Pharmacologic Inhibition of JAK1/JAK2 Signaling Reduces Experimental Murine Acute GVHD While Preserving GVT Effects

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Abstract

Purpose: Immune-mediated graft-versus-tumor (GVT) effects can occur after allogeneic hematopoietic stem cell transplantation (HSCT), but GVT is tightly linked to its main complication, graft-versus-host disease (GVHD). Strategies aimed at modulating GVHD, while maintaining the GVT effect, are needed to improve the cure rate of transplant. Given the emerging role of Janus-activated kinase (JAK) signaling in lymphoproliferative and myeloproliferative diseases and its established function at dictating T-cell differentiation, we postulated that JAKs might be potential therapeutic targets through a pharmacologic approach.

Experimental Design: We examined the effect of JAK1/JAK2 modulation by ruxolitinib in a mouse model of fully MHC mismatched bone marrow transplant comprising in vivo tumor inoculation.

Results: JAK1/JAK2 inhibition by ruxolitinib improved both overall survival (P = 0.03) and acute GVHD pathologic score at target organs (P ≤ 0.001) of treated mice. In addition, treatment with ruxolitinib was associated with a preserved GVT effect, as evidenced by reduction of tumor burden (P = 0.001) and increase of survival time (P = 0.01). JAK1/JAK2 inhibition did not impair the in vivo acquisition of donor T-cell alloreactivity; this observation may account, at least in part, to the preserved GVT effect. Rather, JAK1/JAK2 inhibition of GVHD was associated with the modulation of chemokine receptor expression, which may have been one factor in the reduced infiltration of donor T cells in GVHD target organs.

Conclusions: These data provide further evidence that JAK inhibition represents a new and potentially clinically relevant approach to GVHD prevention.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) represents a potential curative strategy for patients with several hematologic malignancies (1). Donor immune system mediates the beneficial graft-versus-tumor (GVT) effect, but can also result in acute graft-versus-host disease (GVHD) that, despite recent improvements in the transplantation procedure and supportive care (2), remains a major contributor to transplantation-related deaths and the most significant barrier to the success of allogeneic-HSCT (3). In general, attempts to prevent GVHD are often associated with a reduction in GVH effects. Therefore, approaches aimed at separating GVH effect from GVHD are warranted, but are difficult to achieve because of their shared biology (4). Different strategies have been proposed in preclinical mouse models of acute GVHD, including those targeting NK activation, DC maturation, or T cell response, such as T-cell activation, signaling pathway, and homing to GVHD target organs (5). Of note, preserving the GVT effect was not always a prerequisite of these studies. More recently, blockade of visceral lymphocyte chemotaxis resulted in reduced incidence of clinical acute GVHD (6).

The Janus-activated kinases (JAK) family forms one subgroup of nonreceptor protein kinases that are involved in cell growth, survival, and differentiation of various cell populations, mainly through the activation of "signal transducers and activators of transcription" proteins (STATs). JAK2 signaling was shown to be of relevance for the pathogenesis myeloid and lymphoid malignancies (7, 8) and similarly an aberrant activation of the JAK–STAT pathway was linked to the oncogenic process and outcome of Hodgkin and non-Hodgkin lymphomas (9–11). Moreover, a significant improvement for the treatment of patients with myelofibrosis was recently achieved by the introduction of the JAK1/JAK2-specific inhibitor, ruxolitinib (12). Another fundamental role of JAK–STAT signaling is to dictate immune cells activation and differentiation (13). We have previously shown that STAT signaling is not dispensable for development of T-cell alloreactivity (14) in graft rejection experiments and that STAT3 activation is of fundamental importance for the onset of acute GVHD (15). In addition, other reports have extensively studied the role of different proteins of the STATs family for the onset of GVHD (16, 17). More recently, in vitro JAK2 inhibition resulted in reduced activation of dendritic cells (DC) and induced durable tolerance to alloantigen of T cells primed by ruxolitinib-modified DCs (18).
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Translational Relevance

Hematopoietic stem cell transplantation is a commonly used immunotherapy method, but its curative potential, the so-called graft-versus-tumor (GVT) effect, is hampered by graft-versus-host disease (GVHD) and consequent treatment-related mortality. In this study, we demonstrated that ruxolitinib, a JAK1/JAK2 inhibitor, abrogates acute GVHD while sparing the GVT effect. This effect is associated with preserved alloreactivity in vivo and reduced T-cell infiltration of GVHD target organs. Ruxolitinib is approved by the FDA and The European Medicines Agency (EMEA) for the treatment of patients with myelofibrosis, and Janus-activated kinase (JAK) signaling and JAK2 inhibitors play an important role in myeloid and lymphoid malignancies. Thus, these data add to the body of evidence that JAK2 inhibition may represent a new and potentially clinically relevant approach to GVHD prevention. Furthermore, the antitumor effect of this compound may allow inhibiting GVHD while controlling tumor burden, which is an important step toward more effective treatment of patients with hematologic malignancies.

Another report suggested a role for JAK1/JAK2 modulation to reduce GVHD and improve survival in a mouse model of allogeneic transplant (19).

Given the importance of JAK signaling and its inhibitors in the context of myeloid and lymphoid malignancies (12, 20, 21) and because JAK1/JAK2 inhibition might represent a more specific and narrow form of immune modulation relative to standard approaches of immune suppression, we reasoned that ruxolitinib may be a promising drug to be tested in order to preserve GVT and obtain an anti-GVHD effect with a potential fast track for translation into clinical trials. We tested our hypothesis in a mouse model of acute GVHD comprising inoculation of two different tumor cell lines.

Materials and Methods

Animals

Female C57BL/6 (B6, H-2Kb) and BALB/c (H-2Kd) mice were obtained from Charles River Laboratories. Mice were maintained in a specific pathogen-free facility, and treated according to an animal protocol that was approved from CESA (Comitato Etico per la Sperimentazione Animale) of the University of Milan (Milan, Italy) and from the Ethical Committee (study number: INT_21/09) of the Fondazione IRCCS Istituto Nazionale dei Tumori (Milan, Italy).

Cell preparations

B6 bone marrow and spleen cells were purified by magnetic bead-negative depletion in an autoMACS Pro Separator (Miltenyi Biotec) to obtain a purity of 95% to 98%. In detail, donor bone marrow was T cell depleted (TCD) by Miltenyi CD90 [Thy1.2] microbeads, and splenic donor T cells were purified with Miltenyi CD45R [B220] microbeads. Lymphoma A20 cell line was kindly provided from Dr M.P. Colombo’s laboratory (Molecular Immunology Unit at the Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) >6 months after being purchased from a cell bank. Myeloid leukemia RMB-1 cell line was purchased from DMSZ and was confirmed as murine with isoelectric focusing (IEF) of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), malic dehydrogenase (MDH), nucleoside phosphorylase (NP). No authentication was done in our laboratory. Both A20 and RMB-1 cell lines are of H2Kd-positive phenotype. Tumor cells were cultured in complete medium (CM) consisting of RPMI-1640 (Lonza), 10% FCS (Sigma), and Pen–Strep 100 U/mL (Sigma). Tumor-cell lines were cocultured with/without ruxolitinib at the concentration of 10 μmol/L for 24 hours; cell viability was checked by flow cytometry analysis with PI and Annexin V (Miltenyi Biotec).

Bone marrow transplantation and tumor cell inoculation

Recipient BALB/c mice were lethally irradiated (950 Gy total dose by two separate doses 3 hours apart) and reconstituted by intravenous (i.v.) injection of TCD B6 bone marrow cells (10 × 10^6 cells) alone or with the addition of purified B6 T cells (2.5 × 10^6 cells). As per our animal protocol, any transplant recipient that was premorbid (defined by >30% loss in body weight or severe loss in activity) was euthanized. In our facility, this mouse model of GVHD resulted in limited lethality, with only a fraction of the mice dying at later time points (from days 30 to 60). A20 or RMB-1 cells were injected i.v. on day 0 of transplant at the dosage of 2 × 10^6 cells and 0.5 × 10^6 cells, respectively.

Ruxolitinib treatment

Mice treated with ruxolitinib (INCB018424; Selleck Chemicals) received the pharmacologic inhibitor of the JAK1–JAK2 pathway twice-daily by oral gavage from the day of transplant to day 14 after bone marrow transplantation (BMT). Ruxolitinib was solubilized in DMSO and resuspended in water containing 0.5% methylcellulose and 5% N,N-dimethylacetamide to a final volume of 200 μL, as previously described (22). Initial drug dosage, 90 mg/kg/d, was mutated from previous publication where it was used in a mouse model of myeloproliferative disease (22). Other dosages comprised 45 and 22.5 mg/kg/d, as described in the Results.

Histology

For histopathologic grading of GVHD lesions, mice from each cohort were killed at day 14 post-BMT and skin, liver, small intestine, and large intestines were harvested. Tissues were fixed in 10% neutral buffered formalin and paraffin embedded. Tissue sections were routinely stained with hematoxylin and eosin (H&E) and evaluated in a blinded fashion under a light microscope. Skin, liver, small and large intestine were scored semiquantitatively, as previously described (Supplementary Fig. S1; ref. 23). Scores of the individual organs (range, 0–4) were then added to provide a total GVHD score for each mouse (range, 0–16).

Immunohistochemistry

To establish the extent of T-cell and macrophage infiltration in GVHD target organs, formalin-fixed and paraffin-embedded sections from skin, liver, and ileum were immunostained with CD3-epsilon (M20; Santa Cruz Biotechnology) and IBA-1 (Wako) antibodies, and IHC was performed as murine with isoelectric focusing (IEF) of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), malic dehydrogenase (MDH), nucleoside phosphorylase (NP). No authentication was done in our laboratory. Both A20 and RMB-1 cell lines are of H2Kd-positive phenotype. Tumor cells were cultured in complete medium (CM) consisting of RPMI-1640 (Lonza), 10% FCS (Sigma), and Pen–Strep 100 U/mL (Sigma). Tumor-cell lines were cocultured with/without ruxolitinib at the concentration of 10 μmol/L for 24 hours; cell viability was checked by flow cytometry analysis with PI and Annexin V (Miltenyi Biotec).
Flow cytometry analysis

Spleen and bone marrow were harvested on day 14 post-BMT, and single-cell suspensions were labeled with anti-CD62L, -H-2Kb (BioLegend), -CD3, -CD8, -CD4, and -CD44 (Miltenyi Biotec) conjugated with FITC, PE, VioBlue, PerCP, APC-Vio770, and PE-Vio770, respectively. For regulatory T cells (Treg) quantification, spleen cells were fixed and permeabilized with the Foxp3 staining buffer set (Miltenyi Biotec) and labeled in the spleen and bone marrow by FSC/SSC characteristics with CD3 FITC, Gr-1 PerCP, Ter119 APC, and B220 VioBlue, and APC-Vio770. A2O tumor cells were identified both in the spleen and bone marrow by FSC/SSC characteristics together with the following antibodies: anti-H-2Kd-FITC, B220-VioBlue, and CD3-APC (Miltenyi Biotec). RMB1 tumor cells were followed both in the spleen and bone marrow by labeling them with anti-H-2Kd-FITC (BioLegend), CD11b-VioBlue, Gr1-and PerCP Vio770 (Miltenyi Biotec). Six- to seven-color flow cytometry was performed on a MACSQuant Analyzer (Miltenyi Biotec) using MACSQuantify 2.4 software (Miltenyi Biotec).

For mixed lymphocyte reaction experiments, posttransplant splenic single cells were adjusted to 0.5 × 10^5 cells/mL and either not stimulated or stimulated with B6 DC, or BALB/c DCs generated by culturing marrow cells for 6 days in rmGM-CSF and rmIL4 (each at 1,000 IU/mL; PeproTech); bacterial lipopolysaccharide (LPS; 1 μg/mL; Sigma) for 3 hours and incubated with brefeldin (Sigma) for another 4 hours. Cells were permeabilized with the Inside Stain Kit (Miltenyi Biotec) and stained with anti-H-2Kd-FITC (BioLegend), CD4-VioBlue, CD8-PerCP, and IFNγ-PE (Miltenyi Biotec). Allospecific values were expressed in percentage of cells positive for IFNγ secretion and calculated after adjusting for values obtained after stimulation with syngeneic B6 DC.

For quantification of IL17 secretion capacity, single-cell suspension from spleens was activated with PMA (100 ng/mL; Sigma) and ionomycin (1 μg/mL; Sigma) for 3 hours and incubated with brefeldin (Sigma) for another 4 hours. Cells were permeabilized with the Inside Stain Kit (Miltenyi) and stained with anti-H-2Kd-FITC (BioLegend), CD4-VioBlue, CD8-PerCP, IL17 APC (Miltenyi Biotec). For blood cell analysis, blood samples were collected by retro-orbital venipuncture on day +14 and +30 post-BMT and labeled with CD3 FITC, Gr-1 PerCP, Ter119 APC, and B220 VioBlue (Miltenyi Biotec).

ELISA

On day 14 post-BMT, serum was collected from transplanted mice by retro-orbital bleeding. Cytokine production was evaluated using 2-site ELISAs (IL6 and IL12 quantikine ELISA kit purchased from R&D Systems).

Statistical analysis

Survival analysis was performed according to the Kaplan–Meier method, and survival curves were compared using the log-rank testing. Flow and cytokine data were analyzed using the Student two-tailed t tests. Values of P < 0.05 were considered statistically significant.

Results

Ruxolitinib abrogates acute GVHD in vivo

First, we evaluated whether ruxolitinib inhibited acute GVHD in vivo using a fully MHC-mismatched mouse model of BMT (23). We tested ruxolitinib at 90 mg/kg/d; data shown are from three independent experiments comprising 10 to 15 mice per cohort. All experimental cohorts had an initial weight loss due to radiation toxicity (Fig. 1A). Thereafter, mice receiving only TCD bone marrow (BM) cells ("BM only") had a full recovery of their weight, whereas mice in the GVHD group treated with vehicle only ("GVHD + Vehicle") had a slow and constant reduction of their weight up to ~30% on day 48 post-BMT. Treatment with ruxolitinib for 14 days posttransplant (GVHD + ruxolitinib) was associated with an initial additional weight loss and a subsequent slow recovery of weight back to levels reached by the "BM only" cohort. Histologic analysis at day 14 post-BMT revealed reduced lesions of GVHD in all organs of ruxolitinib-treated mice (Fig. 1B), including skin (P < 0.0001), liver (P = 0.001), small intestines (P < 0.0001; Fig. 1C). Unexpectedly, liver examination revealed moderate centrilobular hepatocellular hypertrophy (Fig. 1D), compatible with hepatic toxicity in ruxolitinib-treated animals. In conclusion, ruxolitinib is capable of preventing weight loss and end-organ pathologic changes associated with acute GVHD; however, ruxolitinib therapy caused potential adverse effects at the dose of 90 mg/kg/d, as revealed by hepatic toxicity and early weight loss.

Ruxolitinib treatment has a dose-dependent effect on acute GVHD reduction

Next, we characterized whether reduction of ruxolitinib dosage may abrogate acute GVHD, while sparing mice from adverse drug effects. To test this hypothesis, recipients were treated orally with ruxolitinib at 90 mg/kg, 45 mg/kg, or 22.5 mg/kg daily, respectively: results were combined from at least three different experiments with 12 to 15 recipients per cohort (Fig. 2). Mice receiving ruxolitinib at 45 mg/kg/d had significant improvement of overall survival (OS) relative to GVHD controls (P = 0.03) and relative to ruxolitinib recipients at the 90 mg/kg/d dose (P = 0.01; Fig. 2A). Ruxolitinib at both the 90 and 45 mg/kg/d dosing yielded less weight loss relative to the GVHD cohort (Fig. 2B). The beneficial effect of ruxolitinib was partially lost when the drug was administered at the further reduced dose of 22.5 mg/kg/d, as documented by both survival curves and weight changes (Fig. 2A and B). Histologic analysis at day 14 post-BMT confirmed reduced lesions of GVHD in the examined organs of ruxolitinib-treated mice (Fig. 2C), with a dose-dependent effect of ruxolitinib on GVHD pathology score of skin, small and large intestines. Ruxolitinib delivered at 90 and 45 mg/kg daily doses was effective in this regard, whereas the 22.5 mg/kg dose was only nominally effective against histologically defined GVHD (Fig. 2D). Histologic evidence of GVHD in the liver was relatively refractory to ruxolitinib therapy, as there existed residual presence of portal inflammatory infiltrates still visible at the dose level of 90 mg/kg (Fig. 2C and D). Moreover, we detected a similar dose-dependent effect of ruxolitinib on proinflammatory cytokine production: relative to vehicle-treated mice, posttransplant IL6 levels were significantly reduced at all three ruxolitinib doses (P < 0.05); however, the most significant reductions occurred at the 90 and 45 mg/kg/d doses rather than the 22.5 mg/kg/d dose (11.7 ± 0.1 vs.

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During ruxolitinib treatment the GVT effect is maintained

We next evaluated whether ruxolitinib could abrogate acute GVHD without affecting the GVT effect. To test the hypothesis, in two separate experiments, we inoculated mice with A20 murine B-cell lymphoma cell line and analyzed the effect of ruxolitinib at the dose of 45 mg/kg. As expected, recipients of A20 and TCD-BM (BM only+A20) began to die of tumor starting from day 10 post-transplant. In marked contrast, all mice receiving allogeneic T cells treated with vehicle (GVHD+A20+vehicle) or with ruxolitinib (GVHD+A20+ruxolitinib, 45 mg/kg dose) were alive on day 48 post-BMT (P = 0.04 and P = 0.01, respectively; Fig. 3A).

Because the antitumor effect may be related to a direct effect of the drug, we checked spleen and bone marrow for infiltration by A20 lymphoma cells in recipients of T cell-replete or TCD transplants that were further treated with posttransplant ruxolitinib. Percentages of A20 tumor cells infiltrating the spleen or bone marrow of recipients were similar in mice receiving TCD-BM.

15.9 ± 2.0 vs. 32.0 ± 1.0 pg/mL, P < 0.05). In summary, the best effect of ruxolitinib against GVHD was reached with 90 and 45 mg/kg/d, while a reduced benefit was observed at 22.5 mg/kg/d. Consistent with the existing literature (18, 22), we observed no significant effect of daily administration of ruxolitinib on post-BMT donor blood counts (Supplementary Fig. S2A–S2D).

Indeed, relative to GVHD controls, treatment with ruxolitinib at 45 mg/kg/d was associated with improved myeloid and B-cell reconstitution on day 30 post-BMT. Of note, we found that, relative to the "BM only" cohort, ruxolitinib treatment was associated with similar myeloid and erythroid reconstitution, but reduced B-cell counts. C57BL/6 bone marrow cell engraftment was not delayed by ruxolitinib because the percentage of spleen H2Kb+ cells was similar on day 14 between GVHD controls and mice treated with ruxolitinib at 90 or 45 mg/kg/d (92.2 ± 1.2 vs. 89.5 ± 1.7 vs. 95.1 ± 1.9, respectively; P = 0.2) and improved relative to the "BM only" cohort (72.6 ± 5.2, P < 0.01).

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and signiﬁcantly reduced compared with mice receiving RMB-1 alone (BM only + A20 + vehicle) or with the addition of ruxolitinib (BM only + A20 + ruxolitinib; Fig. 3B, left, and 3C). In marked contrast, recipients of allogeneic T cells with vehicle (GVHD + A20 + vehicle) or ruxolitinib administered at 90 mg/kg/d (GVHD + ruxolitinib 90 mg/kg), 45 mg/kg/d (GVHD + ruxolitinib 45 mg/kg), or 22.5 mg/kg/d (GVHD + ruxolitinib 22.5 mg/kg), from day 0 to day 14 post-BMT. Data were pooled at least three independent experiments results are shown as mean ± SEM (n = 12–15 per cohort). A, the Kaplan–Meier survival curve is shown. B, percentage weight change was assessed on day 14 post-BMT and liver, skin, small and large intestine were harvested to assess GVHD. Representative images of histologic sections of skin, liver, and small intestine are shown: H&E staining. D, for GVHD each organ was scored on a scale of 0 to 4. Data were pooled from two independent experiments (at least n = 5 subjects for each cohort). *p < 0.05; **p < 0.005; ***p < 0.0001.

Ruxolitinib treatment was not associated with reduced alloreactivity

Given the preserved GVT effect, we wanted to analyze the effect of ruxolitinib on donor T-cell function. Previously, JAK-2 inhibition in vitro yielded signiﬁcant reduction of T cell alloreactivity (18); however, the in vivo effect of JAK-2 inhibition has only partially been investigated (24). Data shown are combined from four different experiments with at least 15 to 20 mice per cohort. First, we found that administration of ruxolitinib at 45 mg/kg/d was not associated with a signiﬁcant reduction of total spleen cells, including a full preservation of donor T cells (p = 0.8; Fig. 4A). Of note, the higher dose of ruxolitinib (90 mg/kg/d) yielded a signiﬁcant reduction of spleen size and total cell number (Supplementary Fig. S4A); this result was consistent with
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Figure 3.
Ruxolitinib prevents GVHD while maintaining a GVT effect. Lethally irradiated (950 Gy) BALB/c mice were transplanted with donor B6 cells consisting of 10 x 10^6 TCD-BM cells alone (BM only) or with 2.5 x 10^5 T cells with vehicle or with ruxolitinib at 45 mg/kg/d (GVHD + ruxolitinib 45 mg/kg) administered from day 0 to day 14 post-BMT. Additional mice were intravenously injected on the day of transplant with 2 x 10^5 A20 lymphoma tumor cells. Data were pooled from two separate experiments and results are shown as mean ± SEM (at least n = 10 per cohort). A, the Kaplan-Meier survival curve is shown. B, on day 14 post-BMT, spleen were isolated and analyzed by flow cytometry for A20 infiltration. Representative flow data are shown. C, data from two separate experiments were pooled to evaluate the effect of ruxolitinib alone or in the GVHD cohort on spleen and bone marrow infiltration by A20 lymphoma cells. Results are shown as mean ± SEM (n = 10 per cohort). *P < 0.05; **P < 0.005.

Previous observations (22). Relative to T-cell differentiation, posttransplant ruxolitinib therapy yielded an increase of CD62L^+CD44^+ central memory (CM) T cells (10.8% ± 2.1% vs. 2.3% ± 0.3%; P < 0.0001) and reduction of CD62L^+CD44^+ effector memory (EM) T cells (87.2% ± 2.5% vs. 97.5% ± 0.3%; P < 0.0001; Fig. 4B). Similar results were obtained with ruxolitinib at 90 mg/kg/d (P < 0.0001; Supplementary Fig. S4B). Ruxolitinib did not induce a significant reduction of alloreactive T cells in terms of percentages of CD4^+ IFNγ^+ (7.2% ± 0.8% vs. 5.6% ± 1.1%; P = 0.2) and CD8^+ IFNγ^+ (7.1% ± 0.8% vs. 5.6% ± 0.6%; P = 0.2) cells (Fig. 4C) and in terms of absolute numbers of splenic CD4^+ IFNγ^+ (1.9 ± 0.5 x 10^5 vs. 1.8 ± 0.4 x 10^5; P = 0.8) and CD8^+ IFNγ^+ cells (3.3 ± 0.7 x 10^5 vs. 2.6 ± 0.6 x 10^5; P = 0.4; Supplementary Fig. S5A). Similar results were achieved when ruxolitinib was delivered at the highest dosage (Supplementary Fig. S4C). Of note, mice treated with ruxolitinib at 45 mg/kg had much higher percentages of alloreactive CD4^+ IFNγ^+ and CD8^+ IFNγ^+ cells compared with control mice receiving only TCD-BM (P = 0.0007 and P = 0.0002, respectively; Fig. 4C). Given the role of IL6 for Th17 polarization, we analyzed whether ruxolitinib affected Th17 differentiation in vivo; we found no significant changes relative to vehicle-treated mice both in terms of percentage (0.7% ± 0.2% vs. 0.8% ± 0.2%; P = 0.8; Fig. 4D) and absolute numbers (2.5 ± 0.8 x 10^5 vs. 3.7 ± 1.1 x 10^5; P = 0.4; Supplementary Fig. S5B) of Th17^+ cells. Because it was recently shown that ruxolitinib is associated with preserved Treg differentiation (24), we analyzed the effect of the drug in vivo on CD4^+ CD25^+Foxp3^+ T cells. In our model, we did not observe a...
Ruxolitinib prevents acute GVHD without abrogating T-cell alloseactivity. Lethally irradiated (950 Gy) BALB/c mice were transplanted with donor B6 cells consisting of \(10^5\) CD34+ cells and 2.5 \(10^7\) T cells plus vehicle (GVHD + vehicle) or ruxolitinib 45 mg/kg/d (GVHD + ruxolitinib 45 mg/kg) administered in two separate doses by oral gavage. Results were pooled from four different experiments and shown as mean ± SEM (n = 10–20 mice per cohort). On day 14 post-BMT, spleens were harvested and analyzed by flow cytometry for T-cell composition (A) and T-cell subsets to enumerate central memory (CM), effector memory (EF), and naive T cells (B). Single-cell suspensions were: (C) stimulated with syngeneic or allogeneic DC and analyzed by intracellular cytokine flow cytometry. E, spleen cells were also analyzed to detect percentage of CD4+IL17+ cells capable of secreting IL17 by intracellular flow cytometry. E, spleen cells were also analyzed to detect percentage of CD4+IL17+ cells capable of secreting IL17 by intracellular flow cytometry.

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Discussion

The ideal scenario of allogeneic-HSCT in patients with hematologic malignancies is to promote an immune-mediated GVT effect while preventing the occurrence of GVHD. Acute and chronic GVHD still represent an important complication and the effect while preventing the occurrence of GVHD. Although the precise mechanism of this separation of GVHD from GVT effects will require further investigation, our results indicate that ruxolitinib may operate by preserving alloreactivity (thus allowing a GVT effect) while simultaneously modulating T-cell homing (thus allowing a modulation of GVHD).

Our findings may have important implications for clinical application of ruxolitinib in the contest of allogeneic HSCT. First, ruxolitinib is now available in clinic and is currently used for patients with myelofibrosis (9); this contest may facilitate a fast track for clinical trials evaluating ruxolitinib in the transplant setting. Of note, in our preclinical model, ruxolitinib was safe and not associated with hematologic toxicity. Second, JAK–STAT signaling was recently shown to have a strong relevance not only for the pathogenesis of myeloproliferative diseases, but also for the outcome of patients with diffuse-large B-cell lymphomas where pSTAT3 expression was associated with a poor outcome (33). Moreover, a new JAK2/FLT3 inhibitor, pacritinib, has recently shown promising results both in xenograft mouse models of JAK2V617F-driven diseases (20) and in patients with relapsed lymphoma (21). These recent acquisitions suggest a potential intriguing...
applicability of posttransplant pharmacologic modulation of JAKs in order to both inhibit GVHD and control tumor burden by a drug-mediated effect or by making myeloid and lymphoid diseases more susceptible to allo-responses. Importantly, we found that ruxolitinib not only yields significant anti-GVHD activity, but also preserves the GVT effect against two different tumor cell lines and that this was not due to a direct antitumor effect but rather was likely the result of a sustained posttransplant T-cell alloreactivity. This is consistent with the fact that A20-mediated malignancy is driven by NF-κB mediated polarization of donor T cells toward the Th1 and Th17 phenotypes that are generally recognized as efficient mediators of antitumor effects (36, 37). Moreover, our observations are in agreement with a previous report from Choi and colleagues (19) that described JAK1/JAK2 inhibition by ruxolitinib as an approach to block the IFNα/β-CXCR3 axis and prevent T-cell migration into GVHD organs. Indeed, we also found a reduction of CXCR3 expression, but this effect was more evident on CD8+ cells, and limited on CD4+ subsets. This modest effect of ruxolitinib on chemokine expression in our model suggests that other factors may have contributed to the reduced overall infiltration of donor T cells in the GVHD target tissues. Given the pathophysiologic three-step model of acute GVHD (38), reduced tissue infiltration may also have been a result of reduced alloreactivity and T-cell proliferation. For instance, Betts and colleagues (18) showed that JAK2 inhibition reduced DC-mediated T-cell activation, thereby impairing the activation of central and effector memory T cells as well as the expansion of responder Th1 and Th17 cells. We did not investigate DC activation in this study, but the fact that spleen T-cell infiltration, circulating T-cell numbers, Th1 and Th17 polarization were unchanged in mice receiving ruxolitinib suggests that this might not be the most relevant mechanism in our mouse model.

Of note, prevention of acute GVHD in our experiments was more pronounced in the skin and the intestine. Whether this effect was due to a different modulation of chemokines involved in liver GVHD, such as CXCR6 (39) and CCR5 (40), would be the object of further investigations. Interestingly, our findings partially confirm and also extend a recent study by Spoerl and colleagues (24) who reported a similar anti-GVHD effect of ruxolitinib in a mouse model of transplant. In fact, we demonstrated that ruxolitinib could preserve an immunologic GVT effect, but, differently from the earlier report, we could not identify increased Treg differentiation in vivo. It is possible that methodologic differences between the two experimental models may explain the fact that potential mechanisms of ruxolitinib mechanism of action against GVHD were identified.

In conclusion, our work provides further rationale for evaluating the effect of ruxolitinib on GVHD and GVT effects in clinical trials for patients with hematologic malignancies undergoing allogeneic HSCT. Although our findings point to chemokine modulation as a potential mechanism of action, further mechanistic studies will be required to better understand the precise mechanism of action of ruxolitinib for GVHD prevention. Our results open the way to test the potential application of new JAK inhibitors, such as pacritinib, both for regulating GVHD and directly inhibiting lymphoma cell proliferation after HCT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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