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Ruxolitinib improves hematopoietic regeneration by restoring mesenchymal stromal cell function in acute graft-versus-host disease

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Conflict-of-interest statement

The authors have declared that no conflict of interest exists.
Abstract

Acute graft-versus-host disease (aGVHD) is a severe complication of allogeneic hematopoietic stem cell transplantation. Hematopoietic dysfunction accompanied by severe aGVHD, which may be caused by niche impairment, is a long-standing clinical problem. However, how the bone marrow (BM) niche is damaged in aGVHD hosts is poorly defined. To comprehensively address this question, we employed a haplo-MHC-matched transplantation aGVHD murine model and performed single-cell RNA sequencing of non-hematopoietic BM cells. Transcriptional analysis showed that BM mesenchymal stromal cells (BMSCs) were severely affected with a reduction in cell ratio, abnormal metabolism, compromised differentiation potential and defective hematopoietic supportive function, which were validated by functional assays. We found that ruxolitinib, a selective JAK1/2 inhibitor, ameliorated aGVHD-related hematopoietic dysfunction through direct effect on recipient BMSCs, resulting in improved proliferation ability, adipogenesis/osteogenesis potential, mitochondrial metabolism capacity and crosstalk with donor-derived hematopoietic stem/progenitor cells. By inhibiting the JAK2/STAT1 pathway, ruxolitinib maintained long-term improvement of aGVHD BMSC function. Additionally, ruxolitinib pretreatment in vitro primed BMSCs to better support donor-derived hematopoiesis in vivo. These observations in the murine model were validated in patient samples. Overall, our findings suggest that ruxolitinib can directly restore BMSC function via JAK2/STAT1 pathway and in turn, improve the hematopoietic dysfunction caused by aGVHD.
Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a unique therapy with curative potential for hematologic malignancies. However, acute graft-versus-host disease (aGVHD) remains one of the leading causes of morbidity and non-relapse mortality (NRM) in the early stage after allo-HSCT (1, 2). One of the “secondary disasters” of aGVHD is the failure of donor-derived hematopoiesis, which is typically characterized by myelosuppression with an incidence of 10% to 20% (3, 4). Although several risk factors have been identified, the pathophysiology of myelosuppression is still largely unclear (5).

Our previous research, as well as studies by other groups, has revealed that in addition to the direct suppression of hematopoietic primitive cells, a distorted bone marrow (BM) niche also contributes to secondary hematopoietic dysfunction (4, 6, 7). These findings highlight the importance of deciphering the extrinsic hematopoietic regulators and the BM niche in the context of aGVHD.

The BM niche is a specialized microenvironment that maintains and sustains hematopoietic stem cell (HSC) function through cellular interactions and paracrine cytokines. The niche cellular components mainly include BM mesenchymal stromal cells (BMSC), osteolineage cells (OLC), BM endothelial cells (BMEC), arterioles and sinusoids, sympathetic nerves, non-myelinating Schwann cells, and megakaryocytes (8). BMSCs, which are the major cellular participants in the niche, can differentiate into other cellular components, such as osteoblasts, adipocytes, and chondrocytes (9). Located mainly in the perivascular space, BMSCs produce key niche factors, such as C-X-C motif chemokine ligand 12 (CXCL12) and stem cell factor (SCF), which support the homing and maintenance of HSCs (10). Because these non-hematopoietic BM niche cells are of recipient origin, they can be recognized and attacked as foreign by the alloreactive donor T cells when aGVHD is established (11). However, the mechanisms underlying niche remodeling in aGVHD are largely unknown.

To comprehensively assess the alterations of cellular compartments in the aGVHD niche,
we performed single-cell RNA sequencing (scRNA-seq) and constructed an atlas of niche components. In our murine aGVHD model, we observed a remarkable reduction in the BMSC ratio and function. Notably, we observed upregulation of the JAK/STAT pathway in BMSC isolated from aGVHD hosts, making them potential targets for ruxolitinib, a selective JAK1/2 inhibitor widely used in the prophylaxis and treatment of aGVHD (12, 13). Ruxolitinib directly rescued the BMSC dysfunction caused by aGVHD by inhibiting the JAK2/STAT1 pathway, resulting in enhanced hematopoietic support capacity, differentiation potential, and mitochondrial competence. Moreover, ruxolitinib enhanced the crosstalk between BMSCs and donor-derived hematopoietic stem/progenitor cells (HSPCs) by mitochondrial transfer, in turn forming a better microenvironment for HSC engraftment. These results were also validated with clinical samples. The therapeutic effect of ruxolitinib on BMSC was found to be long-lasting both in our aGVHD murine model and in patients. Therefore, by combining scRNA-seq with functional assays, we revealed a novel mechanism underlying the effect of ruxolitinib on aGVHD niche components and broadened the BMSC-based therapy for aGVHD.
Results

scRNA-seq revealed niche disruption in aGVHD murine model

To dissect the alterations in the cellular composition of the aGVHD BM niche, we utilized a haplo-matched allo-HSCT murine model that recapitulated clinical aGVHD (7). We isolated niche cells from control (BMT) and aGVHD mice by fluorescence-activated cell sorting (FACS) via labeling for CD45, lineage mix, and CD71 to deplete hematopoietic cells and erythrocytes, as well as for Calcein AM and DAPI to eliminate dead cells (Supplemental Figure 1A). These cells were collected on day 21 post-transplantation and analyzed by droplet-based scRNA-seq (10XGenomics) (Figure 1A). We partitioned all enriched cells into 17 clusters (Supplemental Figure 1B). Correlation analysis based on average gene expression and marker gene profiles distinguished non-hematopoietic cells (niche cells) from hematopoietic cells (Supplemental Figure 1, C-H) (14). Six clusters of non-hematopoietic cells were identified as follows: Cluster #1, BMEC (expressing Cdh5); Cluster #2, BMSC (expressing Lepr); Cluster #3, chondrocyte (expressing Acan); Cluster #4, fibroblast (expressing S100a4); Cluster #5, OLC (expressing Bglap) and Cluster #6, pericyte (expressing Acta2) (Figure 1, B and C). The cell ratio of BMSC and OLC decreased significantly under aGVHD conditions (Figure 1D).

aGVHD BMSC presented an aberrant gene profile, including the downregulation of the differentiation-related genes Adipoq and Bmp4 (15, 16), the stemness-related gene Grem1 (17, 18), and the upregulation of the calcification-inhibiting gene Mgp (19) (Figure 2A), suggesting that aGVHD impaired the stemness and differentiation potential of BMSC. Furthermore, aGVHD disrupted niche factor (Cxcl12 and Scf) expression in BMSC (Figure 2B). Gene ontology (GO) term analysis revealed downregulation of differentiation-related pathways (Figure 2C) and upregulation of apoptosis pathways in aGVHD BMSC (Supplemental Figure 1I). We noticed that aGVHD disrupted mitochondrial metabolism in BMSC, including oxidative phosphorylation (OXPHOS), respiratory electron transport chain processes and mitochondrial transfer-related pathways [phosphoinositide 3-kinase (PI3k-Akt signaling and gap junction] (Figure 2C), suggesting that mitochondria play an important role during niche remodeling under
We observed that other niche cellular components were also disrupted by aGVHD. Osteoblast differentiation/maturation-related genes, critical OLC-specification genes (such as Bglap and Sp7) and niche factors were suppressed in aGVHD OLC (Supplemental Figure 1J), indicating an impairment of osteolineage cells in the aGVHD milieu. These results were consistent with the GO term analysis (Figure 2E). Although the frequency of BMECs increased in aGVHD (Figure 1D), their hematopoietic supportive function decreased significantly (Supplemental Figure 1J). Further sub-clustering revealed that aGVHD preferentially disrupted sinusoidal BMECs (sBMECs) (Supplemental Figure 1, K-M). Expression of Cxcl12 and Scf was more abundant in arteriolar BMECs (aBMECs) and substantially inhibited by aGVHD (Supplemental Figure 1N). Additionally, aGVHD caused broad suppression of niche factor expression among fibroblasts, pericytes and chondrocytes (Supplemental Figure 1J). These data allowed us to draft an atlas of the cellular components in the aGVHD BM niche, in which BMSC exhibited impaired differentiation potential, defective metabolism capacity and reduced hematopoietic supportive function (Figure 2F).

BMSC in aGVHD mice and aGVHD patients shared similar features under aGVHD conditions

To validate our transcriptome analysis, we then conducted functional assays in a haplo-matched aGVHD murine model (Figure 3A). The absolute number of niche cells decreased longitudinally from day 7 to 21 in aGVHD mice (Supplemental Figure 2A). Leptin-receptor-positive (LepR+) BMSCs in aGVHD mice exhibited a 36-fold reduction compared with their counterparts in the BMT group by day 21 (Figure 3B, Supplemental Figure 2B). aGVHD BMSC also showed impaired colony-forming ability (Figure 3C, Supplemental Figure 2, C and D), reduced osteogenic (Figure 3D, red arrows) and adipogenic differentiation potential (Figure 3E, yellow arrows). Bulk RNA-seq and quantitative real-time PCR (qPCR) showed downregulation of genes related to stemness
(Lepr, Grem1) and differentiation (Adipoq, PPARγ, Runx2, Alpl, Col1a1 and Col2a1) in aGVHD BMSCs (Supplemental Figure 2, E and F).

To investigate differences in osteolineage dynamics over different stages of aGVHD, we generated Col2.3-GFP-reporter transgenic mice, which specifically expressed GFP in osteoblasts and osteocytes (20). We observed a striking decrease in osteoblasts (Col2.3-GFP<sup>high</sup>) from the early (day 7) to late (day 21) stages of aGVHD, with a 28-fold reduction compared to the BMT group (Figure 3F, Supplemental Figure 2G). Femurs from aGVHD mice also showed much weaker Col2.3-GFP signals in regions of metaphysis and diaphysis (Figure 3, G-I, Supplemental Figure 2, H-J), which was confirmed by the reduction in the proportion of the Col2.3-GFP<sup>high</sup> area (Figure 3J). Calcein labeling showed that bone formation was also severely restricted in aGVHD mice (Figure 3K). These findings suggested that aGVHD severely impairs osteolineage dynamics and bone formation.

In contrast to the diffuse distribution of adipocytes in the femurs of BMT mice, adipocytes of aGVHD mice were almost undetectable (Figure 3, L-N). We observed a 40-fold decrease in adipocytes in aGVHD mice compared to BMT mice (Figure 3O). However, oral administration of two major isoforms of the PPARγ agonists rosiglitazone (thiazolidinedione) and GW1929 (non-thiazolidinedione) did not significantly improve the survival of aGVHD mice (Supplemental Figure 3, A and B), although GW1929 administration increased adipocyte frequency in aGVHD mice (Supplemental Figure 3C). These results indicated that retrieving adipocytes alone was not sufficient to improve the survival of aGVHD.

To validate our observations in human samples, we collected the BM aspirates from patients with or without aGVHD (Table 1). The median platelet count ($P = 0.0062$) and hemoglobin level ($P < 0.0001$) in the aGVHD group were significantly lower compared to their counterparts in the non-aGVHD group. BMSCs from aGVHD patients were defective
in colony-forming ability (Figure 4A), adipogenic and osteogenic differentiation potential (Figure 4, B and C). These data indicated that BMSC in aGVHD mice and aGVHD patients exhibited similar impairment under aGVHD conditions.

**Ruxolitinib directly retrieved BMSC function in aGVHD**

In our transcriptomic analysis, we noticed a significant upregulation of the JAK/STAT pathway in aGVHD BMSC (Figure 5A), which led us to hypothesize that BMSC could be a potential target of ruxolitinib, a selective JAK1/2 inhibitor approved by Food and Drug Administration (FDA) for the treatment of steroid-refractory aGVHD (21). In aGVHD mice, oral administration of ruxolitinib (10 mg/kg or 30 mg/kg, twice a day for 20 days) resulted in a life span extension of 50 days and 150 days, respectively (Figure 5B, Supplemental Figure 4A). Notably, treatment with 30 mg/kg ruxolitinib markedly ameliorated body weight loss and reduced the disease score in aGVHD mice (Figure 5, C and D). We also observed the recovery of hematopoietic health based on increased BM cellularity, HSC reconstitution, and B cell frequency (Supplemental Figure 4B). Furthermore, the effect of ruxolitinib persisted to 150 days post-transplantation (130 days post-ruxolitinib treatment), suggesting that this effect was not transient (Supplemental Figure 4, C and D). LepR⁺ BMSC of aGVHD mice exhibited a >3-fold increase after ruxolitinib treatment compared to matched controls (Figure 5E). BMSC from ruxolitinib-treated mice generated detectable calcium nodules after induction of osteogenesis, while those from the vehicle-treated group did not (Figure 5F), implying an enhanced osteogenic potential of aGVHD BMSC after ruxolitinib treatment. These results suggested that ruxolitinib directly retrieves BMSC function in aGVHD and may also be a potential therapeutic target for the aGVHD niche.

Due to the low frequency of BMSC in aGVHD individuals, we performed scRNA-seq on niche cells (CD45⁻Lineage⁻CD71⁻Calcein AM⁺DAPI⁻) from vehicle- and ruxolitinib-treated aGVHD mice [day 20 and 100 post-transplantation (12 h and day 80 post-ruxolitinib treatment)] to investigate the long-term remodeling of BMSC (Figure 6A). After
removal of contaminating hematopoietic cells (Supplemental Figure 4, E and F),
unsupervised clustering partitioned the stroma into six clusters comprising BMSC, OLC,
BMEC, pericyte, chondrocyte and fibroblast (Figure 6B) according to marker gene
expression patterns (Supplemental Figure 4G). Niche cells exposed to ruxolitinib
exhibited a different transcriptional phenotype to those of vehicle-treated controls (Figure
6C). Cell ratio analysis revealed that BMSCs were significantly boosted after ruxolitinib
treatment, which was consistent with the results of our functional assay (Figure 6D).
Enhanced hematopoietic supportive function and differentiation potential were achieved
for BMSC after ruxolitinib treatment (Figure 6, E and F). Interestingly, ruxolitinib treatment
significantly upregulated mitochondrial metabolism-related genes, such as those
associated with OXPHOS, reactive oxygen species (ROS) and metabolic processes in
aGVHD BMSC (Figure 6G), which was validated by elevated MitoSox levels in BMSC
derived from ruxolitinib-treated mice (Supplemental Figure 4H). In addition, upregulation
of the mitochondrial transfer-related pathway (PI3k-Akt signaling) (Figure 6G) and
downregulation of apoptosis-related pathways were observed in ruxolitinib-treated
aGVHD BMSC (Supplemental Figure 4I). In both the short- and long-term follow-up
analyses post-ruxolitinib treatment, we observed enhanced ROS metabolism in BMSC
(Figure 6H), indicating that ruxolitinib maintains relatively high mitochondrial metabolism
for a long period. Additionally, BMSC shared common features from day 20 to 100 post-
transplantation, including upregulation of differentiation potential (especially osteogenesis)
and the PI3k-Akt pathway (Figure 6I). Our transcriptomic analysis suggested that
ruxolitinib restored niche fitness, which was disrupted by aGVHD and provided long-term
protective effects for BMSC in the aGVHD milieu.

To investigate the function of ruxolitinib-treated BMSC in vivo, aGVHD mice received
injection of primary or cultured BMSCs (Figure 6J, Supplemental Figure 5A).
Intravenous injection of BMSCs did not prolong the survival of aGVHD mice
(Supplemental Figure 5, B and C). However, intratibial injection of 1×10⁶ cultured
BMSCs significantly prolonged the lifespan of aGVHD mice (23 days vs. 17 days, Figure
Ruxolitinib enhanced the colony-forming ability of BMSCs in a dose-dependent manner in vitro (Supplemental Figure 5D); therefore, we preconditioned BMSCs with 0.5 μM ruxolitinib before the injection. Intratibial injection of ruxolitinib-pretreated BMSCs further improved survival of aGVHD mice compared with those administered with vehicle-treated BMSCs (Figure 6K), indicating that ruxolitinib pretreatment in vitro primes BMSC to function better in vivo. Jointly, these data demonstrated that ruxolitinib directly restored BMSC function, resulting in enhanced differentiation potential, hematopoietic supportive function and metabolism capacity.

Ruxolitinib enhanced hematopoietic regeneration by promoting mitochondrial transfer from recipient BMSC to donor-derived HSPC

We noticed that aGVHD mouse-derived BMSC treated with ruxolitinib in vitro showed downregulation of the JAK/STAT pathway and upregulation of gap junction-related genes and the PI3k-Akt pathway (Figure 7A), implying the activation of mitochondrial transfer. We hypothesized that ruxolitinib enhanced the ability of recipient BMSC to create a favorable microenvironment for donor cells by modulating mitochondrial transfer between these two populations. Therefore, we employed mito-Dendra2 reporter mice to track the mitochondrial dynamics. Oral administration of ruxolitinib increased mitochondrial transfer from recipient BMSC to donor-derived BM cells in aGVHD mice (Figure 7B). When co-cultured with ruxolitinib-pretreated BMSCs, the proportion of Dendra2-mitochondria containing HSPCs was higher than that in HSPCs co-cultured with vehicle-pretreated BMSCs (Figure 7C), suggesting that ruxolitinib promoted mitochondrial transfer from BMSC to HSPC. When exposed to a ruxolitinib-treated niche, donor-derived HSPCs exhibited lower ROS levels than those exposed to an untreated niche (Figure 7D). When isolated and co-cultured with HSPCs in vitro, ruxolitinib-pretreated aGVHD BMSCs also decreased the ROS levels in HSPCs (Figure 7E), which may contribute to hematopoietic engraftment. These data indicated that ruxolitinib enhances the ability of BMSC to create a favorable microenvironment for HSC maintenance and engraftment.
Ruxolitinib directly modulated aGVHD BMSC function by inhibiting the JAK2/STAT1 pathway

To clarify the molecular mechanism underlying the pharmacological effects of ruxolitinib, we evaluated the levels of critical proteins in the JAK/STAT pathway. We found that aGVHD induced phosphorylation of JAK2 and STAT1 in BMSC, which was inhibited by ruxolitinib (Figure 8A), while only mild alterations were observed in JAK1 and other members of the STAT family (Supplemental Figure 6, A and B). The selective JAK2 inhibitor Fedratinib elevated expression of differentiation-related genes (Pparγ and Col1a1) and the niche factor Cxcl12, whereas the selective JAK1 inhibitor Itacitinib did not (Supplemental Figure 6C), indicating that ruxolitinib mediated its pharmacological effects via the JAK2/STAT1 pathway. The suppression of JAK2/STAT1 was also confirmed by in vitro treatment of aGVHD BMSC with different concentrations of ruxolitinib (Figure 8B). We then employed lentiviral short hairpin RNA (shRNA) to knock down Jak2 in aGVHD BMSC. The knockdown efficiency was validated by qPCR (Figure 8C) and western blotting (Figure 8D). Ruxolitinib did not enhance osteogenesis/adipogenesis (Figure 8E), mitochondrial metabolism (Figure 8F) and mitochondrial transfer to cocultured HSPCs (Figure 8G) in Jak2-deficient aGVHD BMSC. These data demonstrated that ruxolitinib directly primed aGVHD BMSC function by inhibiting the JAK2/STAT1 pathway.

Ruxolitinib improved BMSC function in aGVHD patients

To investigate whether ruxolitinib treatment directly improves BMSC function in aGVHD patients, we collected BM aspiration samples from aGVHD patients before and after ruxolitinib treatment. The clinical information of the patients is summarized in Supplemental Table 1. Ruxolitinib promoted hematopoietic recovery in aGVHD patients (Figure 9A). In accordance with our findings in the aGVHD murine model, ruxolitinib increased the number of proliferation-competent BMSCs (Figure 9B) and enhanced the adipogenesis and osteogenesis potential of BMSCs in aGVHD patients (Figure 9C). Incubation with ruxolitinib in vitro resulted in a >2.0-fold increase in aGVHD BMSC function.
colonies compared to those incubated with the vehicle (Figure 9D). This increase was accompanied by improvement in adipogenic and osteogenic differentiation potentials (Figure 9E). Notably, ruxolitinib treatment elevated the mitochondrial metabolism of BMSCs in aGVHD patients (Figure 9F). Compared with aGVHD BMSCs treated with vehicle in vitro, those treated with ruxolitinib also presented elevated ROS levels (Figure 9G). In accordance with our findings in the aGVHD murine model, ruxolitinib inhibited activation of the JAK2/STAT1 pathway in aGVHD patient-derived BMSCs (Figure 9H). Following shRNA-mediated knockdown of JAK2 (Figure 9I), ruxolitinib no longer enhanced osteogenesis (Figure 9J) and mitochondrial metabolism of aGVHD BMSC (Figure 9K). In our follow-up of patients up to 6 months post-ruxolitinib administration (Supplemental Table 2), we were surprised to observe continuously enhanced colony-forming ability (Supplemental Figure 7A), osteogenesis (Supplemental Figure 7B), adipogenesis (Supplemental Figure 7, C and D) and mitochondrial metabolism (Supplemental Figure 7E) of BMSC. These data indicated that ruxolitinib acts directly to salvage BMSC function via the JAK2/STAT1 pathway and maintains long-term therapeutic effects.
Discussion

Accumulating evidence has suggested that the BM niche serves as a crucial therapeutic target in aGVHD. However, the biological abnormalities underlying the BM niche in aGVHD are unclear. For the first time, we have constructed an atlas to illustrate the cellular components of the BM niche in aGVHD models, providing a more comprehensive and precise understanding of BM niche remodeling under aGVHD conditions. Through the integration of transcriptional analysis and functional assays, we have systematically identified several remodeling events in the aGVHD BM niche. Specifically, we found that (i) the ratio of cellular components, particularly BMSC and OLC, was significantly reduced; (ii) BMSC exhibited impaired differentiation potential, mitochondrial metabolism, and crosstalk with donor-derived HSPC; (iii) OLC displayed compromised differentiation and maturation ability; and (iv) the hematopoietic support capacity of most niche cells is markedly decreased.

Ruxolitinib has been successfully applied in the treatment of acute and chronic GVHD in recent years (22, 23). The potent therapeutic effect of ruxolitinib was also demonstrated by our clinical and aGVHD model observations. For the first time, we revealed the direct impact of ruxolitinib on BMSC via the JAK2/STAT1 pathway in addition to its well-known systemic immunomodulatory function (13). Our findings were substantiated by several lines of evidence: (i) RNA-seq analysis demonstrated that both in vivo and in vitro exposure to ruxolitinib downregulated the JAK/STAT pathway in aGVHD BMSC (Figure 5A, Figure 7A); (ii) western blotting experiments revealed that aGVHD-induced activation of the JAK2/STAT1 pathway was effectively attenuated by ruxolitinib treatment (Figure 8, A and B, Figure 9H); and (iii) Jak2 knockdown abrogated the therapeutic effect of ruxolitinib on aGVHD BMSC (Figure 8, E-G, Figure 9, J and K). The revelation of the direct protective effects of ruxolitinib on BMSC has pivotal clinical significance for several reasons. First, JAKs function as crucial regulators of immune cells and play pivotal roles in all phases of aGVHD pathogenesis (24). The restoration of BMSC functions by ruxolitinib indicates that BMSC could be a direct sensor of pathological signals. Second,
we discovered that ruxolitinib restores BMSC metabolism, particularly ROS production. Previous studies have shown that JAK/STAT blockade by inhibitors such as tofacitinib, significantly enhanced oxidative phosphorylation, ATP production, the maximal respiratory capacity, and the respiratory reserve in rheumatoid arthritis synovial fibroblasts (25). However, a functional link between JAK/STAT signaling and BMSC metabolism in aGVHD has yet to be established. Our findings have opened a brand-new avenue of research, suggesting that metabolic agents could potentially serve as a viable option for microenvironment-based therapy.

The crosstalk between stromal cells and HSCs has been recognized as an important extrinsic mechanism of HSC regulation. Activated BMSCs were found to abrogate the ROS level of target cells via mitochondrial transfer, with reduced apoptosis and cell death (26), conferring survival benefits in recipient cells (27). One potential mechanism is that ROS-induced oxidative stress regulates the opening of connexin channels mediated by PI3K activation and the transfer of mitochondria from BMSC to HSC (28). To date, the phenomenon of mitochondrial transfer in the context of aGVHD has not been investigated. In our study, we have demonstrated for the first time that ruxolitinib treatment upregulates the PI3k-Akt pathway in BMSC and promotes the transfer of functional mitochondria from BMSC to donor-derived HSC, thereby conferring a protective effect in aGVHD. When co-cultured with BMSCs that were pre-exposed to ruxolitinib, c-Kit+ HSPCs exhibited lower cellular ROS levels. Previous studies have suggested that high levels of cellular ROS in HSPCs can lead to defects in cell-cycle quiescence, disrupted HSPC-osteoblastic niche interactions, and ultimately, HSC exhaustion (29-31). Conversely, HSCs with low ROS levels are thought to have better long-term repopulating capacity (32). Hence, ruxolitinib treatment helps to maintain BMSC function and fosters a favorable microenvironment for donor-derived hematopoiesis, which in turn provides a supportive niche for HSCs and contributes to their mitochondrial fitness. These data indicated that ruxolitinib acts directly on BMSCs and rescues their function, representing a non-canonical pathway that partially contributes to the mechanisms underlying the therapeutic effects of ruxolitinib in aGVHD.
BMSCs possess trophic, homing/migration and immunomodulatory functions that have been demonstrated both in vitro and in vivo (33). However, the key issue of BMSC as second-line aGVHD therapy is that the clinical outcome can be unpredictable. Therefore, there remains a pressing need to optimize BMSC efficiency in aGVHD therapy. In light of the excellent tolerability, efficacy, and safety profile of ruxolitinib observed in clinical trials (22, 34), as well as its direct effects on BMSC, we consider ruxolitinib to be an ideal candidate for in vitro pretreatment of BMSC to prime their function in vivo. The findings of our study have important clinical implications as they shed light on the mechanisms by which aberrant signals from factors such as cytokines and cell-cell interactions within the aGVHD microenvironment can compromise the immunomodulatory and multilineage differentiation potential of adoptively transferred MSCs. By pretreating BMSCs with ruxolitinib, we were able to tune down these aberrant signals, thereby preserving their function in the aGVHD milieu. Our data suggest that administration of ruxolitinib-pretreated MSCs holds great promise for improving hematopoietic dysfunction in aGVHD. Notably, when ruxolitinib-pretreated BMSCs were injected topically, we observed that the megakaryocyte-erythroid progenitor (MEP) frequency in BM was also boosted, implying that functionally retrieved aGVHD BMSCs primed preliminary hematopoietic cells directly. Since MEP give rise to platelets (35), we believed that BMSC injection together with ruxolitinib may overcome the potential risk of thrombocytopenia caused by ruxolitinib (22).

In conclusion, our data provide a comprehensive cellular and molecular landscape of the aGVHD BM niche. We demonstrate that ruxolitinib directly rescues aGVHD BMSC function by inhibiting the JAK2/STAT1 pathway, thereby ameliorating hematopoietic dysfunction secondary to aGVHD. These findings provide novel insights into the mechanisms underlying the therapeutic effects of ruxolitinib in aGVHD.
Methods

Patients

To investigate BM niche alterations in aGVHD, 44 patients (34 patients with aGVHD and 10 without aGVHD) who received allo-HSCT in the Institute of Hematology & Blood Diseases Hospital (Tianjin, China) and Shanghai Institute of Hematology, National Research Center for Translational Medicine (Shanghai, China) were enrolled between September 2018 and September 2021. The patient transplantation procedure used has been described previously (36, 37). Four patients (9.1%) received HLA-matched and related grafts, 38 patients (86.4%) received HLA-mismatched and related grafts and two patients (4.5%) received HLA-matched and unrelated grafts. The median age at allo-HSCT was 35 years (range, 16–67 years). The last follow-up was December 31, 2022 (Table 1). Patients who received HLA-mismatched and unrelated grafts were treated with anti-thymocyte globulin. aGVHD prophylaxis regimens included cyclosporine A, short-term methotrexate, and mycophenolate mofetil. aGVHD staging and clinical responses to treatment were determined according to established guidelines (38).

To determine the effects of ruxolitinib on aGVHD BMSC, seven aGVHD patients who were successfully treated with ruxolitinib as frontline therapy (detailed in Supplemental Table 1) and five aGVHD patients who were followed up to 6 months post-ruxolitinib treatment (detailed in Supplemental Table 2) were included in the analysis. We collected BM aspiration samples before and at least 2 weeks after ruxolitinib treatment (for short-term evaluation) and at least 6 months after ruxolitinib treatment (for long-term evaluation). Samples were immediately suspended in human BMSC culture medium [DMEM/F-12 (Gibco), 20% FBS (Gibco), and 1% penicillin-streptomycin (Gibco), and filtered (0.2 mm pore size; BD Falcon)]. The medium was changed after 3 days of culture and BMSC were harvested for further experiments, including colony-formation, differentiation, and metabolism assays. Institutional databases were reviewed retrospectively to extract the demographic, clinical, and genetic data.
Mice

C57BL/6J (CD45.2*), BALB/C (CD45.2*), and B6.SJL-PtprcaPepcb/BoyJ (B6.SJL, CD45.1*) mice were purchased from the animal facility of the State Key Laboratory of Experimental Hematology (Tianjin, China). Gt (ROSA) 26Sortm1 (CAG-COX8A/Dendra2) Dcc (PhaM excised) mice containing the mitochondrial targeting signal of subunit 8a of cytochrome c oxidase (mito-Dendra2) were obtained from Jackson Laboratories (018397).

Recipient CB6F1 (CD45.2*) mice were first-generation animals obtained by crossing male C57BL/6J with female BALB/C mice. Col2.3-GFP transgenic mice were generally maintained on a CB6F1 background. All mice used in this study were aged 6–8-weeks and maintained in a specific pathogen-free animal facility.

Bone marrow transplantation and establishment of aGVHD model

BM transplantation and establishment of the aGVHD model was performed as previously described (7). Briefly, 4 h after sub-lethal irradiation (4.0 Gy, twice), 5 x 10^6 BM nucleated cells from female B6.SJL mice (CD45.1*) were intravenously injected into female CB6F1 recipients (CD45.2*) to establish the BM transplantation (BMT) model. Additional splenocytes (6 x 10^7) from female C57BL/6 mice were injected into the BMT mice to establish the aGVHD model.

Isolation of mouse niche cells

Mice were sacrificed by CO₂ asphyxia, and the unfractionated BM was crushed in digestion solution [3 mg/ml collagenase I, 3 mg/ml collagenase IV, and 1 mg/ml DNase I mixed in Hank's Balanced Salt Solution (HBSS)] and gently mixed by shaking at 37°C for 30 min to release BMSC. To isolate OLC, the bone matrix was chopped into pieces in another digestion solution (3 mg/ml collagenase I, 1 mg/ml DNase I, Dispase II, mixed in HBSS) and gently mixed by shaking at 37°C for 30 min.

Single-cell RNA sequencing

Niche cells were enriched using a Lineage Cell Depletion Kit (Miltenyi Biotec) and isolated
by FACS after labeling with antibodies for the detection of CD45 (30-F11), Lineage mix [CD3e (17A), CD4 (GK1.5), CD8 (53-6.7), CD45R (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), Ter-119 (TER-119)] and CD71 (RI7217); all antibodies were obtained from BioLegend. Calcein AM and 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) were used to identify and eliminate dead cells. Samples with a cell viability of >80%, a cell concentration ranging from 700 cells/μL to 1,200 cells/μL, and cell diameters ranging from 5 μm to 40 μm were collected and loaded for sequencing. The cell suspension and gel beads contained barcode-sequencing-generated single-cell gel bead-in-emulsions for reverse transcription and library preparation using a Chromium Single Cell 30 v2 Reagent Kit (10XGenomics). Sequencing was performed on an Illumina HiSeq PE150 or NovaSeq PE150 platform with 50,000 to 100,000 reads per cell and a 70%–80% saturation level, as recommended by 10XGenomics.

**Bioinformatics analysis of single-cell sequenced reads**

Sequenced reads were trimmed using Trimmomatic software (Anthony M. Bolger, Trimmomatic) to produce clean data. For dimensionality reduction, cell filtering and t-distributed stochastic neighbor-embedding (t-SNE)/UMAP analysis followed by principal component analysis (PCA) was performed using Cell Ranger (10XGenomics). Graph-based clustering of the PCA-reduced data modified with the Louvain algorithm was visualized on a 2D t-SNE map (39). Non-hematopoietic cells were clustered according to reported transcriptomic signatures (14, 40). The same procedure was performed for sub-clustering. Force-directed layout embedding was based on ForceAtlas2 from the Gephi package. Pearson's correlation analysis was applied based on the average gene expression profile of each pair of clusters. Diffusion trajectory analysis according to a previously described method (41).

**Flow cytometry**

BM hematopoiesis was analyzed using following antibodies (all from BioLegend): CD3e (17A3), CD4 (GK1.5), CD8 (53-6.7), CD45R (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5),
Ter-119 (TER-119), Sca-1 (D7), CD117 (2B8), CD34 (RAM34), CD150 (TC15-12F12.2), CD48 (HM48-1), CD41 (MWReg30), CD16/32 (93), Flk2 (A2F10), IL-7R (A7R34), CD45.1 (A20) and CD45.2 (104). BM niche cells were analyzed using the following antibodies (all from BioLegend, unless indicated): biotin-conjugated leptin receptor (R&D, Cat BAF497), CD31 (390), CD51 (RMV-7), CD45 (30-F11), and Ter119 (TER-119). Streptavidin-APC was used as an indirect labeling reagent for biotinylated antibodies. Dead cells were excluded by DAPI labeling (Sigma-Aldrich).

Colony-forming unit fibroblast (CFU-F) assay

In total, 1,000,000 unfractionated BM niche cells from female CB6F1 mice were added to BMSC culture medium [high-glucose DMEM, 20% FBS (Gibco), 1% penicillin-streptomycin (Gibco), and filtered (0.2 mm pore size; BD Falcon)] in each well of a 6-well plate. After 14 days, the wells were washed with PBS and the cells stained with crystal violet solution (Sigma) for 5 min at room temperature. Colonies containing >40 spindle-shaped cells were counted. For patient samples, 200,000 cells from the BM aspirates were plated immediately, and colonies were counted 14 days after seeding. The ratio of the number of colonies to the initial volume of the BM was used to assess the fibroblast colony-forming unit forming (CFU-F) ability.

In vitro differentiation assay

Unfractionated BM stromal cells were plated into a 10-cm² dish containing BMSC culture medium (described in CFU-F assay method). At 60%–70% confluence, cells were digested with 0.25% trypsin-EDTA and re-plated into collagen-coated 6-well plates. Osteogenic and adipogenic differentiation was induced using osteogenesis and adipogenesis differentiation kits (CYAGEN) according to the manufacturer’s instructions. Calcium nodule staining was performed with the von Kossa method and Alizarin Red S for 20 min at room temperature. Fatty droplets were stained with Oil Red O for 20 min at room temperature.
Bone sectioning and imaging

Mouse femurs and tibias were cleaned of muscle, fixed in 4% paraformaldehyde (Sigma-Aldrich) for 8 h, and dehydrated in 30% sucrose solution for 24 h. Bones were sectioned on a Leica CM1950 microtome and incubated in staining buffer [20% DMSO (Sigma-Aldrich), 5% donkey serum (JACKSON), 0.5% IGEPAL (Sigma-Aldrich) in PBS] containing primary antibodies for 8 h. After washing with PBS and gentle shaking for 4 h, the sections were incubated with secondary antibodies for 8 h in the staining buffer mentioned above. After washing with PBS and gentle shaking for a further 4 h, a rabbit anti-perilipin (Sigma, 1:400) antibody (primary antibody) was used to label adipocytes, which were detected with a donkey anti-rabbit IgG (H+L) Alexa Fluor 555 (Thermo Fisher, 1:400) secondary antibody. DAPI was used to label all nucleated cells. Images were obtained using two-photon excitation microscope (Olympus). A 3-dimensional reconstruction was created using Velocity software (Perkin Elmer).

Calcein labeling

On day 7 after modeling, calcein (10 mg/kg, in PBS) was subcutaneously injected into BMT and aGVHD mice. The mice were sacrificed on day 14 by CO2 asphyxia, and the femurs were isolated, fixed, sectioned, and stained with DAPI as described above. The distances between the calcein-positive lines represented the rate of bone formation.

Drug administration

For PPARγ agonist treatment, rosiglitazone and GW1929 were dissolved in ddH2O and administered orally once a day from day 0 to day 20. Ruxolitinib was administered orally (dissolved in 30% PEG300, 2% DMSO, and ddH2O) twice per day from day 0 to day 20. Control aGVHD mice were administered a vehicle (30% PEG300, 2% DMSO in ddH2O) on the same days. The body weight was recorded every day during the administration period.

Intramedullary injection of BMSC
For intramedullary injection, BMSC isolated from non-treated wild-type C57 mice were plated in BMSC culture medium containing vehicle (DMSO) or 0.5 μM ruxolitinib, cultured and suspended at 1,000,000 cells per 5 μL in injection buffer (10 μM Y-27632 in HBSS; Sigma). For primary BMSC injection, cells were stained for biotin-conjugated leptin receptor (R&D, Cat BAF497), CD45 (BioLegend, 30-F11) and Ter119 (BioLegend, TER-119). Streptavidin-APC (BioLegend) was used as an indirect labeling reagent for biotinylated antibodies. Dead cells were excluded by labeling with DAPI (Sigma-Aldrich). CD45^{-}Ter119^{-}LepR^{+} cells were sorted and suspended at 10,000 cells per 5 μL in the same buffer. Then, BMSC were injected intratibially into aGVHD mice at different time-points using a micro-sample syringe (25 μL; Sangon Biotech).

**BMSC bulk RNA sequencing (RNA-seq)**

Total RNA was extracted from sorted or cultured BMSC using a RNeasy Micro Kit (QIAGEN). Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer’s recommendations. After quantification and quality assessment, the mRNA was purified with oligo-dT magnetic beads for library preparation. A total of 1 μg RNA per sample was used as input material for the RNA sample preparations. Divalent cations were used for fragmentation in NEBNext First Strand Synthesis Reaction Buffer (5x). First strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. After the synthesis both strands and adenylation of the 3’ ends of the DNA fragments, the NEBNext Adaptor with a hairpin loop structure was ligated to prepare the samples for hybridization. The library fragments were purified using an AMPure XP system (Beckman Coulter, Beverly, USA), and then 3 μL of USER Enzyme (NEB, USA) was mixed with the size-selected and adaptor-ligated cDNA (250–300 bp in length) at 37°C for 15 min, then at 95°C for 5 min for PCR preparation. Next, PCR amplification was performed using Phusion High-Fidelity DNA polymerase, universal PCR primers, and an index (X) primer. The PCR products were purified using the AMPure XP system, and the library was
assessed for further clustering and sequencing using an Agilent Bioanalyzer 2100 system. The index-coded samples were clustered using a TruSeq PE Cluster Kit v3-cBot-HS (Illumia), and library preparations were sequenced on an Illumina NovaSeq platform. Differential expression analysis was performed using the DESeq2 R package (1.16.1). Genes with an adjusted $P$-value < 0.05 found by DESeq2 were assigned as differentially expressed and presented in a gene volcano map.

**RNA isolation and real-time quantitative PCR (RT-qPCR)**

RNA was extracted from sorted BMSC using a RNeasy Mini Kit (QIAGEN) and then reverse transcribed into cDNA with the Reverse Transcription System (Roche). RT-qPCR was conducted using SYBR green PCR Master Mix (ROCHE). The housekeeping gene GAPDH served as a positive quantitative control, and the relative quantitation of raw data was based on the $\Delta\Delta CT$ method. The primers used in this study were as follows:

- **Murine Gapdh**: (Forward) 5′-AAGTTCTCAGTCACACCACCCG-3′ and (reverse) 5′-TGCTCAGGTAGTGTTGTCG-3′
- **Murine Pparγ**: (Forward) 5′-ACCACCTACACCTTACGAC-3′ and (reverse) 5′-TGGGTCAGCTCTTGTGAATG-3′
- **Murine Adipoq**: (Forward) 5′-TGTTCTCCTTAAATCCTTGCCCA-3′ and (reverse) 5′-CCAACCTGCACAGTTCTTCA-3′
- **Murine Runx2**: (Forward) 5′-TTACCTACACCCCGCCAGTC-3′ and (reverse) 5′-TGCTGGTCTGGAAGGTCC-3′
- **Murine Col2a1**: (Forward) 5′-GTGGAGCAGCAAGAGCAGCA-3′ and (reverse) 5′-CTTGCCCCATCTTACCATGGAAGGATC-3′
- **Murine Jak2**: (Forward) 5′-CTCTCTGTCACACTTCCTTCA-3′ and (reverse) 5′-TTGGTAAAGTAGAACCTCATGCG-3′
- **Human beta-ACTIN**: (Forward) 5′-AGCGAGCATCCCCCGTTAC-3′ and (reverse) 5′-GGGCACGAAGGCTCATCATT-3′
- **Human ALP**: (Forward) 5′-CAGAGGAAGGACCAACTTGGG-3′ and (reverse) 5′-TTGTATGTTCGAGCAGG-3′.
Human *PPARγ*: (Forward) 5′-GAGCCCAAGTTTGAGTTTGC-3′ and (reverse) 5′-GCAGGTGTCTTGAATGTCTTC-3′.

Human *JAK2*: (Forward) 5′-ATCCACCCAACCATGTCTTCC-3′ and (reverse) 5′-ATTCCATGCGATAGGCTCTG-3′.

**Western blotting**

Western blotting was performed as described previously (42). In brief, total proteins were extracted from cells and resuspended in 5× SDS-PAGE loading buffer. The boiled protein samples were then subjected to SDS-PAGE followed by immunoblotting with the appropriate primary antibodies and secondary antibodies.

**Jak2-knockdown BMSC construction**

For lentivirus production, the pU6-MCS-mcherry-IRES-puromycin vector containing Jak2 shRNA or control lentivirus-shRNA was transfected together with pSPAX2 and pMD2G into HEK293T cell lines using Lipofectamine 2000 (Thermo Fisher). Culture supernatants were harvested after 48 h and 72 h and concentrated using an Amicon filter (100K NMWL, Millipore). aGVHD BMSC (third passage) were subsequently infected with lentiviral shRNA targeting Jak2 or the scramble control and cultured in 10% FBS in DMEM containing 50 U/ml penicillin and 50 mg/ml streptomycin for 8 h. The medium containing lentivirus was replaced by fresh medium and the cells were cultured for a further 24 h. Infected cells were then selected using puromycin (5 μg/mL) for 48 h. The stable cell lines were used in functional assays.

**Mitochondrial ROS and membrane potential analyses**

Mitochondrial ROS and membrane potential were measured by MitoSox red (Molecular probe) and tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) staining, respectively. Following immunostaining of surface markers (CD45/Ter119/LepR and CD45.1/Lineage/Sca-1/c-Kit), single-cell suspensions of BM or co-cultured cells were incubated with MitoSox red or TMRE in HBSS at 37°C for 30 min. Cells were washed
twice with HBSS, labeled with DAPI, and analyzed using a BD FACS Aria III (BD Biosciences). The mean fluorescence intensity data were collated. Unlabeled cells were used as negative controls.

Data analysis

FCS (flow cytometry standard) files were analyzed using Flow Jo software (Flow Jo LLC, Ashland, OR, USA). Data were represented as the mean ± the standard error of the mean (SEM). At least three independent replicates were included for all functional experiments. 1-way ANOVA followed by unpaired t test was employed to test statistical difference. Multiple-testing correction was performed by the Holm-Bonferroni method. Survival was analyzed by Log-rank test. For scRNA-seq data, the significance of gene expression between two group was compared using Wilcoxon Rank-Sum test. In the calculation of the cumulative incidence of non-relapse mortality (NRM), death and relapse were included as competing events and survival was counted as a censored event. The NRM was compared between groups using Gray's method. Hazard ratios were assigned 95% confidence intervals (95% CI). P < 0.05 was considered statistically significant. All calculations were performed using SPSS v.20 (IBM, Armonk, NY) and R v3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) software programs.

Study approval

All human studies were approved by the respective Institutional Review Boards [Shanghai Rui Jin Hospital (Shanghai) and Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin)]. Each patient (or a legal guardian) provided signed informed consent for therapy, and the collection of prospective data was in accordance with the Declaration of Helsinki. Animal experiments were approved by the Institutional Animal Care Committee for the State Key Laboratory of Experimental Hematology and the Institute of Hematology.

Data availability
The single-cell RNA-seq (10XGenomics) and bulk RNA-seq data can be accessed in GEO database. The accession numbers are GSE157389, GSE157326 and GSE165413. The original data could be accessed in the 'Supporting data values' XLS file.

**Author contributions**

Y.L., Q.G., SH.L., ZK.P., and ZN.Y. designed the study, performed the experiments, analyzed the data, and wrote the paper. YPL., SD.Y., ZF.Z., GH.S., FL.G., C.X., XN.Z., FJ.W., and SR.Y., helped with the mouse experiments. YLL. and CC.W helped with single-cell analysis. XB.X. helped with patient sample experiments. WY.G., T.C., H.C., and XX.H. proposed and designed the study, interpreted the results, wrote the manuscript, and oversaw the project.

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Figure 1. scRNA-seq of BM niche cells.

(A) Study procedure. BMT, n = 10; aGVHD, n = 16. (B) t-SNE map of non-hematopoietic cells from BMT and aGVHD mice. Different clusters are colored-coded. (C) Key marker gene expression of niche cells. (D) Ratio of cellular components within BM niche of BMT and aGVHD mice.
Figure 2. scRNA-seq revealed BMSC disruption in aGVHD murine model.

(A) Expression of differentiation-related genes on BMSC. (B) Expression level of niche factors on BMSC. (C) Enrichment of gene ontology (GO) terms in BMSC. (D) Expression of osteoblast differentiation (Bglap) and maturation-related (Sp7) genes in OLC. (E) GO term analysis of significantly downregulated genes in OLC. (F) Graphical summary of niche remodeling under aGVHD. **P < 0.01, ***P < 0.001. The significance of gene expression between the aGVHD and BMT groups was compared using Wilcoxon Rank-Sum test in (A, B and D).
Figure 3. Impaired BM niche in aGVHD murine model.

(A) Overview of murine aGVHD model study. (B) Frequency of leptin-receptor-positive (LepR⁺) cells; n = 10–15 per group. (C) Quantitation of BMSC colony-forming unit fibroblast (CFU-F) assay; three independent replicates. (D and E) Osteogenesis (D) and adipogenesis (E) of BMSC from BMT and aGVHD mice after in vitro induction. Red arrows indicate calcium nodules (D) and yellow arrows indicate fatty droplets (E). (F) Quantitation of Col2.3-GFP⁺ osteoblasts; n = 10–15 per group. (G) Immunofluorescent images of femurs at day 21; three independent replicates. Scale bar, 500 µm. (H and I) High-magnification views of metaphysis areas (H) and diaphysis area (I). White dotted lines represent growth plate and red arrows indicate Col2.3-GFP⁺ osteoblasts. Scale bar, 200 µm. (J) Quantitative analysis of Col2.3-GFP⁺ area vs. DAPI⁺ area in BMT and aGVHD mice. Numbers above columns represent the fold change compared to BMT mice; three independent replicates. (K) Images of calcein-stained femurs; three independent replicates. Scale bar, 50 µm. (L) Immunofluorescent images of adipocytes; three independent replicates. Scale bar, 500 µm. (M and N) High-magnification views of metaphysis (M) and diaphysis (N) areas. Scale bar, 200 µm. (M) Quantitative analysis of perilipin⁺ area vs. DAPI⁺ area. Three independent replicates. **P < 0.01, ***P < 0.001. 1-way ANOVA followed by unpaired t test was used in (B and F). Unpaired t test was performed in C.
Figure 4. Impaired BM niche in aGVHD patients.

(A) Colony forming assay of BMSC from aGVHD patients and allo-HSCT patients without aGVHD; n = 11–18 per group. (B) Representative osteogenesis (upper) and adipogenesis (lower) results; three independent replicates. Arrows indicate calcium nodules (upper) and fatty droplets (lower). (C) Quantification of adipocyte number after in vitro induction; n = 6–8 per group. ***P < 0.001. Unpaired t test was performed in (A and C).
Figure 5. Ruxolitinib enhanced BMSC function in aGVHD mice.

(A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of JAK/STAT signaling in BMSC isolated from BMT and aGVHD mice; n = 30 per group. NES, normalized enrichment score. (B-D) Survival curve (B), body weight (C) and aGVHD score (D) of aGVHD mice after vehicle or ruxolitinib (30 mg/kg in solvent, oral administration, twice a day); n = 6 per group. (E) Alterations to LepR* BMSC frequency after ruxolitinib treatment; n = 4 per group. (F) Osteogenic differentiation assay of BMSC from ruxolitinib or vehicle-treated aGVHD mice; three independent replicates. Scale bar, 200 µm. **P < 0.01, ***P < 0.001. Log-rank test was used in B. 1-way ANOVA followed by unpaired t test was used in (C and D). Unpaired t test was performed in E.
Figure 6. scRNA-seq showed long-term protective effect of ruxolitinib on aGVHD BMSCs.

(A) Procedure for single-cell RNA sequencing during long-term follow-up after ruxolitinib treatment. R 20, day 20 post transplantation (12 h post-ruxolitinib treatment). R 100, day 100 post-transplantation (day 80 post-ruxolitinib treatment). aGVHD, n = 16 per sample; R 20, n = 20 per sample; R 100, n = 10. (B) t-SNE map of niche cells. (C) Pearson’s correlation between the average gene expression profiles of samples. (D) BMSC ratio during long-term follow-up after ruxolitinib treatment. (E and F) Violin plots of expression levels of niche factors (E) and differentiation-related genes (F) in BMSC. (G) GO term enrichment of upregulated genes in ruxolitinib-treated aGVHD BMSC. R 20, day 20 post transplantation. (H) GO term enrichment of ROS metabolic process-related pathway. ROS, reactive oxygen species. (I) Common features of BMSC among samples at different time-points. (J and K) Procedure (J) and survival (K) after intratibial BMSC injection (cultured BMSC, pretreated with vehicle or ruxolitinib); n = 10 per group. ***P < 0.001. Wilcoxon Rank-Sum test was used in (E and F). Log-rank test was used in K.
Figure 7. Ruxolitinib enhanced hematopoietic regeneration by promoting mitochondrial transfer from BMSC to donor-derived HSPC.

(A) Gene set enrichment analysis (GSEA) of the JAK/STAT pathway and critical mitochondrial transfer-related pathway in vehicle- or ruxolitinib-treated aGVHD BMSC. NES, normalized enrichment score. (B) Mitochondrial transfer from recipient BMSCs to donor BM cells (represented by percentage of transplanted BM cells containing host BMSC-derived Dendra2+ mitochondria); n = 3–4 per group. (C) Schematic illustration and percentage of mitochondrial transfer from BMSC to HSPC after in vitro co-culture; n = 6 per group. (D) Relative MitoSox levels in donor-derived HSPCs after transplantation into vehicle- or ruxolitinib-treated aGVHD recipients; n = 3 per group. (E) Strategy for in vitro co-culture and relative MitoSox levels in HSPC after co-culture with BMSC derived from vehicle- or ruxolitinib-treated aGVHD mice; n = 3 per group. *P < 0.05, **P < 0.01, ***P < 0.001. 1-way ANOVA followed by unpaired t test was used in B. Unpaired t test was performed in (C-E).
Figure 8. Ruxolitinib directly modulated aGVHD BMSC function by inhibiting the JAK2/STAT1 pathway.

(A) JAK2, STAT1 and their phosphorylation levels in BMSC derived from BMT, aGVHD and ruxolitinib-treated aGVHD mice. BMT, n = 10; aGVHD, n = 15; aGVHD+R, ruxolitinib-treated aGVHD mice, n = 10. Numbers indicated fold change of protein normalized to BMT.

(B) Inhibition of JAK2/STAT1 pathway in aGVHD BMSC after incubation with different concentration of ruxolitinib for 24 h in vitro; n = 5. p, phosphorylation. R, ruxolitinib. Numbers indicated fold change of protein normalized to control.

(C and D) Jak2 mRNA (C) and protein (D) levels in aGVHD BMSC after transfection with scramble- or Jak2-shRNA. Numbers indicated fold change of protein normalized to scramble (C). (E)
Expression of osteogenesis (*Runx2*) and adipogenesis-related genes (*Ppar*γ) in scramble or *Jak2*-deficient BMSC after ruxolitinib treatment; n = 3–6 per group. (F) Response of BMSC mitochondrial metabolism to ruxolitinib after *Jak2* knockdown; n = 3–9 per group. (G) Relative mitochondrial transfer from *Jak2*-deficient BMSC to HSPC after vehicle or ruxolitinib treatment; n = 7–8 per group. *P < 0.05, **P < 0.01, ***P < 0.001. 1-way ANOVA followed by unpaired t test was used in (E-G).
Figure 9. Ruxolitinib enhanced BMSC function in aGVHD patients via the JAK2/STAT1 pathway.

(A) Hemoglobin levels and platelet counts in aGVHD patients with or without ruxolitinib treatment; n = 7 per group. (B and C) CFU-F assay (B), adipogenesis and osteogenesis (C) of BMSC isolated from aGVHD patients with or without ruxolitinib treatment; n = 3–5 per group. (D and E) In vitro CFU-F ability (D) and differentiation potential (E) of aGVHD BMSC treated with vehicle or ruxolitinib; n = 4–6 per group. (F) Relative cellular ROS
levels in BMSC isolated from aGVHD patients with or without ruxolitinib treatment; n = 3–8 per group. (G) Relative cellular ROS levels in aGVHD BMSC after vehicle or ruxolitinib treatment in vitro; n = 3–4 per group. (H) Inhibition of the JAK2/STAT1 pathway in aGVHD BMSC after incubation with ruxolitinib (1, 5 and 20 μM) for 24 h in vitro. p, phosphorylation. Numbers indicated fold change of protein normalized to control. (I) JAK2 mRNA levels in aGVHD BMSC after transfection with scramble- or JAK2-shRNAs. (J and K) Response of BMSC osteogenesis (J) and mitochondrial metabolism (K) to ruxolitinib treatment after JAK2 knockdown in aGVHD patient-derived BMSC; n = 3 per group. *P < 0.05, **P < 0.01, ***P < 0.001. 1-way ANOVA followed by unpaired t test was used in (J and K). Unpaired t test was performed in (A, B, E, F and G).
Table 1. Characteristics of aGVHD and non-aGVHD patients.

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<tr>
<td>Mismatched</td>
<td>17 (38.6)</td>
<td>14 (41.2)</td>
<td>3 (30.0)</td>
<td></td>
</tr>
<tr>
<td>Conditioning regimen, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIC</td>
<td>9 (20.5)</td>
<td>8 (23.5)</td>
<td>1 (10.0)</td>
<td>0.33</td>
</tr>
<tr>
<td>MAC</td>
<td>35 (79.5)</td>
<td>26 (76.5)</td>
<td>9 (90.0)</td>
<td></td>
</tr>
<tr>
<td>Transplanted cells, median (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+, ×10⁶/kg</td>
<td>4 (1.29–19.92)</td>
<td>5.12 (1.60–19.92)</td>
<td>2.57 (1.29–3.93)</td>
<td>0.0015</td>
</tr>
<tr>
<td>MNC, ×10⁶/kg</td>
<td>10.71 (3.73–37.51)</td>
<td>10.89 (3.73–37.51)</td>
<td>8.96 (7.25–15.00)</td>
<td>0.0995</td>
</tr>
<tr>
<td>aGVHD grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No aGVHD</td>
<td>10 (22.7)</td>
<td>0</td>
<td>10 (100)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>I</td>
<td>1 (2.3)</td>
<td>1 (2.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>12 (27.3)</td>
<td>12 (35.3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Ill–IV</td>
<td>Platelet count, median (range), $\times 10^9$/L</td>
<td>Hemoglobin, median (range), g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------------------</td>
<td>-------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (47.7)</td>
<td>83.5 (39–153)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (61.8)</td>
<td>79 (39–117)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
<td>119.5 (72–153)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Platelet count, median (range), $\times 10^9$/L

- 29.5 (1–252)
- 28 (1–73)
- 55 (23–252)

Hemoglobin, median (range), g/L

- 83.5 (39–153)
- 79 (39–117)
- 119.5 (72–153)

AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CR, complete response; HLA, human leukocyte antigen; RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; MNC, mononucleated cells; NRM, non-relapse mortality; CI, confidence interval.