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ARTICLE

Chronic myeloproliferative neoplasms

Mesenchymal stem cells suppress leukemia via macrophage-mediated functional restoration of bone marrow microenvironment

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Abstract

Bone marrow (BM) mesenchymal stem cells (MSCs) are critical components of the BM microenvironment and play an essential role in supporting hematopoiesis. Dysfunction of MSCs is associated with the impaired BM microenvironment that promotes leukemia development. However, whether and how restoration of the impaired BM microenvironment can inhibit leukemia development remain unknown. Using an established leukemia model and the RNA-Seq analysis, we discovered functional degeneration of MSCs during leukemia progression. Importantly, intra-BM instead of systemic transfusion of donor healthy MSCs restored the BM microenvironment, demonstrated by functional recovery of host MSCs, improvement of thrombopoiesis, and rebalance of myelopoiesis. Consequently, intra-BM MSC treatment reduced tumor burden and prolonged survival of the leukemia-bearing mice. Mechanistically, donor MSC treatment restored the function of host MSCs and reprogrammed host macrophages into arginase 1 positive phenotype with tissue-repair features. Transfusion of MSC-reprogrammed macrophages largely recapitulated the therapeutic effects of MSCs. Taken together, our study reveals that donor MSCs reprogram host macrophages to restore the BM microenvironment and inhibit leukemia development.

Introduction

Mesenchymal stem cell (MSC) therapy has been widely used in treating immune-related graft-vs-host disease and inflammation-related diseases [1, 2]. Over the decades, a lot of evidence demonstrates that MSCs regulate innate and adaptive immune responses largely by secreting distinct sets of cytokines, growth factors, and chemokines depending on different disease contexts [3–7]. Given the short lifespan of donor MSCs after transfusion [8], the underlying molecular and cellular mechanisms by which these cells produce therapeutic effects remain elusive. It is also completely unknown whether donor MSCs can restore the impaired bone marrow (BM) microenvironment and consequently suppress disease progression in leukemia setting.

Macrophages are pivotal for maintenance of the tissue microenvironment, tissue repair, and even the tumor microenvironment [9–13]. BM resident macrophages maintain the homeostasis of HSCs and loss of these macrophages leads to mobilization of HSCs into peripheral blood (PB) [14]. The functions of macrophages are plastic and can be reshaped by distinct sets of soluble factors. When performing tissue repair, macrophages highly express arginase 1 (Arg1) [15], an enzyme that converts L-arginine to urea and L-ornithine. After coculture with MSCs, macrophages can be polarized from proinflammation (M1) to anti-inflammation (M2) type, upregulating IL-10 and CD206 and downregulating IL-6 and IL-1β [16]. Upon

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stimulated by LPS or TNF-α, MSCs can cross-talk with lung macrophages and reprogram these macrophages to secrete IL-10 to alleviate sepsis [17]. Despite these knowledge, whether healthy MSCs can reprogram macrophages from leukemia-bearing host to repair the damaged BM microenvironment is not known.

Using the established mouse model mimicking chronic myeloproliferative neoplasms/myelodysplastic (MPN/MDS) diseases [18–20], we discovered that the deteriorating BM microenvironment was associated with disease progression. Intra-BM instead of systemic transfusion of healthy MSCs restored the local BM microenvironment, improved thrombopoiesis, reduced tumor burden, and prolonged survival of leukemia-bearing mice. Mechanistically, we found that MSCs suppress leukemia development through reprogramming resident macrophages. Our study demonstrates that intra-BM transfusion of MSCs can restore the local BM microenvironment to systemically prevent leukemia progression.

Materials and methods

Mice

All mouse strains were maintained on C57BL/6 genetic background. Mice expressing the conditional oncogenic NrasG12D mutation (a gift from Dr Jing Zhang lab at University of Wisconsin-Madison, WI, USA) were crossed to Vav-Cre mice to generate *LSL Nras*/+; *Vav-Cre* compound mice (NV mice). Genotyping of the adult mice was performed as described previously [18]. Vav-Cre strain (CD45.2), wild-type CD45.2, and CD45.1 strain (C57BL/6) strain were purchased from Jackson lab. GFP strain (CD45.2) was gifted by Guangdong Laboratory Animals Monitoring Institute. All mice were maintained within the SPF grade animal facility of Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (GIBH, CAS, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of GIBH.

NrasG12D leukemia model

White blood cells ($CD45.2^+$, 0.3 million) after depletion of stromal cells from NrasG12D compound mice (*LSL Nras*/+; *Vav-Cre*) or control mice (CD45.2 strain) were sorted and transplanted into sublethally (6.5 Gy, RS2000, Rad Source Inc) irradiated CD45.1 recipient by retro-orbital intravenous injection. Mice were fed with trimethoprim-sulfamethoxazole-treated water for 2 weeks to prevent infection. Hematopoietic lineages in PB were assessed monthly by flow cytometry. During the development of NrasG12D-induced leukemia, the $CD11b^+$ percentage in PB indicated the tumor burden ($CD11b^+$%).

MSC treatment for leukemia-bearing mice

For MSC transfusion, multiple approaches including retro-orbital, tail intravenous, and local intra-BM transfusion were applied independently. For tail vein transfusion, each leukemia-bearing mouse was injected with 2.5×10^7 MSCs/kg (Passage 2) in 100 µl DPBS by tail vein transfusion. For retro-orbital transfusion, each leukemia-bearing mouse was injected with 2.5×10^7 MSCs/kg (Passage 2) in 200 µl DPBS by retro-orbital transfusion. For local intra-BM transfusion, tibia of each leukemia-bearing mouse was injected with 2.5×10^7 MSCs/kg (Passage 2) in 20 µl DPBS by local intra-BM transfusion. MSCs were injected once every 2 weeks and continued in a time window of 16 weeks. Every tibia was treated once per month by switching the injection site every other dose. The control mice were injected with DPBS following the same treatment procedure as MSCs. Analysis of platelets and $CD11b^+$ cells in PB was performed monthly.

Results

Deterioration of BM MSCs accompanies the development of *Nras*-mutant-induced leukemia

Mice carrying an endogenous mutant *Nras* allele develop MDS/MPN-like leukemia with a long latency [18–21]. Here we found the primary BM leukemic cells failed to accelerate the disease in the secondary recipient mice, implying a role of the BM microenvironment in disease etiology (Supplementary Fig. S1A, B). Leukemic MSCs in a mouse T-ALL model suppressed normal hematopoiesis [22]. We hypothesized that the BM microenvironment is impaired by *Nras*-mutant leukemic cells, which in return impedes normal hematopoiesis and accelerates leukemia progression. Indeed, we observed quantitative decreases and functional degeneration of MSCs ($Ter119^-CD45^-CD31^-Sca1^+CD51^+CD146^+$) during disease development and progression (Fig. 1a–c). To further characterize the residual MSCs in mice with leukemia, we performed RNA-Seq analysis of the residual MSCs from leukemia-bearing mice at an early disease phase ($CD11b^+$% in PB: 35–45%). Under leukemia condition, the residual MSCs secreted much less soluble factors, including *Il6, Il11, Ccl2, Ccl7, Cxcl12, Cxcl13*, and *Cxcl14* (padj < 0.05, fold change > 2), compared with MSCs from normal wild-type mice (Supplementary Fig. S1C). Collectively, these results show that the MSCs dramatically deteriorate during the disease development and progression of *Nras*-mutation-caused leukemia.

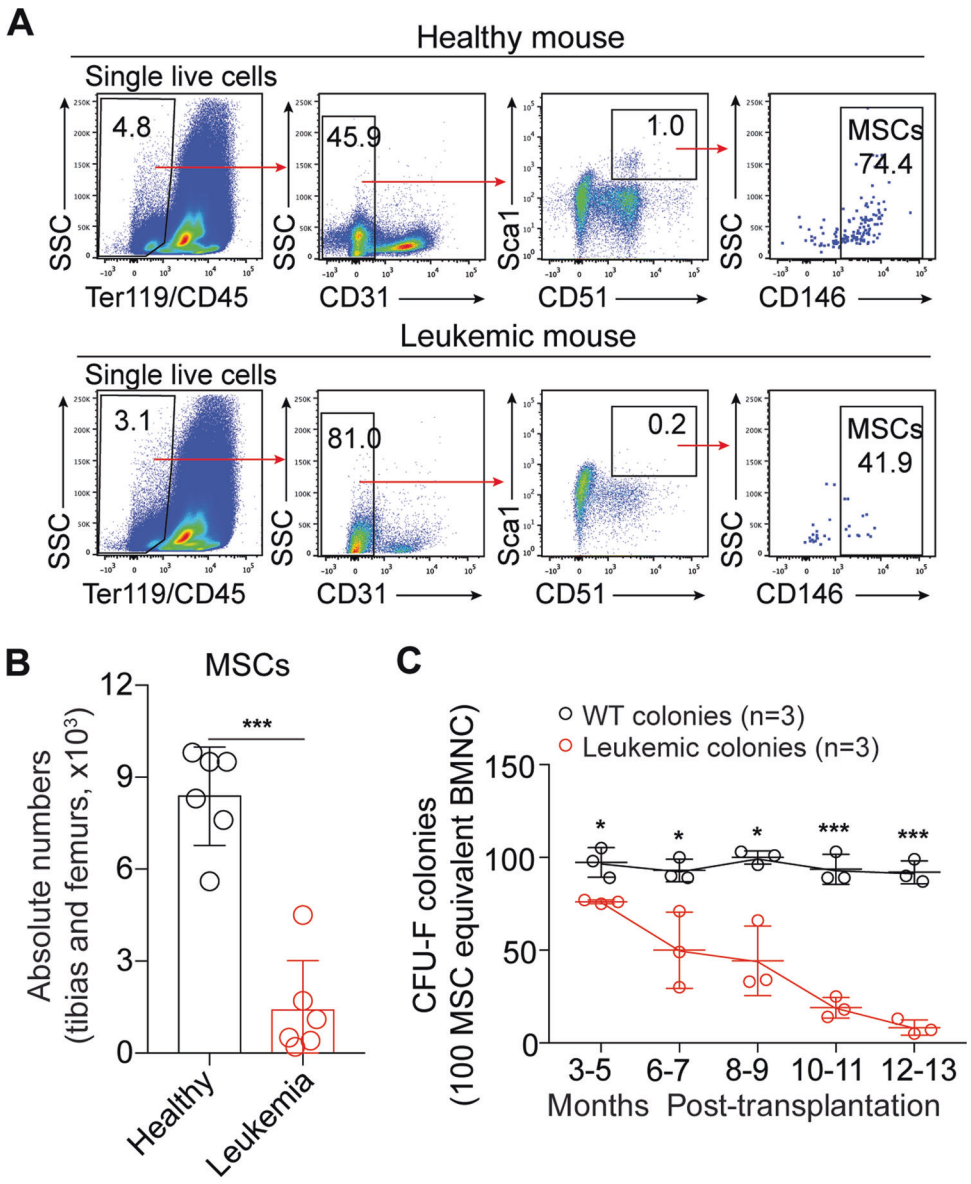

Fig. 1 Impaired bone marrow MSCs in mice with NrasG12D mutation-induced leukemia. **a** Gating strategies for BM MSCs ($Ter119^-CD45^-CD31^-Sca1^+CD51^+CD146^+$). **b** Statistical analysis of the absolute numbers of BM MSCs (mean ± SD, $n = 6$). **c** Kinetic analysis of functional MSCs in CFU-F assay (mean ± SD, $n = 3$). $*p < 0.05$, $***p < 0.001$ (unpaired Student's t test (two tailed)).

Intra-BM transfusion of healthy MSCs improves thrombopoiesis, reduces tumor burden, and improves survival of the leukemia-bearing mice

We hypothesized that restoration of the impaired BM microenvironment in leukemia-bearing mice might suppress/delay the disease progression. To test this hypothesis, we attempted healthy MSC treatment using GFP-tagged MSCs isolated from the tibias and femurs of healthy mice as previously reported [23]. The isolated primary MSCs were expanded shortly in vitro to passage two (P2) and cryopreserved. For MSC treatment, the cryopreserved P2 MSCs were recovered and cultured for 5 days, phenotypically identified ($CD45^-Ter119^-CD31^-CD51^+CD105^+LepR^+PDGFR\alpha^+PDGFR\beta^+Sca1^+$) (Supplementary Fig. S2A), and suspended in DPBS (2.5×10^7/ml) for transfusion. Initially, we adopted a direct delivery procedure by injecting donor MSCs every 2 weeks either via tail vein (dose: 2.5×10^7 MSCs/kg in 100 µl DPBS) (Supplementary Fig. S2B) or retro-orbital (dose: 2.5×10^7 MSCs/kg in 200 µl DPBS) transfusion (Supplementary Fig. S2C) into the leukemia-bearing mice at a late disease phase ($CD11b^+$ cells > 60% in PB). However, these delivery approaches failed to produce therapeutic effects. In vitro cultured MSCs lose their natural homing feature [24], the retro-orbital and intravenous transfusion of cultured MSCs failed to home to BM (Supplementary Fig. S2D, E). Thus, we attempted intra-BM transfusion to overcome the homing defect caused by in vitro culture. Sequential doses of MSCs (2.5×10^7 MSCs/kg per dose in 20 µl DPBS) were injected into the tibia cavities of leukemia-bearing mice with 2-week

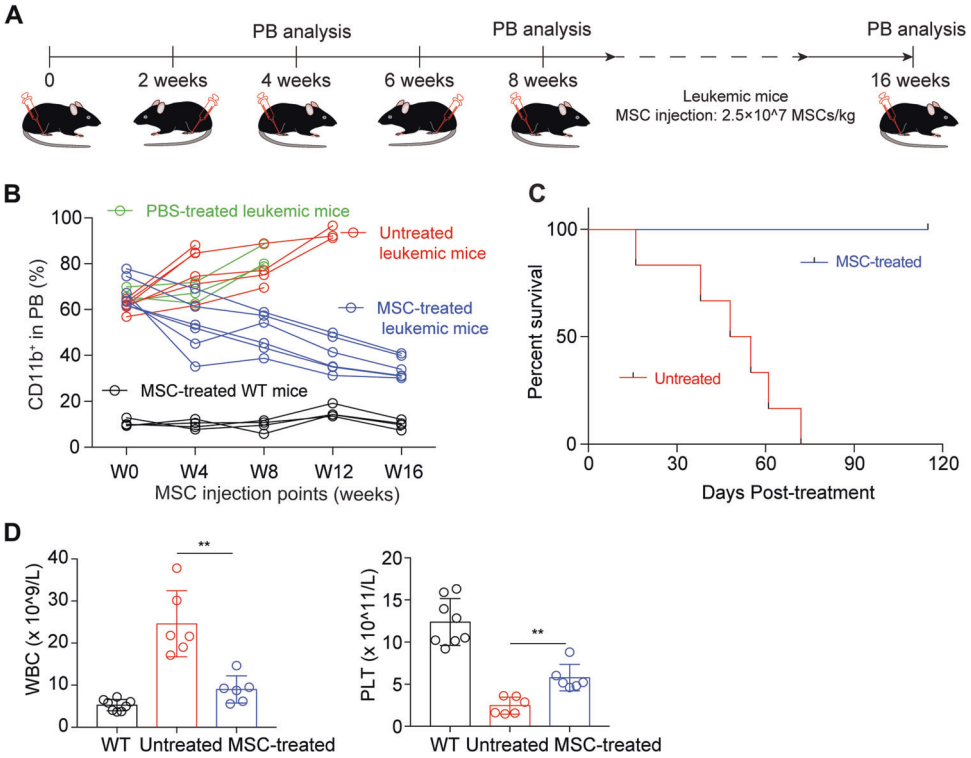

Fig. 2 Intra-BM transfusion of donor MSCs prolongs survival of leukemia-bearing mice. a Schematic diagram of MSC transfusion strategy. **b** Kinetic analysis of tumor burden ($CD11b^+$) of MSC-treated leukemia-bearing mice. MSC-treated leukemia-bearing mice (blue line): $n = 6$. Untreated leukemia-bearing mice were used as disease control ($n = 6$; red line), PBS-treated leukemia-bearing mice were used as injected control ($n = 3$; green line), and MSC-treated WT mice were used as treatment control ($n = 4$; black line). **c** Kaplan–Meier survival of MSC-treated leukemia-bearing mice. Kaplan–Meier survival curves of untreated (red line, $n = 6$, median survival = 51.5 days) and MSC-treated (blue line, $n = 6$, median survival = 115 days) leukemia-bearing mice are shown (log-rank (Mantel–Cox) test: $p < 0.001$). MSC treatment was terminated after 16 weeks. **d** Statistical analysis of white blood cells (WBC) and platelets (PLT) counts in PB (mean ± SD, $n = 6$–8).

intervals for up to 16 weeks (Fig. 2a). Strikingly, the tumor burden continuously decreased during MSC treatment (Fig. 2b). Consequently, the survival of MSC-treated leukemia-bearing mice was significantly prolonged (Untreated: 51.5 days, MSC treated: >115 days, $p < 0.001$) (Fig. 2c). Therefore, intra-BM transfusion of healthy donor MSCs improves the survival of leukemia-bearing mice.

MSC treatment systemically rebalances myelopoiesis and activates megakaryopoiesis

We next investigated the underlying mechanisms associated with the systemically decreased tumor burden. We found that the hematopoiesis in the MSC-treated leukemia-bearing mice was rebalanced, demonstrated by significant decreases of white blood cells (untreated vs. MSC treated: 23.04 vs. 8.876, $p = 0.009$), and significant elevation of platelets (untreated vs. MSC treated: 2.64 vs. 6.01, $p = 0.004$) (Fig. 2d) in PB. On the contrary, the PBS-treated leukemia-bearing mice exhibited neither improved hematopoiesis (Supplementary Fig. S2F) nor prolonged survival. Collectively, these results indicate that intra-BM transfusion of healthy donor MSCs systemically improves hematopoiesis and prolongs the survival of leukemia-bearing mice.

To further investigate the systemic effects of the local MSC treatment on hematopoiesis in leukemia-bearing mice, we analyzed the ratios of myeloid progenitor subpopulations in MSC-treated leukemia-bearing mice. Consistent with the elevated platelet levels and reduced myeloid cells in PB, the MSC-treated leukemia-bearing mice showed increased proportions of megakaryocyte-erythroid progenitors (>1.6 folds) ($p < 0.001$) and decreased ratios of granulocyte-macrophage progenitors (>1.5 folds) ($p < 0.001$) in both injected and noninjected sites than those sites in PBS-treated leukemia-bearing mice (Supplementary Fig. S3A, B). In addition, we observed increased (>1.3 folds) ratios of mature megakaryocytes (≥8N) in both injected and noninjected sites in MSC-treated leukemia-bearing mice (Supplementary Fig. S3C, D) in comparison with PBS-treated leukemia-bearing control mice ($p < 0.001$). Thus, these data demonstrate that MSC treatment systemically rebalances myelopoiesis and activates megakaryopoiesis in leukemia-bearing mice.

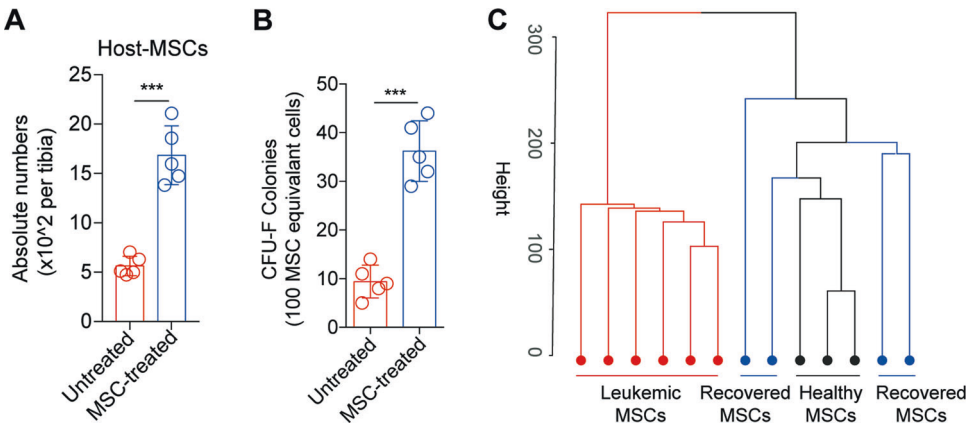

Fig. 3 Characterization of recovered host MSCs from MSC-treated leukemia-bearing mice. a Statistical analysis of the absolute numbers of host MSCs (GFP$^-$, host-derived MSCs) in tibias from untreated and MSC-treated leukemia-bearing mice (mean ± SD, $n = 5$). **b** Statistical analysis of CFU-F colonies (mean ± SD, $n = 5$). **c** Unsupervised hierarchical clustering of RNA-Seq data of healthy MSCs, leukemic MSCs, and recovered MSCs (GFP$^-$, recipient derived) ($n = 3$–6). ***$p < 0.001$ (unpaired Student's t test (two tailed)).

Recovered host MSCs are functional as healthy counterparts

To investigate whether the improved hematopoiesis is associated with restoration of the BM microenvironment, we analyzed the MSC-treated tibias 8 weeks after MSC treatment. Interestingly, host MSCs (GFP negative) were partially recovered (Fig. 3a), but restricted to the locally treated tibias (Supplementary Fig. S4A, B). Functionally, the recovered host MSCs formed markedly more CFU-F colonies than the residual MSCs from untreated leukemia-bearing mice (>3.8 folds) ($p < 0.001$) (Fig. 3b). To characterize the recovered MSCs at the transcriptome level, we sorted the recovered MSCs for RNA-Seq analysis. Unsupervised hierarchical clustering analysis showed that the recovered MSCs clustered closer to healthy MSCs (Fig. 3c). Further, the expression of cytokines and chemokines, including *Il6*, *Ccl2, Ccl7, Ccl19, Cxcl12, Cxcl13*, and *Cxcl14*, was restored in the recovered MSCs compared with that in MSCs from untreated leukemia-bearing control mice (padj < 0.05, fold change > 2) (Supplementary Fig. S4C). Therefore, donor MSC treatment results in local functional restoration of host MSCs.

The donor MSCs reprogram macrophages to execute tissue-repair function

We further investigated the cellular mechanism underlying the restored BM microenvironment mediated by donor MSCs under leukemia condition. BM macrophages play a pivotal role in maintaining the BM niche [9]. To study whether donor MSCs reprogram BM macrophages, we performed coculture assay of healthy MSCs with BM macrophages (L-Mac) sorted from the leukemia-bearing mice in vitro for 12 h and resorted the macrophages (E-Mac) for RNA-Seq analysis. GSEA illustrated that angiogenesis-related genes, including *Vegfa, Hif1a, Serpine1, Eng, and Thbs1* [25], were enriched among the differentially expressed genes in E-Mac (Fig. 4a and Supplementary Fig. S5A). Genes associated with cell migration, including *Sirpa* [26], were also enriched in E-Mac (Fig. 4a and Supplementary Fig. S5B). Further, gene-ontology analysis demonstrated features of positive regulation of cell migration and angiogenesis in E-Mac (Supplementary Fig. S5C). RNA-Seq analysis showed that the expression of Arg1, an indicator of tissue-repair function [15], was dramatically upregulated over thousand folds in E-Mac (Fig. 4b) after direct coculture with MSCs in vitro. However, the *Arg1* expression in macrophages after transwell coculture was barely elevated (Fig. 4b), indicating that direct cell–cell interaction instead of MSC-secreted soluble factors is essential for the functional reprogramming. Consistent with the observation in vitro, the expression of *Arg1* was also significantly increased in BM macrophages directly isolated from MSC-treated leukemia-bearing mice (Fig. 4c). Furthermore, intracellular flow cytometry staining confirmed that the ratios of Arg1^{high} macrophage subpopulation significantly increased in E-Mac at the MSC-treated sites ($p < 0.01$) (Fig. 4d, e). Collectively, these results indicate that the donor MSCs reprogram BM macrophages from leukemia-bearing mice to execute tissue-repair function.

The E-Mac treatment largely recapitulates the therapeutic effects of MSC treatment

Given the short lifespan of donor MSCs in vivo [8], we speculated that MSCs mediate the restoration of the BM microenvironment of leukemia-bearing mice by reprogramming macrophages. We isolated macrophages from leukemia-bearing mice and cocultured them with healthy MSCs for 12 h, and then transplanted these E-Mac back into

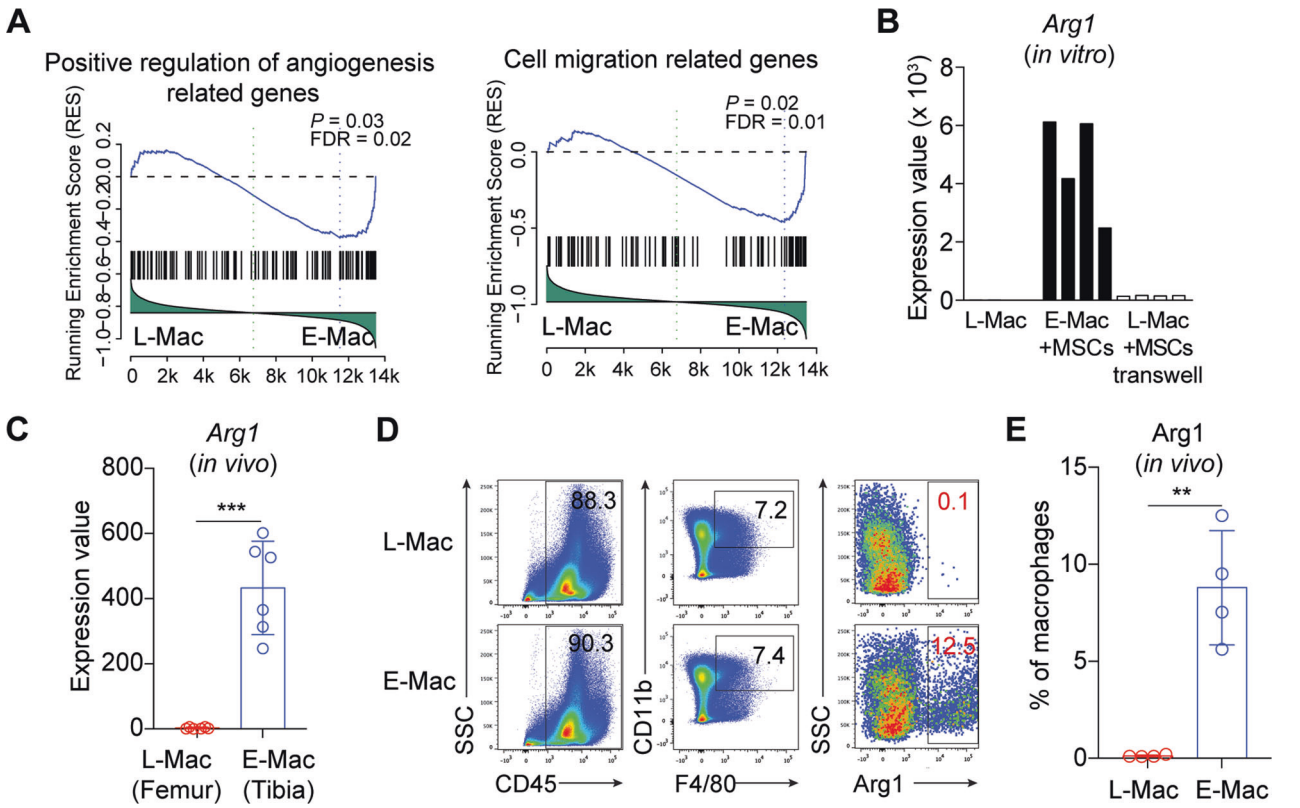

Fig. 4 Characterization of MSC-reprogrammed BM resident macrophages isolated from leukemia-bearing mice. a Gene set enrichment analysis (GSEA) of the positive regulation of angiogenesis and cell migration-related genes in L-Mac and E-Mac. L-Mac indicates leukemic macrophages. E-Mac indicates MSC-reprogrammed leukemic macrophages, which were cocultured with MSCs in vitro for 12 h. **b** RNA-Seq analysis of *Arg1* in L-Mac and E-Mac in vitro. **c** RNA-Seq analysis of *Arg1* in leukemic macrophages sorted from MSC-treated leukemic mice in vivo. **d** Representative intracellular staining plots of Arg1 proteins in L-Mac and E-Mac. **e** Statistical analysis of the ratios of $Arg1^{high}$ macrophage subpopulation in L-Mac and E-Mac (mean ± SD, $n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired Student's *t* test (two tailed)).

leukemia-bearing mice by intra-BM transfusion (Fig. 5a). We indeed found that the thrombopoiesis was significantly improved (>6 folds) after E-Mac treatment ($p < 0.001$) (Fig. 5b, c). Host MSCs were also significantly increased (>3 folds) in E-Mac-treated leukemia-bearing mice ($p < 0.001$) (Fig. 5d, e). Collectively, these results demonstrate that MSC-reprogrammed macrophages largely recapitulate the therapeutic effects of MSCs.

Discussion

Deteriorating BM microenvironment accompanies chronic leukemia progression. Here we unravel a de novo approach of reverting the impaired BM microenvironment by intra-BM injection of donor MSCs. Upon injection, the donor MSCs quickly reprogrammed local host BM macrophages to repair the niche, thus improving normal hematopoiesis and suppressing leukemia development. Our studies reveal de novo mechanisms underlying MSC-mediated local BM microenvironment restoration that systemically suppress leukemia development.

Given the short-term lifespan of the exogenous MSCs in vivo, it is surprising that local injection of donor MSCs results in long-term improvement of thrombopoiesis and reduction of tumor burden. Following injection, exogenous donor MSCs immediately reprogram host resident macrophages that further organize the overhaul of local BM microenvironment, including restoring the functions of host MSCs. There is a lot of evidence supporting the pivotal roles of macrophages in tissue repair [27, 28]. Donor MSCs can transiently release a key wave of tissue-repair factors, such as CCL7 [29] and CXCL12 [30], and reprogram host macrophages, subsequently resulting in the recovery of host MSCs. Recovered host MSCs further secreted much higher level of CCL7 and CXCL12 that can further facilitate BM niche repair. Donor MSCs could also directly modulate the other niche cells, in addition to macrophages, to participate in BM niche repair [3]. Consequently, the restored local BM microenvironment outputs abundant hematopoiesis-improving cytokines. Thus, despite the short lifespan, donor MSCs provide long-term thrombopoiesis improvement and tumor burden reduction through the stepwise microenvironment restoration.

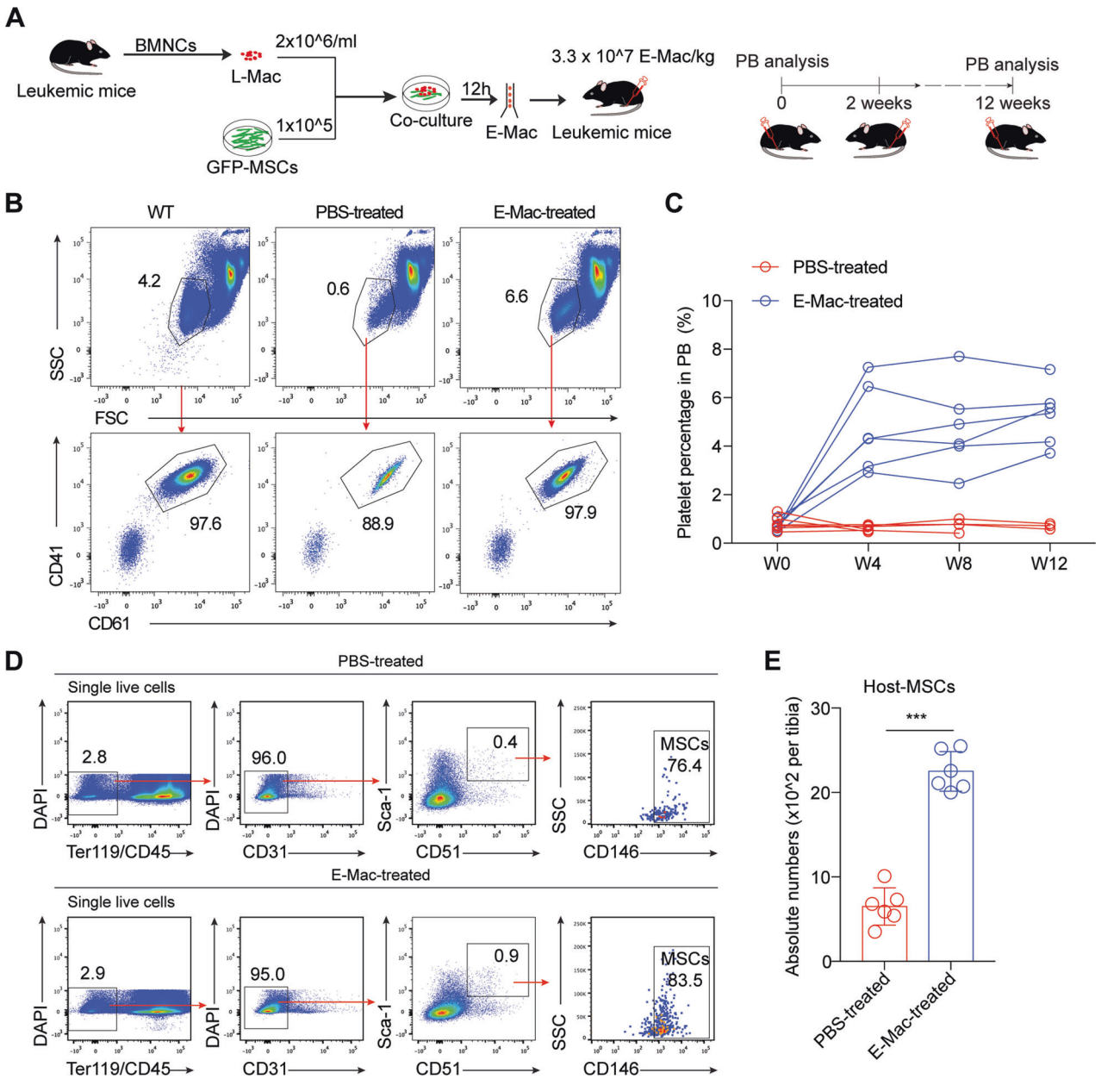

Fig. 5 Intra-BM transfusion of MSC-reprogrammed macrophages largely rescues the therapeutic effects of MSC treatment in leukemic mice. a Schematic diagram of MSC-reprogrammed macrophages transfusion strategy. **b** Representative dot plots of platelet populations and quantitative gating. **c** Kinetic analysis of platelets in PB of leukemia-bearing mice treated with PBS or E-Mac ($n = 6$). **d** Flow cytometry analysis of MSCs in leukemia-bearing mice post PBS/E-Mac treatment. **e** Statistical analysis of the absolute numbers of host MSCs in tibias from PBS/E-Mac treated leukemic mice (mean ± SD, $n = 6$). $***p < 0.001$ (unpaired Student's t test (two tailed)).

Of note, reprogramming host macrophages by MSCs is required for MSC-mediated microenvironment restoration and leukemia inhibition. The molecular features involved in angiogenesis and cell migration signaling demonstrated by MSC-reprogrammed macrophages suggest that the impaired BM macrophages might be therapeutic targets for improving normal hematopoiesis and suppressing leukemia in MDS/MPN patients with NRAS mutations.

In addition, many studies have reported that MSCs-derived extracellular vesicles are being examined for their biological effects in MSC-based cellular therapy. For example, in models of kidney regeneration, biological effect of systemic delivery of MSCs could be replaced by systemic infusion of microparticles/exosomes isolated from MSCs [31, 32]. Thus, to further investigate the potential mechanisms, it is worth testing the potential therapeutic effects of systemic transfusion of MSCs-derived microparticles/exosomes in NRAS mutant leukemia.

MSC treatment inhibits leukemia development in the *Nras*-mutation-induced MPN/MDS-like disease model. We

also attempted to broaden MSC treatment for acute leukemia in the MLL-AF9-initiated model (Supplementary Fig. S6A), in which impaired MSCs results in the reduction of osteogenesis and CXCL12 production [33]. Despite a mild elevation of platelet level, the intra-BM transfusion of donor MSCs failed to significantly improve normal hematopoiesis or suppress acute leukemia development (Supplementary Fig. S6B–H). Therefore, the intra-BM MSC treatment might be beneficial for MPN/MDS leukemias, such as JMML and CMML, but insufficient for suppressing acute leukemia.

In conclusion, we establish a novel approach of reverting the deteriorated BM microenvironment by intra-BM transfusion of healthy MSCs under leukemia condition. This study also unveils de novo mechanisms underlying MSC-mediated local BM microenvironment restoration that systemically suppress leukemia development.

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Author contributions CXX performed research, analyzed data, and wrote the manuscript; YD and QTW analyzed RNA-Seq data; TJW, HC, PQZ, KTW, XFL, YG, SHM, LX, and YXG performed experiments; SH, JD, XD, YQL, XFZ, YFS, and SX discussed the manuscript; DW discussed the project and wrote the manuscript; and TC and JYW designed the research and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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