pacritinib treatment (Figure

5D-F). Conversely, pacritinib significantly increased the ratio of iTreg to activated conventional T cells (Tconv) compared to DMSO (Figure 5G). The suppressive potency of pacritinib- or DMSO-pretreated iTregs was similar, suggesting JAK2 is not required for human iTreg function (Figure 5H).

IV.4.g. Pacritinib reduces xenograft rejection but maintains CD8+ CTL activity against tumor

The immune suppressive effect of pacritinib on human T cells was tested in vivo. Immunodeficient NSG mice received a dorsally positioned, 1cm2, split-thickness human skin graft. After a 30-day rest period, $5x10^6$ human PBMCs, allogeneic to the skin donor, were injected into the mouse[172,176]. Mice received pacritinib (100mg/kg) or vehicle twice a day by oral gavage from day 0 until day +14. Pacritinib significantly delayed skin graft rejection by allogeneic T cells compared to vehicle (Figure 6A,B). Skin xenografts from pacritinib-treated mice therefore exhibited significantly less pathologic rejection at day +21 (Figure 6C,D), compared to the vehicle-treated controls. IHC analysis suggested a modest reduction in Th1 cells (CD4+, T-BET+) in the skin of pacritinib treated mice (Figure 6E,F). We used an established method to generate human anti-tumor CTL in vivo and then test their specific killing in vitro[172]. NSG mice received human PBMCs ($30x10^6$) and were inoculated with irradiated U937 cells ($10x10^6$) on days 0 and +7. Mice were treated with pacritinib, ruxolitinib (30mg/kg twice a day, or vehicle as described[161]. Human CD8+ T cells were isolated from the spleens of euthanized recipients during days +10-12 and cultured against fresh U937 target

cells without further drug exposure in vitro. Ruxolitinib significantly impaired anti-tumor activity, while CTL function was preserved among mice treated with pacritinib or vehicle controls (Figure 6G).
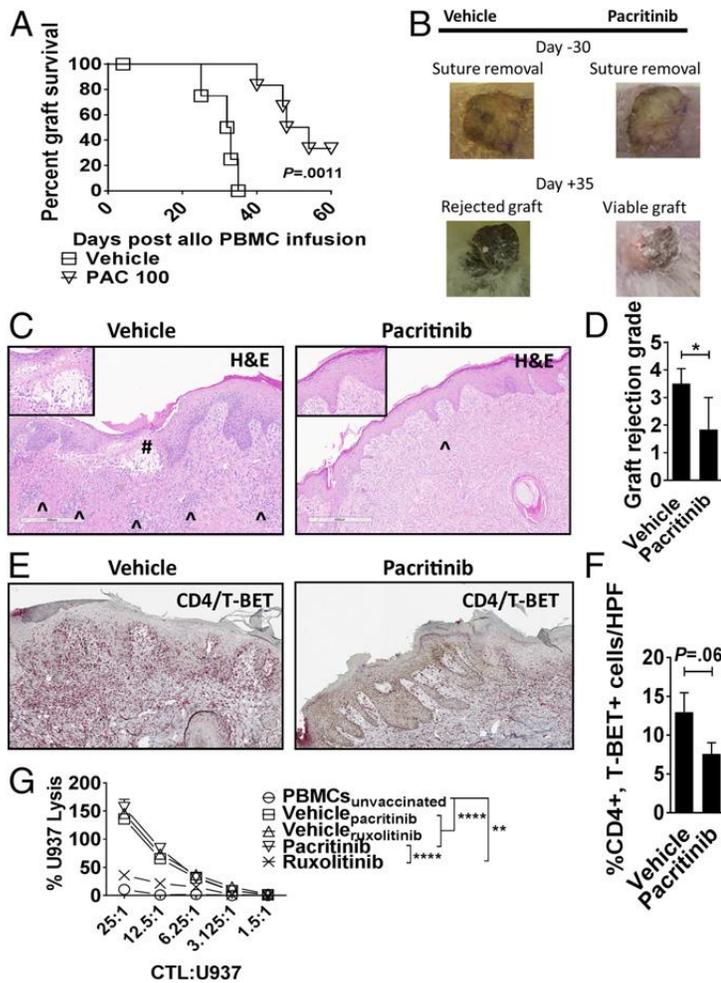
Figure 6. Pacritinib reduces xenograft rejection. NSG mice received a 1-cm^2 split thickness human skin graft. After 30 d of rest to ensure engraftment, an inoculum of 5×10^6 human PBMCs (allogeneic to the skin) were administered by i.p. injection. Unique pairs of donor skin and allogeneic PBMCs were used for each set of experiments. Pacritinib 100 mg/kg or vehicle was given twice a day from days 0 to +14. (A) Graph shows human skin graft survival among pacritinib- or vehicle-treated NSG hosts (log-rank test). (B) Representative im- ages show skin at time of suture removal (day −30) and at day +35. (C) His- tologic representations of the skin grafts uniformly harvested on day +21 demonstrate that pacritinib reduces lymphocytic infiltration (^) and severe basal vacuolar changes of the graft, such as the subepidermal blister (#). Low power at 6✕, high-power Inset at 20✕. (Scale bar, 400 μm.) (D) Bar graph shows pathologic skin graft rejection scores at day +21 among vehicle- and pacritinib- treated mice. (E and F) Immunohistochemistry and accompanying bar graph shows skin-resident Th1 cells at day +21 by dual staining of CD4 (red) and T-BET (brown). n = 2 experiments, 5–6 mice per arm. (G) Graph depicts mean specific lysis ±SEM by human CD8+ CTL generated in vivo using NSG mice transplanted with human PBMCs (30×10^6) and vaccinated with irradiated U937cells (10×10^6) on days 0 and +7. Mice were treated with pacritinib (100 mg/kg twice a day), ruxolitinib (30 mg/kg twice a day), or vehicle from day 0 up to day +12. Human CD8+ T cells were harvested from euthanized mice between days +10 to +12. Results shown are from one of two independent experiments. U937 lysis was measured by colorimetric assay after 4 h. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$. (Magnification: C and E, slides were scanned using a 20✕/0.8 N.A. objective and viewed using a 6.5✕ digital zoom in ImageScope software.)

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IV.5. Discussion

While it is known that JAK2 inhibition spares Tregs and reduces Th1 responses[26,172], we demonstrate that genetic ablation of JAK2 on donor T cells or treatment with pacritinib significantly enhances Th2 differentiation after allo-HCT. Strategies to modulate Th2 over Th1 have proven effective in reducing GVHD lethality in rodents. IL-18 promotes STAT6-dependent Th2 polarization, suppresses Th1s, and reduces GVHD yet spares GVL[182]. Th2 cells are also required for MDSC-mediated immune suppression and GVHD prevention[183]. Additionally, the GVHD biomarker, soluble suppression of tumorigenicity 2 (sST2), orchestrates alloreactivity in part by binding IL-33 and preventing Th2 differentiation[184]. Therefore, we surmise that Th2 polarization contributes to the immune suppressive activity of JAK2 inhibition.

The recipients of JAK2-/- T cells survived longer, higher body weight maintenance, and less GVHD pathology in their small intestine, large intestine, liver and skin compared to WT controls. We also observed significantly less Th1 differentiation and CXCR3 expression among JAK2-/-T cells. Further, JAK2-/- donor T cells had reduced expression of the α4β7 integrin that is required for gut homing by Th1 cells. Our data suggest targeting JAK2 may reduce GVHD in part by limiting Th1 differentiation and the migratory capacity of alloreactive T cells[28,179].

Species-specific immune effects were observed among the murine GVHD experiments compared to the human in vitro and xenogeneic tissue rejection experiments. Pacritinib significantly reduced human Th17 differentiation, which is similar to the published results using the JAK2 inhibitor, TG101348[26,172]. Conversely, recipients of JAK2-/- T cells and those treated with pacritinib exhibited moderately increased Th17 cells after allo-HCT. This is distinct from STAT3 KO donor T cells, which results in reduced Th17 differentiation in transplanted mice[185]. TGFβ can promiscuously activate STAT3 in T cells to facilitate murine Th17 development[186,187]. The loss of JAK2 countered by STAT3 activity via alternative pathways may enhance Th17 differentiation in mice but not humans due to the species-specific effects of TGFβ[188]. This is further supported by the observation that JAK2-/- T cells exhibited significantly increased Treg differentiation compared to WT controls, which may serve as a source of TGFβ in vivo[189]. Our in vitro human data demonstrated that JAK2 inhibition with pacritinib spared Treg differentiation, but did not increase the amount of Tregs compared to controls (Figure 5F). However, the ratio of Tregs to activated Tconv was increased with pacritinib (Figure 5G).

An important limitation of these data is that pacritinib is a multikinase inhibitor with effects on pathways potentially relevant to GVHD[175]. Pacritinib potently inhibits JAK2, FLT3, IRAK1, TNK1, ROS, and HIPK (IC50 <50nM), and also has activity against JAK3 and TYK2 (IC50 50-100nM) in the JAK-STAT system[175]. Such activity by pacritinib may be important to differences seen between JAK2-/-T cells

and the studies using pacritinib as a JAK2 inhibitor in GVHD prevention. However, the anti-JAK3 activity of pacritinib did not prevent IL-2-mediated phosphorylation of STAT5, which is required by Tregs and CTL. Tyk2 and JAK2 regulate p40 cytokine receptor signal transduction[190], and Tyk2 inhibition by pacritinib might also impact Th1 and Th17 differentiation beyond the effects of JAK2 blockade. Additionally, suppression of IRAK1 and IL-1β receptor activity may modulate donor alloresponses[191]. In comparison, ruxolitinib inhibits JAK1 and JAK2 equally[161]. Ruxolitinib is known to suppress STAT5 phosphorylation in human T cells and NK cells via JAK1 inhibition[169,171]. Our group and others have observed that ruxolitinib, as opposed to pacritinib, significantly impairs human CTL function[169], although this is not observed among murine T cells in vivo (Supplemental Figure 6A-C)[168]. These data suggest that human and mouse T cells have somewhat different sensitivity to Jak inhibition. Similar to ruxolitinib, we show pacritinib also limits human NK cell proliferation and function in vitro (Supplemental Figure 9). While pacritinib spares JAK1, we surmise its effect on NK cells is due to suppression of Tyk2 and impaired IL-12 activity[192]. Given that pacritinib permits common gamma chain cytokine signal transduction, such as IL-2, its effect on JAK3 does not explain the drug's inhibition of NK cells. Our data also supports ruxolitinib induces profound immune suppression compared to pacritinib, yet potentially at the cost of CTL function required for anti-tumor activity.

Our data support that neutralization or pharmacologic blockade of JAK2 with pacritinib preserves donor T cell mediated GVL activity. We propose that targeting

JAK2, but sparing JAK1, with agents such as pacritinib is sufficient to control alloreactivity without impairing normal T-effector and Treg function. A phase I/II acute GVHD prevention trial combining pacritinib with standard immune suppression after allo-HCT is actively being investigated (NCT02891603). Therefore, such an approach has direct translational implications in GVHD and solid organ rejection prophylaxis.

V. Conclusions and future directions

Targeting p40, a shared subunit of IL-12 and IL-23 cytokines, consistently mitigates GVHD in clinical and preclinical studies. Our group and others have demonstrated that neutralization of the p40, using genetically deficient mice and pharmacological inhibition, alleviated acute and chronic GVHD in murine models through reducing Th1 and Th17 differentiation[145,152]. Recent data from Pidala, et al. demonstrates *in vivo* IL-12/IL-23p40 neutralization with ustekinumab blocks the Th1/Th17 response and improves overall survival in patients after allo-HCT[150]. Notably, in other models of autoimmunity, in which Th17 is the major mediator of diseases, much of the originally allocated inflammatory actions of IL-12 have since been shown to be influenced by IL-23, as many studies prior to IL-23 identification were conducted via targeting p40[91,193]. Regarding clinically translatable approaches targeting the IL-12 family, ustekinumab targets the p40 shared subunit between IL-23 and IL-12. Ustekinumab added to tacrolimus and rapamycin was shown to be safe and effective for GVHD prophylaxis after related or unrelated allo-HCT. In a randomized, blinded, placebo-controlled study, ustekinumab significantly improved overall survival and CRFS (Conditional Random Fields Score), a novel composite endpoint including moderate/severe cGVHD and relapse-free survival[150]. Guselkumab and tildrakizumab, two monoclonal antibodies against p19, were approved for treatment of plaque psoriasis[194,195]. However, these specific neutralizing antibodies against p19 have yet to be evaluated in GVHD patients. Recent findings have emphasized the need to

develop therapeutics methods that enable targeting of IL-12 and IL-23 signaling. A key point of our data is the potential involvement of IL-39. Given the novel nature of this cytokine, much more research is required to fully understand the role of IL-39, especially with regards to GVT. However, our work does provide a baseline by which to begin evaluating whether better outcomes can be achieved by targeting p19 in GVHD. As mentioned above, p40 blockade has been shown to be clinically effective in reducing GVHD. Interestingly, we found IL-12Rβ1 to be required for cGVHD, consistent with our results targeting p40. Further, in the study by Pidala et al. in 2018, they found an overall improvement in GVHD outcomes when results from acute and chronic GVHD patients were combined, yet p40 blockade was much more effective when treating patients with cGVHD compared to acute. Therefore, future studies should focus on the biological differences of IL-12 and IL-23 in order to determine why IL-12 can exacerbate GVHD in some contexts yet suppress it in others, yet pharmacologically targeting p40 can be efficacious in reducing GVHD severity in experimental and clinical settings.

Interestingly, not only do the cytokines IL-12 and IL-23 share the same cytokine subunit, p40, but also the cognate receptor, IL-12Rβ1. Thus, these shared motifs provided the rationale for blocking Th1 and Th17 responses simultaneously through targeting p40/IL-12Rβ1. Our data indicates that IL-39 could be responsible for the similar outcomes between WT and IL-12Rβ1KO observed aGVHD models. However, p40 itself has a diverse set of functions. Therefore, it is important to consider these functions as alternative explanations/ additional factors for our

results. For example, p40 has a chemo attractant role for macrophages mediated by IL-12Rβ1 alone, which is dependent on the intracellular domain of IL-12Rβ1 to signal; these reports were published with regard to IL-12Rβ1 signal transduction in response to a p40 homodimer[196,197]. With respect to alloreactive T cells, this p40 homodimer was demonstrated to have antagonistic activity for CD4^{+} IFNγ production, yet could amplify IFNγ production by CD8^{+} T cells[198]. Hence, targeting p40 in the context of GVHD may result in enhanced Th1 responses and potentially hinder CD8 mediated GVT responses[138]. IL-12Rβ1 promiscuity among the IL-12 family has both assisted in the development of pharmaceuticals to target both pathways (as in the case of p40), yet also illuminated their complexity. Th1 and Th17 differentiation and stability converge at IL-12 and IL-23 signaling, respectively, as both signaling motifs share p40 at the cytokine level and IL-12Rβ1 for downstream signal transmission. Supported by studies done in mice and men, IL-12 is documented to induce IFNγ production by Th17 cells with respect to cytokines in the milieu, *in vivo* and *in vitro* respectively[199,200]. This Th1/Th17 subset was shown in Crohns disease[200].

The use of any immunosuppressive agent carries the theoretical risk of impairing host defense responses to pathogens and/or decreased tumor surveillance. Relative risks of targeting IL-12 and/or IL-23 are well documented with respect to potential risk of infections. When challenged with *Mycobacterium*, *Salmonella* or *Candida*, mice lacking IL-12p35, IL-12p19 and IL-12/23p40 have phenotypes that generally mirror what has been observed in humans. Studies of IL-12/23p40 and

IL-12Rβ1 deficiencies indicate that human IL-12 and IL-23 are redundant in host defense to many pathogens. Importantly, allo-HCT recipients treated with ustekinumab did not experience any increase in opportunistic infections or reactivation of CMV, EBV, or HHV6 compared to the placebo arm[150]. One important factor to consider going forward is whether a similar safety profile is observed using p19 inhibitors.

Our group has demonstrated both Th1 and Th17 subsets are required to induce GVHD[25]. Pharmacological inhibition of IL-23p19 results in reduced GVHD, and recent evidence suggests that IL-23R drives GVHD pathogenesis[52–54,56]. These studies show that a CD4+CD11c+IL-23R+ T cell population induces colonic inflammation during GVHD, indicating a key role for IL-23R expression on donor T cells in mediating damage to the gut after allo-HCT. Consistently, the gene expression levels of *IL-23* and *IL-23R* were upregulated in murine colons after allo-HCT[54]. These studies demonstrate that the colon is specifically protected via IL-23p19 signaling blockade, and that GVL activity is maintained. In a patient cohort, Liu et al. observed IL-23 mRNA expressions in patients with aGVHD were significantly higher than those in healthy donors, and IL-23 and IL-23R expression were positively correlated with IL-17 expression[201]. These studies additionally showed that IL-23 serum levels were elevated during the onset of aGVHD yet decreased during disease remission (Table 1). In aGVHD, two out of three independent studies in patients found that a single nucleotide polymorphism (rs11209026) in IL-23R of donor origin reduced incidence of GVHD; the third study

did not observe any effect[30,202]. Hence, blocking either p19 or p40 reduces aGVHD and IL-23R deficiency in donor T cells results in abrogated GVHD. These results indicate IL-23 also plays a key role in GVHD pathogenesis. Albeit, a recent paper demonstrated genetic inactivation of IL-23R, or the transcription factor RORγt, within donor T cells similarly ablated Th17 cell formation *in vivo* but preserved the T cells' ability to induce intestinal GVHD in an indistinguishable manner compared to wild-type controls[24]. Hence, further studies are required to dissect this conflicting result.

As with p40, IL-12Rβ1 has also been implicated to have a regulatory role, although there remains a lack of data in this area. IL-12Rβ1 was identified in 1994 by Chua et al. as a member of the hemopoietin receptor superfamily, an amino acid type I transmembrane protein that resembled the IL-6 signal transducer, gp130[203]. It was not until 1996 that IL-12Rβ2 was identified, which subsequently led to the identification of a high affinity IL-12 receptor complex when IL-12Rβ1 and IL-12Rβ2 were coexpressed[41]. Notably, the existing data with respect to IL-12Rβ1 in murine models of GVHD is sparse, although there is an abundance regarding its role in conferring immunity to mycobacteria and other infections[204–206] in human. However, given that deficiency of IL-12Rβ1 is relevant in patients, there are a plethora of case studies documenting related T cell responses[207]. The IL12Rβ1 promoter, when deficient of the -265 to -104 region, suggested the existence of an important regulatory element. Furthermore, the -111A/T substitution appeared to cause decreased gene transcriptional activity, such that cells from -111A/A

individuals were observed to have increased IL12Rβ1 mRNA levels compared with those from -111T allele carriers. Thus, in individuals with the -111T/T genotype, reduced IL12Rβ1 expression may lead to augmented Th2 cytokine production in the skin, and subsequently contribute to the development of atopic dermatitis and other associated allergic diseases[208].

Of particular interest is the role of IL-12/IL-12Rβ1 pathway in the induction of highly suppressive antigen-specific Th1-like Tregs from naïve Tregs[209]. It was recently described that, in two patients with IL-12Rβ1 deficiency, features of systemic autoimmunity and photosensitivity were observed[210]. These features are similar to transgenic mice deficient for IL-12Rβ2, which develop an autoimmune syndrome consisting of anti-DNA positivity, immunocomplex glomerulonephritis, and multiorgan lymphoid infiltrates with features of vasculitis. However, IL-12Rβ1 deficient patients displayed substantially less circulating memory Tfh and memory B cells than healthy controls[85]. In humans, TGFβ cooperates with IL-12 and IL-23 for expression of Tfh molecules: CXCR5, ICOS, IL-21, and the transcriptional regulator Bcl6[189]. Hence, data taken from studies in IL-12Rβ1 deficient patients suggests a regulatory role for IL-12, perhaps derived from Treg function, which may explain the contradictory results observed in murine models. Albeit, the role of IL-12 in Tfh/B cell axis seems at baseline consistent among experimental and clinical studies. Our data also supports a key role for IL-12 in Tfh function, at least with respect to IL-21 production and BCL6 expression in cGVHD. While the aforementioned discrepancies are preliminary in comparison to the mass of

studies documenting proinflammatory roles of IL-12, there is still much to learn in terms of IL-12 function; especially with respect to differences versus IL-23.

While promiscuity among IL-12 cytokine and/ or receptor family is a common theme, the degree of association with glycoprotein 130 (gp130), better known for its role in IL-6 signaling, has become an intriguing area of research. Gp130 forms the link between "IL-6R/IL-12R" families, which collectively include Leukemia Inhibitory Factor Receptor (LIF-R), IL-12Rβ1, IL-12Rβ2, Granulocyte Colony-Stimulating Factor Receptor (GCSF-R), and Oncostatin-M Receptor (OSM-R); and serves as a shared signal-transducing subunit for IL-6, IL-11, Leukemia Inhibitory Factor (LIF), Oncostatin-M (OSM), Cilliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1), Cardiotrophin-like cytokine (CLC), and IL-27[211,212]. Importantly, gp130 is well documented for its capacity to transduce signals, especially for IL-6, a staple cytokine involved in inflammation. The complex of IL-6 and IL-6R binds to the ubiquitously expressed receptor subunit gp130, which forms a homodimer and thereby initiates intracellular signaling via the JAK/STAT and the MAPK pathways. IL-6R expressing cells can cleave the receptor protein to generate a soluble IL-6R (sIL-6R), which can still bind IL-6 and can associate with gp130 and induce signaling even on cells, which do not express IL-6R. This paradigm has been called IL-6 trans-signaling whereas signaling via the membrane bound IL-6R is referred to as classic signaling[211–213]. This well documented promiscuity is of gp130 is one of the primary reasons we

hypothesized that IL-23R signaling may be able to circumvent IL-12Rβ1 through IL-39.

Other than cytokine blockade, which may target a wide variety of cell types, an alternative strategy to inhibit the IL-23 pathway is by targeting the receptor component. The crystal structure of IL-23Rα was recently reported[214]. Hence, development of pharmaceutical compounds capable of specifically binding/ inhibiting the IL-23R has been stagnant since its discovery in 2002. It appears that IL-23 binds IL-23R with an affinity of 44 nM, while binding IL-12Rβ1 with an affinity of 2 μM; nonetheless, the affinity of the IL-23:IL-23R complex for IL-12Rβ1 has been described as 25 nM, despite no apparent interaction of IL-23R with IL-12Rβ1, implying that there is a cooperative effect which is likely to be due to a conformational change of IL-23 upon binding IL-23R, which is indeed observed crystallographically[214–218]. In a recent publication, hydrogen−deuterium exchange mass spectrometry (HDX-MS) was used to demonstrate IL-23 binding to the N-terminal immunoglobulin domain of IL-23R in both the solid state as well as under more physiologically relevant conditions. This data allowed specific identification of a binding epitope using a macrocyclic small molecule against IL-23R for the first time[217]. However, IL-23R antagonism is not a new concept, as a peptide antagonist was shown to reduce inflammation in different models of autoimmune disease[219]. The aforementioned data presents exciting new possibilities for future studies, yet efficacy of such prototype molecules requires vigorous testing in preclinical models.

Both IL-12R and IL-23R have been demonstrated to signal via JAK2; JAK2 deficient donor T cells or JAK2 inhibition with pacritinib were demonstrated to significantly alleviate GVHD in murine models via spared Treg differentiation and reductions in Th1 and Th17 differentiation in in mouse and human T cells[220]. This is consistent with reports describing a common reliance on JAK2 by both IL-12 and IL-23. A key difference in downstream signaling is that IL-12 phosphorylates primarily STAT4, while IL-23 mainly induces STAT3 phosphorylation. Betts et al. reported that at 20 days' post allo-HCT, pSTAT3 was significantly increased in CD4+ T cells among patients who would later develop aGVHD; a signaling pathway known to directly drive the transcription of Th17 lineage-specific genes[26].

JAK2 signal transduction is implicated in human autoimmune syndromes and GVHD. IL-6, IL-12, and IL-23 mediate inflammation and activate T cells via JAK2[26,158–161]. Blocking the IL-6 receptor with the monoclonal antibody tocilizumab has demonstrated efficacy in a phase II GVHD prevention trial[162]. Tocilizumab, however, does not fully impair pathogenic Th1/Th17 responses[163], which may be attributed to the IL-12 and IL-23 receptor signaling-induced JAK2 activation to promote Th1 and Th17 differentiation, respectively. Neutralization of these p40 cytokines prevents GVHD in murine models, and may have activity in treating patients with steroid refractory GVHD[139].

JAK2 inhibition is an alternative approach to suppress IL-6 and p40 receptor signal transduction and induce durable tolerance to alloantigens. JAK2 inhibitors are clinically efficacious in myelofibrosis, a hematological disease often driven by constitutive JAK2 activation[164]. The existing evidence regarding JAK2 as a therapeutic target for acute GVHD is primarily supported by observations using ruxolitinib, an equimolar inhibitor of JAK1 and JAK2[165–168]. Ruxolitinib has been previously demonstrated as efficacious in treating steroid refractory GVHD and is clearly immunosuppressive. In part, JAK1 mediates the biologic effects of common gamma chain cytokines, including IL-2 and IL-15. Ruxolitinib suppresses host-reactive T cells in mice and humans. Although not observed in murine transplant studies, ruxolitinib treatment reduces the quantity of Tregs as well as the beneficial effects of NK cells in myelofibrosis patients[169–171]. Therefore, a JAK2 inhibitor has the potential to prevent GVHD without conceding JAK1-mediated functions provided by donor lymphocytes. Further research determining the differential effects of JAK1 and JAK2 is required to resolve these conundrums.

Given the recent discovery of IL-39 (p19/EBI3) and its cognate receptor (IL-23Ra/gp130), the question pertaining to the individual requirement for each particular cytokine/ receptor complex becomes much more complex. IL-39R was shown to signal via STAT1/ STAT3 pathways, which overlaps with IL-27 and IL-23 signaling, respectively. The manner by which IL-39R and IL-23R on T cells may differentially or similarly impact the T cell response in allo-HCT requires further investigation.

While inhibiting JAK2 signal transduction by IL-12 and IL-23 is a promising strategy, the question pertaining to how the shared or disparate receptors contribute to signal transduction and the consequential effect on T cell differentiation in allo-HCT remains unclear. The advancement of targeted pharmacological compounds specific for IL-12 or IL-23 signaling will be required to adequately dissect these scientific questions appropriately across species.

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