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Author manuscript *Leukemia.* Author manuscript; available in PMC 2018 October 03.

Published in final edited form as:

Leukemia. 2018 November ; 32(11): 2483-2494. doi:10.1038/s41375-018-0123-z.

# Baricitinib-induced blockade of interferon gamma receptor and interleukin-6 receptor for the prevention and treatment of graft-versus-host disease

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# Abstract

The therapeutic benefits of allogeneic hematopoietic stem cell transplantation (allo-HSCT) are derived from the graft-versus-leukemia (GvL) effects of the procedure. There is a strong association between the GvL effects and graft-versus-host disease (GvHD), a major lifethreatening complication of allo-HSCT. The limiting of GvHD while maintaining the GvL effect remains the goal of allo-HSCT. Therefore, identifying optimal therapeutic targets to selectively suppress GvHD while maintaining the GvL effects represents a significant unmet medical need. We demonstrate that the dual inhibition of interferon gamma receptor (IFN $\gamma$ R) and interleukin-6 receptor (IL6R) results in near-complete elimination of GvHD in a fully major histocompatibility complex–mismatched allo-HSCT model. Furthermore, baricitinib (an inhibitor of Janus kinases 1 and 2 [JAK1/JAK2] downstream of IFN $\gamma$ R/IL6R) completely prevented GvHD; expanded regulatory T cells by preserving JAK3-STAT5 signaling; downregulated CXCR3 and helper T cells 1 and 2 while preserving allogeneic antigen-presenting cell–stimulated T cell proliferation; and suppressed the expression of major histocompatibility complex II (I-Ad), CD80/86, and PD-L1 on host antigen-presenting cells. Baricitinib also reversed established GvHD with 100%

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Conflict of Interest: no conflicts of interest.

Author Contributions: Conceptualization: JC, MLC, and JFD; methodology: JC, MLC, and JFD; investigation and validation: JC, MLC, KS, KA, KRV, BW, LM, JN, JR, and BA; formal analysis: JC, MLC, KS, and LM; resources: SA and IT; writing – original draft: JC, MLC, and JFD; writing – review and editing: JC, MLC, KS, SA, IT, MAS, and JFD; visualization: JC, MLC, KS, KRV, and LM; supervision: JC and JFD; funding acquisition: JC and JFD.

survival, thus demonstrating both preventive and therapeutic roles for this compound. Remarkably, baricitinib enhanced the GvL effects, possibly by downregulating tumor PD-L1 expression.

# Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the only curative therapy for relapsed and refractory hematological malignancies. The therapeutic benefits of allo-HSCT are primarily derived from its graft-versus-leukemia (GvL) effects, which are mediated by mature T cells present in the donor graft. Unfortunately, the same donor T cells that mediate the GvL effects can also cause graft-versus-host disease (GvHD), the major source of non-relapse morbidity and mortality among allo-HSCT patients. There is a lack of optimal therapeutic targets for preventing GvHD while preserving the beneficial GvL effects. Current GvHD treatment strategies that broadly suppress T-cell expansion and activity may also reduce the GvL effects, thereby increasing the frequency of malignancy relapse, graft rejection, and infection.<sup>1</sup> Despite prophylactic immunosuppression, approximately 50% of allo-HSCT recipients still develop GvHD.<sup>2</sup> Thus, an ideal allo-HSCT therapeutic strategy would potentiate the GvL effects and hematopoietic reconstitution (especially of B and T cells) while eliminating GvHD.

Our previous studies suggested two targetable GvHD signaling pathways: interferon gamma receptor (IFN $\gamma$ R) and downstream Janus kinases 1 and 2 (JAK1/JAK2). The genetic deletion of IFN $\gamma R^3$  or the pharmacologic inhibition of downstream JAK1/JAK2 using ruxolitinib<sup>3, 4</sup> mitigates GvHD while preserving T-cell number and function as well as GvL effects in major histocompatibility complex (MHC)-mismatched allo-HSCT mouse models. Since then, other groups have reported comparable results using ruxolitinib in mouse models and in selected patients outside of clinical trials. $^{5-7}$  In addition, we and two other groups have reported that the off-label use of ruxolitinib results in overall response rates of 83% (48 of 58 subjects) and 86% (48 of 56 subjects) for acute and chronic GvHD, respectively.<sup>5, 7, 8</sup> Thus, the pharmacologic inhibition of IFN $\gamma R$  and potentially of other JAK-STAT-mediated pathways mitigates GvHD while preserving the GvL effects, thereby indicating a promising therapeutic strategy for allo-HSCT patients. Although ruxolitinib has high selectivity for JAK1/JAK2, it also has a significant affinity for JAK3 and Tyk2.9 Because these four JAK family members control approximately 40 cytokine receptor signaling pathways,<sup>10</sup> ruxolitinib likely affects many cytokine signaling pathways to some degree, which results in off-target effects that may modulate its therapeutic efficacy. Although ruxolitinib has provided compelling preclinical and clinical evidence for pursuing JAK-STAT inhibition for the treatment of GVHD, we hypothesized that the further identification of the specific cytokine receptor signaling pathways necessary and sufficient for GvHD would permit the development of more efficacious prophylaxis for or treatment of GvHD after allo-HSCT.

We demonstrate here that the genetic deletion of *Ifngr* in combination with interleukin-6 receptor (IL6R)–blocking antibody completely prevents GvHD. Likewise, we show that baricitinib—a best-in-class JAK1/JAK2 inhibitor—inhibits IFN $\gamma$ R and IL6R signaling, prevents GvHD with 100% survival, and reverses ongoing GvHD in a fully MHC-mismatched allo-HSCT preclinical model. We further demonstrate that baricitinib is superior

to a structurally related JAK1/JAK2 inhibitor, ruxolitinib, in mouse preclinical GvHD models: it dramatically increases regulatory T cells (Tregs) in vivo while decreasing helper T cell 1 and 2 (Th1 and Th2) cell differentiation and reducing the expression of MHC II (I-Ad) and costimulatory molecules CD80/86 on allogeneic antigen-presenting cells (APCs). In addition, baricitinib preserves in vivo T-cell expansion and GvL effects. Our findings support the need for clinical trials that examine baricitinib and other JAK1/JAK2 inhibitors for GvHD prevention and treatment, with broad implications for inflammatory diseases such as solid organ transplant rejection and non-transplant autoimmune diseases.

# Materials and Methods

# Mice

All mice (7–12 week old males) were obtained from Jackson Laboratory (Bar Harbor, ME), except for the IFN $\gamma$ R–/– (*Ifngr1*–/–) mice,<sup>3, 11</sup> which were provided by Herbert Virgin of the Washington University School of Medicine (St. Louis, MO), and the GATA3 Tg mice, which were provided by Ikuo Tsunoda of the Louisiana Health Sciences Center (Shreveport, LA). Animal care and euthanasia protocols were approved by the Washington University School of Medicine (WUSM) Animal Studies Committee.

# Allo-HSCT

Allo-HSCT was performed as previously described.<sup>3, 4</sup> Full details can be found in Supplementary Information. All mice were randomly assigned to a treatment group in blinded fashion. Clinical GvHD was assessed as described by Cooke et al.<sup>12</sup>

### In vivo bioluminescence imaging

The assessment of in vivo T-cell expansion and GvL effects using the in vivo bioluminescence imaging of animals was done as previously described.<sup>3, 4, 13</sup> Full details can be found in Supplementary Information. A20 cell line [TIB-208] (ATCC, Manassas, VA) was mycoplasma tested by the Tissue Culture Support Center (WUSM).

# JAK1/JAK2 inhibitors and anti-IL6Ra antibody

Ruxolitinib and baricitinib were purchased from Selleck Chemicals (Houston, TX) and ApexBio Technology (Houston, TX), respectively. Anti-IL6Ra antibody (clone 15A7) and its isotype control (Rat IgG2b, clone LTF-2) were purchased from Bio X Cell (West Lebanon, NH).

### Cell culture

Mouse CD4+CD25- T cells were isolated from mouse spleens using Miltenyi mouse pan Tcell kits supplemented with biotinylated anti-CD8 (clone 53–6.7; BD Pharmingen, San Diego, CA), anti-CD25 (clone 7D4; BD Pharmingen), and an AutoMACS (Miltenyi Biotech, Auburn, CA). The isolated CD4+CD25– T cells were activated for 3 days in the presence of anti-CD3/CD28 antibody-coated beads (bead:cell = 1:1; Invitrogen, Carlsbad, CA) and Xcyte media with 10 U/ml of hIL-2.<sup>13</sup>

### Flow cytometric analysis

The antibodies used for flow cytometric analyses for mouse cells can be found in Supplementary Information. All cells were analyzed on a FACScan cytometer (BD Biosciences, Mountain View, CA) or a Gallios system (Beckman Coulter Life Sciences, Indianapolis, IN).

# Cytokine analysis

Mouse blood was collected from the allo-HSCT recipients at day 6 after allo-HSCT, and plasma was separated by centrifugation. The amounts of the cytokines were analyzed using the bead-based 36-plex Luminex Assay kits (R&D Systems, Minneapolis, MN) at the CHiiPs Immunomonitoring Laboratory at Washington University School of Medicine in St. Louis, MO.

### Statistical analysis

The determination of sample size and data analysis for this study followed the general guideline for animal studies.<sup>14</sup> The significance of the differences in the survival of the treatment groups was analyzed using the log-rank test. When extra weight to death for early time points is necessary, the Gehan-Breslow-Wilcoxon test was used (WT versus GATA3 Tg T cells). For all other analyses, the unpaired *t*-test was used. The normality of data was assessed graphically using residuals and the similarity of variance across groups was also assessed visually by checking the estimated variance of each group. All analyses were two-sided and *p* values of less than .05 were considered significant.

# Results

#### Co-blockade of IFNγR and IL6R signaling prevents GvHD

We were the first to demonstrate that ruxolitinib reduces GvHD while preserving GvL effects in mouse models of allo-HSCT.<sup>3, 4</sup> As we reported previously, small molecules that primarily inhibit JAK2 over JAK1, such as TG101348 and AZD1480, failed to reduce GvHD.<sup>4</sup> In addition, we have found that INCB039110 (a JAK1 inhibitor), LY2784544 and pacritinib (JAK2 inhibitors), and tofacitinib (a JAK3 inhibitor) significantly reduce GvHD in preclinical allo-HSCT models but that they are not as effective as ruxolitinib (Supplementary Figure 1). Thus, we reasoned that balanced JAK1/JAK2 inhibition would optimally control GvHD. The major cytokine receptor signaling pathways mediated by JAK1/JAK2 are IFN $\gamma$ R and IL6R. In our previous study, mice that received *Ifngr<sup>-/-</sup>* T cells and that were also treated with ruxolitinib demonstrated 100% survival in a fully MHC-mismatched allo-HSCT model,<sup>4</sup> which suggests that ruxolitinib's benefit relies on the JAK1/JAK2-mediated inhibition of IL6R signaling. Previous reports have shown that ruxolitinib potently inhibits IL6R signaling,<sup>9</sup> that the anti-IL6R-blocking antibody tocilizumab reduces acute GvHD in allo-HSCT patients,<sup>15</sup> and that donor IFNG and IL6 single nucleotide polymorphisms correlate with gastrointestinal GvHD severity.<sup>16</sup> Together, these observations suggest that IFNyR and IL6R signaling critically mediate GvHD. To test this hypothesis, we performed allo-HSCT using *Ifngr*<sup>-/-</sup> T cells and anti-mouse IL6Ra antibody as described in Supplementary Figure 2. Remarkably, as compared with controls, recipients of Ifngr<sup>-/-</sup> T

cells that had been combined with IL6Ra-blocking antibody demonstrated dramatically reduced GvHD clinical scores and a survival rate of more than 95% (Figure 1a).

# Co-blockade of IFN $\gamma$ R and IL6R signaling enhances hematopoietic reconstitution and increases Tregs

Allo-HSCT recipients of both Ifngr<sup>-/-</sup> T cells and IL6Ra-blocking antibody demonstrated significantly enhanced total peripheral blood cell count recovery-including white blood cells, lymphocytes, and platelets—as compared with recipients who received only wild-type (WT) T cells or IL6Ra-blocking antibody on day 27 after allo-HSCT (Figure 1b). In addition, the co-blockade of IFNyR and IL6R signaling also resulted in significantly higher lymphocyte counts as compared with Ifngr<sup>-/-</sup> alone (Figure 1b). We found that Ifngr<sup>-/-</sup> with or without anti-IL6Ra antibody resulted in full donor chimerism (i.e., <1% host-derived cells; Supplementary Figure 3), which suggests that the blocking of IFN $\gamma R$  enhances donor engraftment. In addition, the co-blockade of IFNyR and IL6R resulted in higher percentages of donor bone marrow-derived B and T cells in the peripheral blood (Supplementary Figure 3), which is consistent with less severe GvHD.<sup>3, 13</sup> We also observed dramatic increases in both percentages and absolute numbers of donor T-cell-derived Tregs (identified by FOXP3+CD45.2+ H-2Kd- in mature splenic pan T cells) in recipients of both Ifngr<sup>-/-</sup> T cells and IL6Ra antibody as compared with control groups (Figure 1c). Given the critical role of Tregs in the mitigation of GvHD,<sup>13, 17, 18</sup> these data suggest a potential mechanism by which the co-blockade of both IFNyR and IL6R signaling mitigates GvHD.

### Baricitinib phenocopies the co-blockade of IFN<sub>y</sub>R and IL6R signaling

Because baricitinib efficiently inhibits the phosphorylation of STATs, which are mediators of IFN $\gamma$ R and IL6R signaling (Supplementary Figure 4), we tested the effect of baricitinib on GvHD (see Supplementary Figure 2 for our schema). Baricitinib-treated recipients of WT T cells demonstrated 100% survival (Figure 2a) and reduced early stage intestinal GvHD (Supplementary Figure 5). Allo-HSCT mice treated with both ruxolitinib and anti-IL6Ra. antibody did not demonstrate any difference in overall survival as compared with ruxolitinib-treated mice (Figure 2a). These data suggest that ruxolitinib is an efficient inhibitor of IL6R signaling and that baricitinib's superior efficacy to ruxolitinib for the prevention of GvHD relies on mechanisms other than the inhibition of IL6R signaling. As compared with control mice, baricitinib-treated mice demonstrated significantly improved blood cell count recovery (including white blood cells, lymphocytes, and platelets [Figure 2b]), full donor chimerism, and significantly higher percentages of donor bone-marrow–derived B cells and T cells (Figure 2c).

#### Baricitinib increases Tregs and prevents GvHD while preserving donor T-cell expansion

Baricitinib group significantly increased donor T-cell–derived Tregs compared to vehicle- or ruxolitinib-treated mice in the spleen at days 6 and 17 after allo-HSCT (Figure 3a), with a trend toward an increase in donor bone-marrow–derived Tregs (Supplementary Figure 6). Because IL2R-mediated JAK1/JAK3-STAT5 signaling is important for Treg proliferation and survival, we next evaluated the effect of JAK1/JAK2 inhibition on STAT5 phosphorylation. We found that the baricitinib treatment group showed significantly increased phosphorylated STAT5 (pSTAT5) compared to vehicle control or ruxolitinib group

in the spleen at day 6 after allo-HSCT (Supplementary Figure 7a). In contrast, baricitinib was a more potent pSTAT1 inhibitor than ruxolitinib while no difference was observed in pSTAT3 between ruxolitinib and baricitinib at this time point. The low level of pSTAT3 at day 6 after allo-HSCT in vehicle control group (thereby no difference between the control and JAK inhibitor groups) is likely due to shedding of IL6R in donor T cells following T cell activation.<sup>19</sup> Consistent with these data, a two-fold greater amount of pSTAT5 was observed in human primary T cells treated with baricitinib as compared with cells treated with ruxolitinib in response to IFNa (Supplementary Figure 7b; this was also seen in Jurkat cells, as shown in Supplementary Figure 4c). Considering that ruxolitinib's IC50s for JAK1 (3.3 nM) and JAK3 (428 nM) are lower than baricitinib's (5.9 nM and 560 nM, respectively), <sup>9, 20, 21</sup> we speculated that the preservation of IL2R-JAK1/JAK3-STAT5 signaling may contribute to improved Treg proliferation and survival in vivo. Thus, we compared the effects of baricitinib versus ruxolitinib on in vivo Treg expansion using two independent methods. First, we labeled pan T cells isolated from Foxp3GFP KI mice (C57BL/6) with Violet Proliferation Dye 450 (VPD450; BD Biosciences) before allo-HSCT. We found that Tregs (CD4+FOXP3GFP+) in the baricitinib-treated group expanded significantly more than those in the vehicle- or ruxolitinib-treated groups, whereas there was only a trend toward an inhibitory effect of baricitinib on effector T-cell (CD4+FOXP3GFP-) proliferation in the spleen at day 5 after allo-HSCT (Figure 3b). Interestingly, baricitinib does not induce T-cell anergy. Donor T-cell-derived CD4 T cells harvested from the allo-HSCT recipients (harvested on day +29) treated with baricitinib proliferated at the same rate as naive C57BL/6 CD4 T cells in response to BALB/c whole splenocytes as measured by in vitro mixed lymphocyte reactions (Supplementary Figure 8). These data suggest that baricitinib treatment in vivo did not result in the clonal deletion of alloreactive T cells. Next, we performed bioluminescence imaging after allo-HSCT in which luciferase transgenic (luc+) C57BL/6 mice served as Treg donors. Baricitinib enhanced in vivo Treg expansion throughout the duration of the experiment (as assessed on days 10, 18, and 31)—especially on day 10 after allo-HSCT—as compared with ruxolitinib and vehicle control (Figure 3c). Ruxolitinib-treated allo-HSCT mice showed increased Treg expansion only at later time points (days 18 and 31) as compared with vehicle-treated controls (Figure 3c). These data are consistent with the increased Treg levels seen in the IFN $\gamma R$  and IL6R co-blockade group (see Figure 1c). To determine whether baricitinib converts effector T cells into Tregs, we performed allo-HSCT after the depletion of natural Tregs from donor grafts. We found that baricitinib neither increased Tregs in allo-HSCT recipients of Treg-depleted T cells nor altered Th17 differentiation (Figure 3d). In fact, Tregs in the donor graft were essential for optimal GvHD prevention, because Treg-depleted T-cell recipients demonstrated significantly reduced overall survival rates and increased GvHD clinical scores as compared with Treg-replete recipients (Figure 3e). Even in the absence of donor Tregs, allo-HSCT recipients treated with baricitinib still had a survival rate of approximately 70%, which suggests that mechanisms independent of the enhanced in vivo expansion of Tregs contribute to the drug's GvHD protective effect.

Despite our observations of enhanced Treg expansion, we considered the idea that IFN $\gamma R$  and IL6R signaling blockade may prevent GvHD simply by inhibiting allogeneic T-cell expansion. Therefore, we performed in vivo bioluminescence imaging using luc+ T cells as

donors in allo-HSCT. Although IFN $\gamma$ R knockout T cells expanded more than T cells in other groups (which is consistent with our previous report<sup>3</sup>), the co-blockade of IFN $\gamma$ R and IL6R signaling with baricitinib or *Ifngr*<sup>-/-</sup> in combination with anti-IL6Ra antibody preserved donor T-cell expansion in vivo (Figure 3f).

# Baricitinib reduces Th2 cytokine IL5 and alters donor T-cell differentiation

In humans, serum levels of IL5 and ratios of IL5:IL2, IL5:IL4, and IL6:IL4 correlate with GvHD severity.<sup>22</sup> With the use of a cytokine multiplex assay, we found that baricitinibtreated allo-HSCT mice demonstrated lower levels of IL5 and lower ratios of IL5:IL2 than the ruxolitinib group and lower ratios of IL5:IL2, IL5:IL4, and IL6:IL4 than the vehicle control group (Figure 4a). In addition, ruxolitinib and baricitinib both suppressed the expression of T-bet, GATA3, and CXCR3 (Figure 4b). It is of note that IFNyR signaling regulates CXCR3-mediated T-cell trafficking to GvHD target organs.<sup>3</sup> Furthermore, previous reports have shown that IFNyR-STAT1 signaling regulates T-bet expression and that the genetic deletion of Tbx21 (encoding T-bet) or Stat1 mitigates GvHD.<sup>23-25</sup> Given that baricitinib reduced plasma levels of the Th2 cytokine IL5 in addition to reducing GATA3 expression in vitro, we examined whether baricitinib's effect on the reduction of GvHD was in part dependent on reduced Th2 activity in vivo using GATA3-overexpressing transgenic T cells. Baricitinib treatment reduced GATA3 expression in WT T cells but failed to reduce its expression in GATA3-transgenic T cells (Supplementary Figure 9). Furthermore, transplantation with GATA3-transgenic T cells resulted in a lower median survival rate as compared with the use of WT T cells (Figure 4c). However, baricitinibtreated allo-HSCT recipients of either GATA3-transgenic or WT T cells demonstrated equivalent survival rates and GvHD scores (see Figure 4c), thereby suggesting that baricitinib's mitigation of GvHD does not depend on the decreased expression of GATA3.

#### Baricitinib reduces the expression of MHC II, CD80/86, and PD-L1 on allogeneic APCs

To examine potential host-dependent baricitinib effects, we assayed MHC II (I-Ad), costimulatory molecules CD80/86, and PD-L1 expression on recipient CD11c+ and B220+ APCs. As compared with ruxolitinib and vehicle control, baricitinib reduced I-Ad and CD80/86 expression on CD11c+ or B220+ cells (Figure 5a and Figure 5b), which suggests that baricitinib may modulate host antigen presentation and activity of APCs. Likewise, baricitinib potently suppressed PD-L1 expression on CD11c+ and B220+ cells (Figure 5c). IFN $\gamma$ R signaling promotes PD-L1 expression,<sup>26</sup> and Tregs are more susceptible than effector T cells to PD-L1<sup>27</sup>, which suggests a plausible mechanism by which baricitinib can reduce PD-L1 expression on recipient APCs and thus enhance Treg expansion and function.

### Baricitinib effectively treats ongoing GvHD

To test baricitinib as a GvHD treatment (rather than as prophylaxis), we delayed baricitinib treatment until mice developed clinically apparent GvHD (day 10). Despite this latency, baricitinib given at both 200  $\mu$ g/day and 400  $\mu$ g/day produced 100% overall survival and reduced clinical GvHD scores (Figure 6a). The higher dose of baricitinib (as compared with control) resulted in substantially improved blood cell count recovery, including white blood cells, lymphocytes, and platelets (Figure 6b); full donor chimerism (Figure 6c); and higher

percentages of donor bone-marrow-derived B cells but not T cells (Figure 6c) or Tregs (Supplementary Figure 10) at day 27.

### Baricitinib preserves and enhances the GvL effects

Finally, we evaluated whether baricitinib could preserve GvL effects. Many agents or approaches used to mitigate GvHD reduce or at best maintain GvL seen after the infusion of alloreactive T cells. Because BALB/c-derived B-cell lymphoma A20 cells can be exquisitely sensitive to cytotoxic allogeneic donor T cells, we infused A20 cells and T-cell-depleted bone marrow cells into lethally irradiated BALB/c recipients, waited 11 days for leukemia to become established, and then performed delayed donor T lymphocyte infusion (Figure 7a). It has been demonstrated in this model of GvL that A20 cells, when stably established demonstrated resistance to the cytotoxicity of alloreactive T cells.<sup>13</sup> Although baricitinib treatment alone (in the absence of donor T cells) did not inhibit tumor growth, baricitinib enhanced GvL effects of donor T cells as compared with control animals receiving A20 and delayed donor T cells, thereby resulting in significantly lower leukemic tumor burden (Figure 7b). We hypothesized that enhanced GvL effects in baricitinib-treated mice may depend on the reduced expression of PD-L1 on A20 cells. Indeed, we found that baricitinib reduces PD-L1 expression in A20 cells (Figure 7c) and inhibited IFNy-induced PD-L1 expression in the human cell lines MOLM-13 and RAMOS (Supplementary Figure 11). Although provocative the significance of PD-L1 downregulation by baricitinib on tumor clearance requires further investigations.

# Baricitinib treatment does not increase the expression of PD-L1 and CD80 on T cells or PD-L1 on intestinal epithelial cells

The Zeng group recently demonstrated that CD4 T cell depletion after allo-HSCT resulted in an increase of serum levels of IFN $\gamma$  but decrease of IL2.<sup>28</sup> The altered serum levels of these two cytokines subsequently increased PD-L1 and CD80 expression on donor T cells in lymphoid tissues and PD-1 on donor T cells and PD-L1 on parenchymal cells in GvHD target tissues.<sup>28</sup> While *trans*-interaction between PD-L1 and CD80 on donor CD8 T cells in lymphoid tissues augmented donor CD8 T cell expansion and GvL, interaction between PD-L1 on parenchymal cells of GvHD target organs and PD-1 on donor CD8 T cells induced CD8 T cell tolerance in GvHD target organs.<sup>28</sup> Thus, we examined the expression of PD-L1, and CD80 on donor T cells in the spleen and PD-L1 on epithelial cells and PD-1 on donor T cells in intestine. As shown in Supplementary Figure 12a, we found that baricitinib group showed decreased PD-L1 expression in CD8 T cells (no significant difference in CD4 T cells) but no statistically significant difference in CD80 expression in CD4 or CD8 T cells in the spleen compared to vehicle control or ruxolitinib group. Likewise, PD-L1 expression in intestinal epithelial cells (CD326+CD45-H-2Kd+) in baricitinib group was significantly lower than that in vehicle control and ruxolitinib group (Supplementary Figure 12b; we were unable to obtain statistically reliable analyses of PD-1 expression on donor T cells in intestine due to insufficient infiltrating donor T cells in baricitinib and ruxolitinib groups see below). The reduction of PD-L1 in both T cells and intestinal epithelial cells is consistent with the data in Figure 5c. All of these data suggest that the interactions between PD-L1, PD-1, and CD80 might not be a major mechanism underlying the baricitinibinduced complete prevention of GvHD while preserving/enhancing GvL. In contrast, we

found that baricitinib group had significantly reduced intestine-infiltrating T cells (both CD4 and CD8 T cells) (Supplementary Figure 12b), suggesting that baricitinib inhibits T cell trafficking to GvHD organs through the reduction of CXCR3 (Figure 4b).<sup>3</sup>

# Discussion

Our study demonstrates that IFN $\gamma$ R/IL6R or downstream JAK1/JAK2 are optimal targets to prevent GvHD and to reverse ongoing GvHD while preserving or enhancing both GvL effects and multi-lineage hematopoietic reconstitution after MHC-mismatched allo-HSCT. Possible mechanisms for the complete prevention and reversal of GvHD include increased Tregs, reduced CXCR3 expression on donor T cells, altered helper T cell differentiation, and decreased allogeneic antigen presentation and costimulatory molecule expression on recipient APCs.

Baricitinib is superior to ruxolitinib for the expansion of Tregs at early time points after allo-HSCT. Mechanistically, baricitinib may exert less inhibition on IL2 signaling and STAT5 phosphorylation as compared with ruxolitinib. Franceschini and colleagues demonstrated that Tregs are more susceptible than effector T cells to PD-L1 and that the blocking of PD-L1 increases pSTAT5 in Tregs.<sup>27</sup> Consistent with that report is the idea that reduced PD-L1 expression on allogeneic APCs and increased pSTAT5 in T cells may correlate with increased Tregs in the baricitinib group. The superiority of baricitinib over ruxolitinib for multi-lineage hematopoietic and especially platelet recovery (see Figure 2b) may be the result of two factors. First, baricitinib has a higher IC50 (5.7 nM) as compared with ruxolitinib (2.8 nM) for JAK2 (the mediator of thrombopoietin/MPL signaling), which functions both as an early acting hematopoietic growth factor and as a positive regulator of platelet production and mass.<sup>29, 30</sup> Second, baricitinib completely prevents GvHD, which itself directly correlates with delayed blood count recovery after allo-HSCT in mice and humans. Thus, our preclinical data support baricitinib as a GvHD prophylactic therapy without significant concern for interference with hematopoietic recovery.

This is the first study to demonstrate the potent effect of the blocking of both IFN $\gamma$ R and IL6R on the prevention and treatment of ongoing GvHD in mouse allo-HSCT models. However, this study has some important limitations. Although baricitinib blocks IFN $\gamma$ R and IL6R signaling, it may still exert off-target effects on molecules other than JAK1 and JAK2, which we have not identified here. In addition, we have not distinguished the roles of the host-tissue–specific expression of PD-L1 and indoleamine 2,3-dioxygenase, both of which potently suppress GvHD.<sup>31, 32</sup> IFN $\gamma$ R signaling positively regulates the expression and function of these two molecules, whereas IL6R signaling negatively regulates them.<sup>33–35</sup> Thus, certain GvHD target organs (e.g., the lungs) may demonstrate increased sensitivity to alloreactive T cells when IFN $\gamma$ R signaling is inhibited.<sup>36</sup> However, the additive effect of IL6R blockade, as seen in our genetic and pharmacologic studies, may protect against the loss of IFN $\gamma$ R signaling in these organs. Therefore, our ongoing studies aim to quantify PD-L1 and indoleamine 2,3-dioxygenase expression in GvHD target organs to determine whether the differential regulation of these molecules can, in part, explain baricitinib's superiority to ruxolitinib for the prevention of GvHD.

The proof-of-concept that JAK1/JAK2 blockade is effective in humans with steroid refractory acute GvHD has recently been reported,<sup>7</sup> and the first prospective clinical trial of a JAK1 selective inhibitor, itacitinib (INCB039110), has been completed.<sup>37</sup> Both studies suggest a class effect of JAK/STAT inhibition for the treatment of acute GvHD. In the light of their prior preclinical studies, their emergent clinical experience, and their recent observations, Alam and colleagues have suggested that specific donor IFN $\gamma$  and IL6 SNP/ genotypes correlate strongly with the risk of steroid refractory GvHD.<sup>16</sup> Our findings corroborate the observation that IFN $\gamma$  and IL6 are critical to the development and progression of acute GvHD. In addition, the need to selectively block both IFNyR and IL6R to prevent and treat GvHD further supports the rationale for combining an anti-human IFN $\gamma R$  antibody with tocilizumab. Therapeutic strategies involving these antibodies are expected to eliminate unwanted and unexpected off-target or side effects of JAK1/JAK2 inhibitors on molecules other than JAK1 and JAK2. Similarly, our data provide compelling preclinical evidence to support future clinical trials testing baricitinib for the treatment and prevention of GvHD. Baricitinib is currently being developed by Eli Lilly for the treatment of rheumatoid arthritis. The use of JAK1/JAK2 inhibitors such as ruxolitinib and baricitinib for GvHD prophylaxis may limit the need for expensive and labor-intensive ex vivo cellular manipulations<sup>38</sup> and may also reduce the use of broadly immunosuppressive agents that may themselves contribute to disease relapse, morbidity, and mortality after allo-HSCT. In fact, our studies suggest enhanced-not reduced-multi-lineage engraftment (including B and T lymphocytes and platelets) when mouse allo-HSCT recipients are treated with baricitinib as compared with vehicle control or ruxolitinib. In conclusion, this study provides mechanistic insight into GvHD that may have broad implications for other inflammatory disorders, including solid organ transplant rejection and other non-transplant autoimmune diseases.

# **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

# Acknowledgments

JC is supported by the Amy Strelzer Manasevit Research Program, which is funded through the Be The Match Foundation and the National Marrow Donor Program; the Rays of Hope St. Baldrick's Research Grant (St. Baldrick's Foundation); Washington University SPORE-CDP (P50 CA171963-01); Washington University DDRCC grant (P30 DK052574); Alvin J. Siteman Cancer Center Siteman Investment Program (supported by the Foundation for Barnes-Jewish Hospital Cancer Frontier Fund, National Cancer Institute Cancer Center Support Grant, P30 CA091842, and Barnard Trust); and the Bryan Thomas Campbell Foundation. JFD is supported by the National Cancer Institute (P50 CA94056-09, R35 CA210084-01, and P50 CA171963-01) and the Bryan Thomas Campbell Foundation. LM and SA are supported by the National Institutes of Health (P50 CA94056). IT is supported by the National Institute of General Medical Sciences COBRE Grant (P30-GM110703) and the Japan Society for the Promotion of Science (Grants-in-Aid for Scientific Research-KAKENHI, 16H07356). We thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, MO for the use of the Immunomonitoring Laboratory, which provided the Cytokine analysis service. The Siteman Cancer Center is supported in part by an NCI Cancer Center Support Grant #P30 CA091842. We thank Jennifer Gann of Gann Editorial Group (St. Louis, MO) for editorial assistance.

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Figure 1. Allo-HSCT with IFN $\gamma R$  knockout T cells and anti-IL6Ra antibody treatment results for the prevention of GvHD with enhanced hematopoietic reconstitution and an increase of donor T-cell-derived Tregs

(a) Shown are clinical GvHD scores represented as mean ± standard deviation and survival after allo-HSCT. (b) White blood cells, lymphocytes, and platelets in peripheral blood at day 27 after allo-HSCT as compared with mice in other groups. Dotted lines indicate the lowest limit of normal ranges. (c) In this C57BL/6 to BALB/c model, donor bone-marrow–derived cells (H-2<sup>b</sup>, CD45.1<sup>+</sup>), donor splenic T cells (H-2<sup>b</sup>, CD45.2<sup>+</sup>), and host-derived cells (H-2<sup>d</sup>, CD45.2<sup>+</sup>) were distinguished by flow cytometry using CD45.1, CD45.2, H-2K<sup>b</sup>, and H-2K<sup>d</sup> fluorophore-conjugated antibodies. The percentages of donor splenic T cell-derived FOXP3+ Tregs among CD4 T cells as well as the absolute counts of Tregs in the peripheral blood were measured using flow cytometry at day 27 after allo-HSCT. All error bars are represented as mean ± standard deviation.



Figure 2. In vivo administration of baricitinib results for the complete prevention of GvHD with enhanced hematopoietic reconstitution

(a) Survival after allo-HSCT. A pool of three independent experiments. (b) White blood cells, lymphocytes, and platelets counts. The percentages of (c) recipient-derived cells and donor bone-marrow–derived B220+ B cells and CD3+ T cells in the peripheral blood were measured using flow cytometry at day 27 after allo-HSCT. All error bars are represented as mean  $\pm$  standard deviation.



Figure 3. Baricitinib is superior to ruxolitinib for the expansion of natural Tregs in vivo and preserves in vivo donor T-cell expansion

(a) Baricitinib treatment increased donor T-cell–derived Tregs in the spleen at days 6 and 17 after allo-HSCT. The data shown here represent multiple independent experiments with the same results without the later Treg increases (day 27 in the peripheral blood or spleen) unlike the results seen with dual genetic IFNyR inhibition and IL6R antibody blockade. (bc) The effect of baricitinib versus ruxolitinib on in vivo Treg proliferation and expansion using (b) Violet Proliferation Dye 450 and (c) bioluminescence imaging weekly after allo-HSCT. Photon flux was measured with a region of interest drawn over the entire body of each mouse. (d) Intracellular staining of FOXP3 (Tregs) and IL17 (Th17) to determine the effect of baricitinib on donor T-cell-derived Tregs and Th17 cells in the spleen at day 6 after allo-HSCT. (e) Survival rates and clinical GvHD scores after allo-HSCT in which pan T cells or Treg-depleted (TrD) pan T cells were used. (f) The effect of baricitinib and the dual inhibition of IFN $\gamma$ R and IL6R signaling on in vivo T-cell expansion using bioluminescence imaging weekly after allo-HSCT in which luciferase transgenic (luc+) C57BL/6 mice were used as T-cell donors. Photon flux was measured with a region of interest drawn over the entire body of each mouse. Shown are actual images of one representative mouse from each group at days 1 and 15 after allo-HSCT. D, Dorsal image; V, ventral image. All error bars are represented as mean  $\pm$  standard deviation.





(a) Mouse plasma levels of IL2, IL4, IL5, and IL6 were measured using multiplex cytokine assays. (b) Both ruxolitinib and baricitinib were found to equally inhibit the expressions of T-bet, GATA3, and CXCR3 in activated CD4+CD25– T cells. (c) Survival rates and clinical GvHD scores after allo-HSCT in which GATA3 Tg mice were used as T-cell donors. The results of a pool of two independent experiments are shown. All error bars are represented as mean  $\pm$  standard deviation.



# Figure 5. The effect of baricitinib on MHC II, CD80/CD86, and PD-L1 Baricitinib suppresses the upregulation of (a) I-Ad, (b) CD80/CD86, and (c) PD-L1 on recipient CD11c+ and B220+ APCs in the spleen on day 5 after allo-HSCT. *MFI*, Geometric mean fluorescence intensity relative to the vehicle control was used. All error bars are represented as mean $\pm$ standard deviation.



### Figure 6. Baricitinib reverses established GvHD

Baricitinib treatment was started on day 10 after allo-HSCT. (**a**) Survival rates and clinical GvHD scores after allo-HSCT. A "+" indicates that only one mouse was surviving and scored in the vehicle control group. Data are representative of two independent experiments. (**b**) White blood cells, lymphocytes, and platelets as well as the percentages of (**c**) recipient-derived cells and donor bone-marrow–derived B220+ B cells and CD3+ T cells in peripheral blood were measured using flow cytometry at day 27 after allo-HSCT. Baricitinib (200 µg or 400 µg) was administered once a day, 5 days a week, for 3 weeks for a total of 15 injections.



Figure 7. Baricitinib preserves GvL effects in vivo and inhibits the expression of PD-L1 in tumors in vitro

(a) The schema of the experiments. Luc+ A20 cells were injected at day 0 along with T-celldepleted bone marrow cells. Donor T cells were transplanted 11 days after bone marrow transplantation. Baricitinib (200 or 400 µg) was administered subcutaneously from days 12 through 32 once a day, 5 days a week, for 3 weeks. Weekly bioluminescence imaging was performed to measure tumor burden starting 1 day before donor T lymphocyte infusion (day 10 after allo-HSCT). (b) Photon flux was measured with a region of interest drawn over the entire body of each mouse. Shown are actual images of one representative mouse from each group at 1 day before donor T lymphocyte infusion (day 10 after allo-HSCT) and at 20 days after donor T lymphocyte infusion (day 31 after allo-HSCT). D, Dorsal image; V, ventral image. (c) PD-L1 expression in A20 cells in vitro. The result of a pool of two independent experiments are shown. Splenic pan T cells were isolated from B6 mice and activated with anti-CD3/CD28 antibody-coated beads (cell:bead = 1:1) in the presence of baricitinib (0-1  $\mu$ M) for 2 days. Actively growing A20 cells were added to the T cell culture (A20:T cell = 1:1) for 1 day before PD-L1 in A20 cells was measured with the use of flow cytometry. MFI, Geometric mean fluorescence intensity relative to the vehicle control was used. All error bars are represented as mean  $\pm$  standard deviation.