Impaired immunosuppressive role of myeloid-derived suppressor cells in acquired aplastic anemia

by Peiyuan Dong, Lingyun Chen, Hongfei Wu, Jiali Huo, Zhongxing Jiang, Yingqi Shao, Xiang Ren, Jinbo Huang, Xingxin Li, Min Wang, Neng Nie, Jing Zhang, Peng Jin, Yizhou Zheng, and Meili Ge

Received: November 2, 2021.
Accepted: June 13, 2022.

Citation: Peiyuan Dong, Lingyun Chen, Hongfei Wu, Jiali Huo, Zhongxing Jiang, Yingqi Shao, Xiang Ren, Jinbo Huang, Xingxin Li, Min Wang, Neng Nie, Jing Zhang, Peng Jin, Yizhou Zheng, and Meili Ge. Impaired immunosuppressive role of myeloid-derived suppressor cells in acquired aplastic anemia. Haematologica. 2022 June 23. doi: 10.3324/haematol.2021.280292. [Epub ahead of print]

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Impaired immunosuppressive role of myeloid-derived suppressor cells in acquired aplastic anemia

Peiyuan Dong¹²#, Lingyun Chen¹#, Hongfei Wu¹, Jiali Huo¹, Zhongxing Jiang², Yingqi Shao¹, Xiang Ren¹, Jinbo Huang¹, Xingxin Li¹, Min Wang¹, Neng Nie¹, Jing Zhang¹, Peng Jin¹, Yizhou Zheng¹, Meili Ge¹*

¹State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, 300020, China
²Department of Hematology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, China

# Peiyuan Dong and Lingyun Chen contributed equally.

Running heads: Myeloid-derived suppressor cells in aplastic anemia

Corresponding author:
Meili Ge, M.D., Ph.D., State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, 300020, China
Tel: 86-022-23909121.
FAX: 86-022-23909093.
E-mail: gemeili503@126.com

Author Contributions
J.-b.H, X.-x.L, M.W, N.N, J.Z and P.J contributed to the clinical data collection, and sample preparation. J.-l.H, Y.-z.Z and M.-l.G revised the manuscript. All authors made significant contributions to, reviewed, and approved the final version of the manuscript.

**Conflict of Interest Statement**

None.

**Data sharing statement**

All data generated and/or analyzed in this study are included in this published article and its supplementary files. Meanwhile, the datasets used and analyzed during the current study are also available from the corresponding author on reasonable request.

**Word count**

The category of the manuscript: original article

The main text’s word count: 3437

The abstract’s word count: 207

The figures count: 7

The tables count: 0

Supplementary files:1

**Acknowledgement**

The authors would like to thank all the doctors and nurses in the Therapeutic Centre of Anemic Diseases and the researcher team of the Clinical Laboratory Centre for their professional assistance. This work was supported by grants from the National Natural Science Foundation of China (No.81700120, No.81770119 and No.81970104) and the Haihe Laboratory of Cell Ecosystem Innovation Fund (No. HH22KYZX0041).
Abstract

Myeloid-derived suppressor cells (MDSCs) are a group of heterogeneous immature myeloid cells and display immunosuppressive function. In this study, MDSCs populations were evaluated in acquired aplastic anemia (AA) (n=65) in which aberrant immune mechanisms contributed to bone marrow destruction. Our data demonstrate that both the proportion and immunosuppressive function of MDSCs are impaired in AA patients. Decreased percentage of MDSCs, especially monocytic-MDSCs, in the blood of AA patients (n=15) is positively correlated with the frequency of T regulatory cells, bone marrow level of WT1 and decreased plasma level of arginase-1. RNA sequence analyses reveal that multiple pathways including DNA damage, interleukin (IL)-4, apoptosis, and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) are upregulated, whereas transcription, IL-6, IL-18, glycolysis, transforming growth factor and reactive oxygen species are downregulated in MDSCs of AA (n=4), compared with that of healthy donors (n=3). These data suggest that AA MDSCs are defective. Administration of rapamycin significantly increases the absolute number of MDSCs and levels of intracellular enzymes, including arginase-1 and inducible nitric-oxide synthase. Moreover, rapamycin inhibits MDSCs from differentiating into mature myeloid cells. These findings reveal that impaired MDSCs are involved in the immunopathogenesis of AA. Pharmacologically targeting of MDSCs by rapamycin might provide a promising therapeutic strategy for AA.

Key words: aplastic anemia, myeloid-derived suppressor cells, immunosuppression, RNA sequence analysis, rapamycin
Introduction

Myeloid-derived suppressor cells (MDSCs), derived from myeloid cells, are a group of heterogeneous cells featured by immature state and inhibiting T cells mediated immune response, which expand during cancer, infection and some autoimmune diseases. Based on phenotypic and morphological features, MDSCs are classified into two major subsets: polymorphonuclear (PMN) and monocytic (M)-MDSCs. Recently, a novel small group of MDSCs that comprised more immature progenitors were defined as “early-stage MDSCs” (eMDSCs). Multiple lines of evidence indicate that the suppressive activity of MDSCs has been associated with the expression of interferon (IFN)-γ and the metabolism of L-arginine. L-arginine is the substrate for two enzymes: arginase (Arg)-1, which converts L-arginine into urea and L-ornithine, and nitric oxide synthase 2/inducible nitric-oxide synthase (NOS2/iNOS), which generates nitric oxide (NO). NO and shortage of L-arginine could suppress T-cell function through a variety of mechanisms including the inhibition of JAK3-STAT5 pathway, the induction of apoptosis and the restraint expression of CD3ξ. MDSCs could also promote M2 macrophage polarization and T regulatory cell (Treg) induction, probably through IL-10. In tumors, M-MDSCs could rapidly differentiate into tumor-associated macrophages.

Acquired aplastic anemia (AA) is an immune-mediated bone marrow failure syndrome, in which activated cytotoxic T cells and intrinsically impaired Tregs are involved. Moreover, innate immune cells such as dendritic cells and macrophages also contribute to the pathological mechanism of AA.

In the steady state, immature myeloid cells didn’t display immune inhibitory functions. Under pathologic conditions such as inflammation, cancer and autoimmune diseases, populations of immature myeloid cells are expanded and converted into immunosuppressive MDSCs. Nevertheless, little is known about the role of MDSCs in AA. In this study, we discovered that the impairment of MDSCs played a role in the pathogenesis of AA. Furthermore, numerous genes associated with apoptosis, JAK3/STAT5 and abnormal immune-related genes were found differentially expressed in AA MDSCs. Our results provided novel insights into a possible mechanism of AA.

Rapamycin has been successfully applied to AA patients in clinical therapy. Previous literature confirmed that rapamycin expanded Treg and inhibited CD8+ T-cell function in AA. In mice, rapamycin significantly induced MDSCs expansion and enhanced their immunosuppressive function. Nevertheless, little has been defined about the precise role of rapamycin on MDSCs in patients with AA. Our data elucidate that rapamycin could expand MDSCs and restore their function in vitro.

Methods

Patients

Sixty-five acquired AA patients (41 severe AA and 24 non-severe AA; age, 13-70 years) and twenty-eight age-matched healthy donors (HDs) (12 male and 16 female; age, 17-68 years) were included after signing written informed consent which was approved by the Medical Ethics Committee of Institute of Hematology & Blood
Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College in accordance with the Declaration of Helsinki (KT2017031-EC-2). The characteristics of patients were listed in supplemental Table 1. All patients were newly diagnosed. The diagnosis and disease severity classification were abided by Camitta criteria. Cases complicated with active infection, pregnancy and other autoimmune diseases were excluded.

**Flow cytometric analysis**
Peripheral blood mononuclear cells (PBMNCs) were isolated through Ficoll-paque PLUS reagent (GE Healthcare, Sweden) centrifugation and were analyzed within 6h after collection. The phenotype of MDSCs was analyzed for the cell surface markers, including CD33, CD11b, human leukocyte antigen-D-related (HLA-DR), CD14, CD15 and lineage-specific markers (Lin), as described in Supplemental Data. This marker combination allows the identification of MDSCs (CD33+CD11b+HLA-DR−) and three MDSC subsets: PMN-MDSCs (CD33+CD11b+HLA-DR+CD15+), M-MDSCs (CD33+CD11b+HLA-DR-CD14+) and eMDSCs (CD33+CD11b+HLA-DR-Lin−). Intracellular expression of Arg-1 and iNOS were also determined as described before. All stained cells were detected by a FACS Canto II flow cytometer (BD Biosciences) and data were analyzed with FlowJo V10 (BD Biosciences).

**Cytometric bead array (CBA) for cytokines**
Plasma levels of interferon (IFN)-γ, Arg-1, tumor necrosis factor (TNF)-α and interleukin (IL)-10 in AA patients and HDs were quantitatively detected using CBA kits (Biolegend, San Diego, CA, USA), according to manufacturer’s instructions.

**MDSCs isolation**
HLA-DR+ cells were removed from PBMNCs by a negative selection using HLA-DR microbeads according to the manufacture’s protocols (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by further isolation of CD14+HLA-DR+flow cells by positive selection with anti-CD14 microbeads (Miltenyi Biotec). The purity of the CD14+HLA-DR+low cell population was >85%, as detected by flow cytometry.

**T cell proliferation and activation assay**
CD3+T cells were isolated from PBMNCs of HDs by anti-CD3 microbeads (Miltenyi Biotec) and labeled with CellTrace™ Violet Cell Proliferation kit (5μM; Invitrogen, Waltham, USA). Isolated MDSCs were co-cultured with allogeneic CD3+T cells for 72 h at ratios of 1:32, 1:16, 1:8, 1:4, 1:2 or 1:1 in the presence of anti-CD3/anti-CD28 Dynabeads® (Gibco, Grand Island, USA). Cells were then stained with APC-Cy7-conjugated anti-human CD3, PerCP-Cy5.5-conjugated anti-human CD4 and APC-conjugated anti-human CD8 (Biolegend) antibodies. The proliferation of CD3+, CD3+CD4+ or CD3+CD8+T cells was evaluated by flow cytometry. For activation assay, cells were stained with APC-Cy7-conjugated anti-human CD3, FITC-conjugated anti-human CD69 and PE-conjugated anti-human CD25 (Biolegend).
antibodies.

**Th1 cells induction in vitro**

CD4+ T cells were isolated from PBMCs of HDs by anti-CD4 microbeads (Miltenyi Biotec) and were seeded into 96 well culture plates at a density of 1×10^5 cells per well. CD4+ T cells were stimulated with plate-bound anti-human CD3 (OKT-3, Biolegend) and soluble anti-human CD28 mAb (CD28.2, Biolegend) in the presence of MDSCs at a ratio of 2:1 for 4 days. The cells were further stimulated with PMA (50ng/ml, Sigma-Aldrich), ionomycin (1μg/ml, Sigma-Aldrich) and 0.4μL BD GolgiStop™ Protein Transport Inhibitor (BD Biosciences) for 5 hours and then stained intracellularly with FITC-conjugated anti-human IFN-γ and PE-conjugated anti-human IL-4 (Biolegend) antibodies.18

**RNA sequencing and functional annotation analyses**

MDSCs from AA patients and HDs were isolated as described before. Both RNA extraction and sequencing were undertaken at Novogene Inc, as previously reported.19 The construction of heatmaps, volcano plot analysis, principal component analysis (PCA), gene ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on NovoMagic platform (https://magic.novogene.com). Protein-protein interaction networks (PPI) of differentially expressed genes were conducted on the STRING database (https://string-db.org/). The selected signaling pathways were mapped on KEGG website (https://www.kegg.jp). Gene set enrichment analysis (GSEA) was performed on GSEA 4.1.0 software (GSEA, Inc., Massachusetts, USA).

**The function of rapamycin on the generation of human MDSCs in vitro**

Both PBMCs from HDs and AA patients were cultured in RPMI Medium 1640 containing 10% fetal Bovine Serum (Gibco, Thornton, Australia) and 1% glutamine (Gibco, Thornton, Australia) for 6 days. Each culture was supplemented with recombinant human IL-6 (10ng/ml, Peprotech, Rocky Hill, USA) and granulocyte-macrophage–colony-stimulating factor (GM-CSF) (10ng/ml, Peprotech, Rocky Hill, USA) in the presence or absence of rapamycin.17 Adherent cells were harvested using cell detachment solution ACCUTASE™ (Stemcell, Canada). Cell surface and intracellular markers of MDSCs were analyzed as described before. To determine MDSCs differentiation, cells were then stained with BV421-conjugated anti-human CD80 and PerCP-Cy5.5-conjugated anti-human CD86 (Biolegend) antibodies.

**Statistical analysis**

All results were expressed as mean ± standard deviation of the median (range). Data were analyzed with SPSS 20.0 (SPSS, Inc., Chicago, USA) statistical software. An unpaired student’s t test was performed to compare the two independent groups. For non-normally distributed data, Mann-Whitney U was used for analysis. P<0.05 was considered statistically significant.
Results

Decreased MDSCs in the peripheral blood of AA patients

To examine MDSCs proportion in the peripheral blood (PB) of AA patients and HDs, the cell surface markers of CD33, CD11b, HLA-DR, CD14, CD15 and Lin on PBMNCs were determined. Results showed that the percentage of CD33+CD11b+HLA-DR-MDSCs in PB was significantly decreased in AA patients compared with that in HDs. However, there was no difference between patients with SAA and NSAA (Figure 1A, B, Supplemental Table 2). CD33+CD11b+HLA-DR-MDSCs were further divided into CD15+ PMN-MDSCs, CD14+ M-MDSCs and Lin+ eMDSCs. Compared with HD, M-MDSCs in AA were significantly decreased (Figure 1A, C), whereas PMN-MDSCs and eMDSCs in AA were only slightly declined (Supplemental Figure1, Supplemental Table 2).

The level of intracellular Arg-1 and iNOS in circulating CD33+CD11b+HLA-DR-MDSCs was analyzed by flow cytometry. The mean fluorescence intensity (MFI) of Arg-1 and iNOS were significantly lower in AA MDSCs compared with HD MDSCs (3154.25±1472.09 vs 1696.00±403.74, P=0.037, Figure1D) and (4611.13±1160.01 vs 2519.33±403.02, P=0.001, Figure1E).

Compared with newly diagnosed AA patients, not only percentage of MDSCs, but also MFI of Arg-1 and iNOS were elevated (Supplemental Figure1) in patients with partial or complete response. Thus, after treatment, both quantity and function of AA MDSCs were improved.

Relationship between MDSCs and clinical characteristics of AA

The percentage of MDSCs was higher in male patients with AA than that in females (1.20±1.05% vs 0.57±0.63%, P=0.106, Figure 2A), while it was independent of age (Supplemental Figure 2A). WT1, mostly expressed in CD34+ HSPCs, was reported as a surrogate marker of cell proliferation.20, 21 Our team previously confirmed that WT1 was positively associated with disease severity and clinical outcomes in AA patients.22 In this study, we discovered that percentages of MDSCs, especially M-MDSCs and eMDSCs, were positively correlated with WT1 level (Figure 2B, Supplemental Figure 2B, C).

Serum levels of TNF-α, IL-10 and IFN-γ were higher in AA patients compared with HDs (11.88±4.02 pg/ml vs 4.02±3.24 pg/ml, P=0.001; 2.25±1.43 pg/ml vs 0.74±0.58 pg/ml, P<0.001 and 95.09±63.63 pg/ml vs 20.09±30.35 pg/ml, P<0.001, Supplemental Figure 2D-F), whereas Arg-1 level was lower in AA patients (8.54±6.96 ng/ml vs 29.87±16.87 ng/ml, P<0.001; Figure 2C). In addition, decreased level of Arg-1 was positively correlated with MDSC proportion (Figure 2D). However, there was no significant relationship between MDSC proportion and TNF-α, IL-10 or IFN-γ levels (Data not shown). It was reported that MDSCs could inhibit T cell proliferation and induce Treg expansion.1, 7 In this study, we found that MDSC proportion was positively correlated with the frequency of Treg, while it was negatively associated with the frequency of CD8+ T cells (Figure 2E, F).

Decreased immunosuppressive functions of MDSCs in AA patients
To examine the capacity of MDSCs in inhibiting T cell proliferation, we cocultured MDSCs with CellTrace\textsuperscript{TM} Violet Cell Proliferation kit-labeled CD3\(^+\)T cells at ratios of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 or 1:64 in the presence of anti-CD3/anti-CD28 Dynabeads for 3 days. MDSCs could significantly suppress CD3\(^+\)T cell proliferation at a ratio of 1:1 and 1:2. As the ratio of MDSCs to CD3\(^+\)T cells decreased, the immunosuppressive functions of MDSCs declined (Figure 3A, B). In the following experiments, MDSCs were cocultured with CD3\(^+\)T cells at a ratio of 1:2. As expected, AA MDSCs strongly inhibited the production of IFN-\(\gamma\) in CD3\(^+\)T cells (10.67±2.28\% vs 2.83±0.82\%, \(P=0.02\), Figure 3C).

MDSCs show a prominent ability to suppress T-cell responses mediated in part by the secretion of Arg-1.\(^1\) Previously, we have demonstrated that the intracellular level of Arg-1 was significantly lower in AA MDSCs compared to HD MDSCs. To further evaluate the immunosuppressive functions of MDSCs, CD3\(^+\)T cells were co-cultured with MDSCs from HDs and AA patients separately. Compared to HD MDSCs, the inhibitory capacities of AA MDSCs on proliferation of CD3\(^+\)T, CD3\(^+\)CD4\(^+\)T and CD3\(^+\)CD8\(^+\)T cells were strikingly impaired (81.71±4.18\% vs 34.35±11.67\%, \(P=0.009\); 85.59±4.62\% vs 43.66±19.60\%, \(P=0.002\); and 75.39±7.10\% vs 51.07±13.86\%, \(P=0.008\), respectively; Figure 3D-F). Moreover, the ability of AA MDSCs to inhibit CD3\(^+\)T cells activation was also decreased compared to HD MDSCs (CD25: 53.71±16.33\% vs 81.47±15.26\%, \(P=0.048\); CD69: 25.90±20.42\% vs 78.52±21.54\%, \(P=0.012\); Figure 4A, B).

We then assessed the effect of MDSCs on Th1 cell differentiation in CD4\(^+\)T cells. In vitro, the suppression assay revealed that AA MDSCs were less potent than HD MDSCs in suppressing Th1 cell differentiation (82.79±17.53\% vs 97.38±3.07\%, \(P=0.025\); Figure 4C, D). Taken together, our results implicated the immunosuppressive function of AA MDSCs was impaired.

**RNA sequence of MDSCs in AA patients**

To explore the molecular mechanism underlying impaired MDSCs in AA patients, we performed genome-wide RNA sequence (RNA-seq) of MDSCs from randomly selected treatment-naïve AA patients (n=4) and HD (n=3). MDSCs (CD14\(^+\)HLA-DR\(^-\)/low) were enriched by magnetic cell sorting as referred before. Person correlations between samples were \(\geq0.92\) (Supplemental Figure 3A). The hierarchical clustering of differentially expressed transcripts showed 702 upregulated and 658 downregulated transcripts in AA MDSCs compared with HD MDSCs (fold change \(>2\) and \(P\) value \(<0.05\)) (Figure 5A, B). The volcano plot showed numerous genes were enriched in HD MDSCs (FOS, JAK3, etc) and AA MDSCs (HLA-DRA, HLA-DQB1, etc), respectively (Figure 5B). PCA of the transcriptome showed that MDSCs from AA patients and HD were clustered separately, which represented the significant differences in the overall gene expression (Supplemental Figure 2B). Though the PCA plot showed moderate variability in the expression of MDSCs among AA patients (Supplemental Figure 2B), it should not affect the downstream analyses due to the cluster separation of AA and HD MDSCs. GO analysis revealed that enriched genes were related to immunoregulation (e.g., adaptive immune
response) and cellular process (e.g., chemotaxis, migration, cell-cell adhesion, differentiation, and activation) (Figure 5C). Accordingly, immunologically relevant signaling pathways [graft-versus-host disease (GVHD), IL-17 signaling pathway, and TNF signaling pathway], biological processes (necroptosis, galactose metabolism and apoptosis) were enriched as well by utilizing KEGG pathway analysis (Figure 5D).

Functional annotation analyses showed that upregulated pathways in AA MDSCs were related to DNA damage, apoptosis, IL-4 and allograft rejection (Figure 6A, B). Besides, we have confirmed that late apoptotic cells rate in AA MDSCs was higher than that in HD MDSCs (Supplemental Figure 4). Interestingly, the genes responsible for regulation of transcription, protein secretion, glycolysis, IL-6 and immunoregulation (TGF-β, ROS and IL-18) were downregulated in AA MDSCs (Figure 6A, B and supplemental Figure 2C). These findings might partly explain the dysfunction of AA MDSCs in immune regulation.

Reports revealed that both growth hormone (GH) and leptin could activate intracellular tyrosine kinases (JAKs) and the latent cytoplasmic transcriptions factors (STAT), which further induced proliferation, differentiation, cell cycle and anti-apoptosis pathways in normal cells. In this study, we found that leptin receptor (LEPR) and genes related with GH receptor (GHR) pathway were upregulated in AA MDSCs (Figure 6C). In addition, PPI analysis showed that 19 genes related to JAK/STAT pathway formed an interaction network. STRING database identified 18 nodes and 60 edges with PPI enrichment P value <1e-16, average clustering coefficient of 0.79, and average node degree of 6.67 (Figure 6C, D). Interestingly, anti-apoptosis pathways related genes (JAK3, STAT3, PIM1 and SOCS3) were also upregulated in AA MDSCs (Figure 6E). Collectively, these data suggest that MDSCs reduction could be associated with the upregulation of apoptosis and DNA damage, as well as downregulation of gene expression and development-inducing factors in AA. Moreover, our data imply that upregulated JAK/STAT pathway in AA MDSCs may be negative feedback of decreased MDSCs numbers.

Rapamycin treatment increased MDSCs and improved their immunosuppressive function

Wang et al reported that treatment with rapamycin induces MDSCs recruitment. Human MDSC is roughly CD33+CD11b+HLA-DRlow-. Cytokine-induced CD33+ cells showed high expression of CD11b+ and low to intermediate HLA-DR expression. Thus, these cytokine-treated CD33+ cells phenotypically resembled human MDSCs.

To investigate the effect of rapamycin on MDSCs expansion, changes in surface expression of CD33, CD11b, CD14, HLA-DR on cytokine-induced MDSCs from HD or patients with AA were determined in vitro. After different concentrations of rapamycin (0, 10, 20, 50, 100 and 1000nM) were added, absolute number of CD33+ cells as well as the percentages of HLA-DR+ in CD33+CD11b+ cells and CD14+ cells were evaluated. As shown in Figure 7A, rapamycin with the concentration of 10nM could significantly increase the percentage of HLA-DR+ cells (72.80%±7.84% vs 47.52%±17.73%, P=0.019). Conversely, higher concentrations of rapamycin failed to promote MDSCs expansion. Therefore, 10nM rapamycin was applied in the following
experiment. Together, the absolute number of CD33+ cells and percentage of HLA-DR cells showed a significant increase after the addition of rapamycin in vitro compared with control ($P<0.05$, Figure 7B-D). Moreover, rapamycin markedly suppressed lipopolysaccharide-induced CD80 expression in MDSCs ($P<0.05$, Figure 7F). However, rapamycin didn’t significantly reduce CD86 expression (Supplemental Figure 5). These data suggest that rapamycin increases MDSCs by promoting their proliferation and suppressing their differentiation into mature myeloid cells.

MDSC-mediated suppression on T-cell responses was correlated with expression of iNOS and Arg-1. To better characterize the function of rapamycin-treated MDSCs, the above enzymes were evaluated by flow cytometry. Results showed that rapamycin treatment significantly augmented the expression level of iNOS and Arg-1 in cytokine-induced MDSCs (Figure 7G, H). Therefore, rapamycin modulation proved to reinforce certain suppressive pathways involving iNOS and Arg-1 levels in MDSCs.

Discussion

T-cell mediated autoimmunity targeting marrow leads to impaired hematopoiesis in AA. Defective Tregs and mesenchymal stem/stromal cells were involved in the pathogenesis of AA. MDSCs, identified as regulators of the immune system, show a remarkable ability to suppress T-cell responses partly mediated by the production of Arg-1 and iNOS. MDSCs consist of two major subsets: PMN-MDSCs and M-MDSCs. M-MDSCs have upregulated expression of iNOS, STAT1 and NO; PMN-MDSCs increase the activity of STAT3 and NADPH. Both subsets have elevated levels of Arg-1, which could suppress the immune response of T cells by deletion of arginine.

Our data verified that MDSCs, especially M-MDSCs, were reduced in the PB of AA patients. The decreased intracellular levels of Arg-1 and iNOS in AA MDSCs might contribute to the impaired immunosuppressive function. Consistently, plasma levels of Arg-1 in AA patients were lower than that of HDs. In addition, the percentage of peripheral AA MDSCs was positively correlated with the frequency of Tregs and negatively correlated with CD8+ T cells, which coincided with the defected immunosuppressive function of AA MDSCs. Interestingly, the percentage of MDSCs in male AA exceeded that of female AA. It was reported that MDSCs were susceptible to sex hormones and androgen suppression therapy inhibited the expansion of MDSCs. Herein, we speculated that androgen might play a role in the development of MDSCs, which provided evidence for applying androgen in AA therapy. WT1 was reported to control the growth and differentiation of CD34+ HSPCs. Former study revealed that bone marrow WT1 level in patients with AA was related to disease severity and could predict the response to immunosuppressive therapy. Our data illustrated that the percentage of MDSCs was positively associated with bone marrow WT1 level, which further indicated the involvement of MDSCs in the impaired hematopoiesis of AA.

To elucidate the distinct signaling pathways and biological mechanisms
regulated by MDSCs in the circulation of AA patients, we performed comparative analyses of the transcriptomic profiles between AA and HD MDSCs. Our data showed that critical pathways associated with MDSCs expansion, such as transcription, IL-6, IL-18 and glycolysis, were downregulated in AA MDSCs. Additionally, genes related to DNA damage and apoptosis were upregulated in AA MDSCs, which shed light on the reduced number of MDSCs in AA patients. Interestingly, the JAK-STAT pathway, which mediated anti-apoptosis, was also found to be upregulated in AA MDSCs. Occurring in 7% AA patients, STAT3 mutation was associated with the presence of human leukocyte antigen-DR15 and predicted better responses to immunosuppressive therapy. Moreover, both GH and leptin could activate JAK-STAT. In agreement with these reports, we found that STAT3, LEPR and genes related to the GHR signaling pathway were upregulated in AA MDSCs. Furthermore, components of JAK-STAT signaling pathway (STAT1, STAT5, etc) could expand the immunosuppressive-cell subsets such as MDSCs and Tregs. Collectively, these data implied that the JAK-STAT signaling pathway in AA MDSCs was important to trigger their proliferation and resist apoptosis. Upregulated JAK-STAT signaling pathway may be negative feedback of decreased MDSC numbers in AA.

Apart from Arg-1 and iNOS, the immunosuppressive property of MDSCs could also be mediated by the production of TGF-β and ROS. In this study, TGF-β and ROS pathways were found downregulated in AA MDSCs, which further explained the dysfunction of AA MDSCs. MDSCs were confirmed to promote immune tolerance in bone marrow transplantation and show a protective effect in GVHD regulation. Our data indicated that the allograft rejection pathway was upregulated in AA MDSCs whereas GVHD-related genes (IL1A, PRF1, GZMB and KLRD1) were downregulated. Collectively, there might be an interesting trend for the decreased incidence of GVHD in AA patients with bone marrow transplantation.

Many factors affecting MDSCs expansion and function were reported. However, to date, no clinically effective therapy targeting MDSCs has been developed yet. Rapamycin, serving as an inhibitor of the intracellular kinase mTOR, was clinically applied in AA as an immunosuppressive agent. Consistent with previous reports, our results demonstrated that rapamycin treatment in vitro increased the number of MDSCs and significantly improved the Arg-1 and iNOS levels in MDSCs. In addition, the increased proportion of HLA-DR after rapamycin intervention indicated that rapamycin might affect the differentiation of MDSCs. Indeed, rapamycin significantly suppressed lipopolysaccharide-induced MDSCs differentiation into macrophages. This is one of the mechanisms in which rapamycin plays a therapeutic role in AA.

Conclusions
In summary, impaired MDSCs are involved in the immunopathogenesis of AA. We have revealed intrinsic defects of MDSCs in AA and provided new overwhelming evidence of rapamycin in AA treatment.
References:

Figure 1. Decreased myeloid-derived suppressor cells (MDSCs) in the peripheral blood of aplastic anemia (AA) patients. A. The representative cytograms of CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup> MDSCs and MDSC subsets CD33<sup>+</sup>CD11b<sup>+</sup>HLA-DR<sup>-</sup> CD14<sup>+</sup> monocytic (M)-MDSCs within the gate of peripheral blood mononuclear cells (PBMCs). B-C. The percentage of MDSCs (B) and M-MDSCs (C) in PBMCs from non-severe AA (NSAA) patients (n = 8), severe AA (SAA) patients (n = 7) and healthy donors (HDs) (n = 17). D-E. The expression of arginase (Arg)-1 (D) and inducible nitric-oxide synthase (iNOS) (E) in MDSCs compared between AA patients (n = 6) and HDs (n = 7). *P<0.05, ** P<0.01; NS, not significant; SSC, side scatter; HLA-DR, human leukocyte antigen-D-related.

Figure 2. Relationship between myeloid-derived suppressor cells (MDSCs) and clinical characteristics of aplastic anemia (AA). A. The percentage of MDSC was higher in male patients with AA (n=10) than in female patients (n = 11). B. MDSC proportion was positively correlated with WT1 level (n = 15). C-D. Plasma levels of arginase (Arg)-1 of AA (n = 15) and healthy donors (HDs) (n = 17) were determined by cytometric bead array. The Arg-1 level was positively associated with the percentage of MDSC (n = 15) (D). E-F. MDSC proportion was negatively correlated with the percentage of CD8<sup>+</sup>T cells (n = 9) (E), while positively associated with the frequency of T regulatory cells (Treg) (n = 9) (F). *P<0.05, ** P<0.01, *** P<0.001.

Figure 3. Impaired inhibitory capacities of myeloid-derived suppressor cells (MDSCs) in aplastic anemia (AA) on the proliferation of T cells. A. The inhibitory effect of MDSCs on T-cell proliferation. The number of peaks represents cell division process in different ratios of MDSC and T-cell groups. B. The divided cell proportion of T cells cocultured with MDSCs at different ratios. C. IFN-γ in CD3<sup>+</sup>T cells was detected by flow cytometry (n = 4). D-F. Healthy donors (HD) and AA MDSCs were cocultured with CellTrace™ Violet Cell Proliferation kit-labeled CD3<sup>+</sup>T cells separately at a ratio of 1:2 in the presence of anti-CD3/anti-CD28 Dynabeads for 3 days (HD: n = 5; AA: n = 5). The proliferation of CD3<sup>+</sup>T (D), CD4<sup>+</sup>T (E) or CD8<sup>+</sup>T (F) cells was analyzed by flow cytometry. *P<0.05, ** P<0.01, *** P<0.001.

Figure 4. Impaired inhibitory capacities of myeloid-derived suppressor cells (MDSCs) in aplastic anemia (AA) patients on activation and differentiation of T cells. A, B. Compared to Healthy donors (HD) MDSC (n=4), AA MDSC (n=4) showed a defective capacity to inhibit the activation of T cells. C-F. The capacity to inhibit T cells towards Th1 (CD4<sup>+</sup>IFN-γ IL-4<sup>-</sup>; D) was markedly decreased in AA MDSC, while there was no difference in inhibiting T cells towards Th2 (CD4<sup>+</sup>IFN-γ IL-4<sup>+</sup>; E) and the ratio of Th1/Th2 (F) (HD: n=6; AA: n=6). *P<0.05, ** P<0.01, *** P<0.001. NS, not significant; SSC indicates side scatter; HLA-DR, human leukocyte antigen-D-related.

Figure 5. Gene expression pattern of myeloid-derived suppressor cells (MDSCs) in aplastic anemia (AA) patients (n=4) and healthy donors (HDs) (n=3). A. Hierarchical clustering of AA MDSCs and HD MDSCs on differentially expressed RNA transcripts from RNA-Seq data. Each column represents a sample, and each row represents a transcript. The
color gradient reveals the expression level of each transcript. B, Volcano plot analysis shows differentially expressed genes; Fold changes (>2 or < -2) with significant P values (<0.05) are highlighted in red (for upregulated genes) and green (for downregulated genes). C, D, Gene ontology (C) and Kyoto Encyclopedia of Genes and Genomes (D) analysis show significantly upregulated and downregulated pathways involved in AA MDSCs based on their functional categorization. GVHD, graft-versus-host disease; GnRH, gonadotropin-releasing hormone; TNF, tumor necrosis factor; IL, interleukin; NO, genes with no change in expression.

Figure 6. Dysregulated pathways in AA MDSCs. A, Heat map shows that DNA damage and regulation of transcription were dysregulated in AA MDSCs compared with HD MDSCs. B, Gene set enrichment analysis reveals six dysregulated pathways in AA MDSCs. C, Heat map shows genes associated with JAK-STAT and GHR pathways were differentially expressed in AA MDSCs. D, PPI network analyses shows the interaction network of dysregulated genes related to JAK-STAT signaling pathway by using the STRING database. The overall network statistics are shown in the boxes. E, Kyoto Encyclopedia of Genes analysis shows upregulated genes (marked with red boxes) involved in the JAK-STAT pathway. AA, aplastic anemia; HD, healthy donors; GHR, growth hormone receptor; ES, enrichment score; IL, interleukin.

Figure 7. Rapamycin treatment increases MDSCs and improves their immunosuppressive function. A, Rapamycin with concentration gradients from 10 to 1000nM stimulated the percentage of HLA-DR- in CD33+CD11b+ cells in vitro (n = 5). B, Rapamycin significantly increased the absolute number of CD33+ cells (HD: n = 4; AA: n=4), while there was no difference for the percentage of CD33+CD11b+ cells (HD: n = 5; AA: n = 4). C, D Rapamycin significantly increased the percentage of HLA-DR- in CD33+CD11b+ cells and CD14+ cells (HD: n = 5; AA: n = 4). E, Peripheral blood mononuclear cells were treated with rapamycin or DMSO (control) for 6 days before lipopolysaccharide (1μg/ml) stimulation for 24 hours. F, The expression of CD80 from patients with AA and HD was detected by flow cytometry (HD: n = 5; AA: n = 5). G-H, Expression levels of arginase (Arg)-1 (G) and inducible nitric-oxide synthase (iNOS) (H) with or without treatment of rapamycin from patients with AA and HD (HD: n = 5; AA: n = 4). All quantitative data represent mean±SEM. *P<0.05, ** P<0.01. AA, aplastic anemia; HD, healthy donor; SSC, side scatter; HLA-DR, human leukocyte antigen-D-related; Rap: Rapamycin.
Supplementary Materials and Methods

Characterization of cell phenotype
Cell staining was performed using fluorescein isothiocyanate (FITC) conjugated anti-Lin (CD3, CD14, CD16, CD19, CD20, CD56), phycoerythrin (PE) conjugated anti-HLA-DR, allophycocyanin (APC) conjugated anti-CD33, and phycoerythrin-cyano dyes 7 (PE-Cy7) conjugated anti-CD11b, peridinin chlorophyl II protein-cyano dyes 5.5 (PerCP-Cy5.5) conjugated anti-CD14, Brilliant Violet 510 (BV510) conjugated anti-CD15 mouse anti-human monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA, USA). To determine the expression level of Arg-1 and iNOS, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience, USA) according to the manufacturer’s instructions. The monoclonal Brilliant Violet 421 (BV421) conjugated anti-Arg-1 and FITC conjugated anti-iNOS antibodies (Biolegend, San Diego, CA, USA) were incubated for 1h.

The phenotype of in vitro generated MDSCs was evaluated for the cell surface markers of CD33, CD11b, HLA-DR and CD14. Changes in PBMC subpopulations during cytokine induction and rapamycin modulation, as well as intracellular molecular of iNOS and Arg-1, were measured by flow cytometry.
## Supplemental Table 1. Clinical characteristics of patients with AA

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male, No. (%)</td>
<td>30(46.20%)</td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>35(53.80%)</td>
</tr>
<tr>
<td>Median age (years, range)</td>
<td>33(13-70)</td>
</tr>
<tr>
<td>Severity of disease, No. (%)</td>
<td></td>
</tr>
<tr>
<td>NSAA</td>
<td>24(36.90%)</td>
</tr>
<tr>
<td>SAA</td>
<td>27(41.50%)</td>
</tr>
<tr>
<td>VSAA</td>
<td>14(21.50%)</td>
</tr>
<tr>
<td>Bone marrow hypoplasia, No. (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;10%</td>
<td>29(44.60%)</td>
</tr>
<tr>
<td>10-20%</td>
<td>15(23.10%)</td>
</tr>
<tr>
<td>20-30%</td>
<td>6(9.20%)</td>
</tr>
<tr>
<td>30-40%</td>
<td>4(6.20%)</td>
</tr>
<tr>
<td>40-50%</td>
<td>7(10.80%)</td>
</tr>
<tr>
<td>NA</td>
<td>4(6.20%)</td>
</tr>
<tr>
<td>Cell counts (median, range)</td>
<td></td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>2.31(0.11-4.38)</td>
</tr>
<tr>
<td>ANC (×10⁹/L)</td>
<td>0.64(0.00-2.35)</td>
</tr>
<tr>
<td>ARC (×10⁹/L)</td>
<td>23.40(2.60-94.90)</td>
</tr>
<tr>
<td>PLT (×10⁹/L)</td>
<td>18(2-80)</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>72(33-137)</td>
</tr>
</tbody>
</table>

AA: aplastic anemia; NSAA: non-severe aplastic anemia; SAA: severe aplastic anemia; VSAA: very severe aplastic anemia; WBC: white blood cell; ANC: absolute neutrophile granulocyte; ARC: absolute reticulocyte; PLT: platelet; Hb: hemoglobin.
Supplemental Table 2. The number of MDSC subsets in the peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>HD</th>
<th>NSAA</th>
<th>SAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>23</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>MDSC mean±SEM (%)</td>
<td>3.57±1.99</td>
<td>1.11±1.10</td>
<td>0.67±0.76</td>
</tr>
<tr>
<td>P value</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M-MDSC mean±SEM (%)</td>
<td>3.11±1.75</td>
<td>0.87±0.87</td>
<td>0.58±0.68</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.001&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PMN-MDSC mean±SEM (%)</td>
<td>0.16±0.42</td>
<td>0.05±0.79</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>P value</td>
<td>0.415&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.284&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>eMDSC mean±SEM (%)</td>
<td>0.12±0.03</td>
<td>0.03±0.05</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td>P value</td>
<td>0.276&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.661&lt;sup&gt;h&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

HD: healthy donor; NSAA: non-severe aplastic anemia; SAA: severe aplastic anemia; MDSC: myeloid-derived suppressor cell; M-MDSC: monocytic-myeloid-derived suppressor cell; PMN-MDSC: polymorphonuclear-myeloid-derived suppressor cell; eMDSC: early-stage myeloid-derived suppressor cell. <sup>a,b</sup>, The percentage of MDSC in NSAA (a) and SAA (b) patients were lower than HD; <sup>c,d</sup>, The percentage of M-MDSC in NSAA (c) and SAA (d) patients were lower than HD; <sup>e,f</sup>, The percentage of PMN-MDSC in NSAA (e) and SAA (f) patients was not distinguishable with HD; <sup>g,h</sup>, The percentage of eMDSC in NSAA (g) and SAA (h) patients was not distinguishable with HD.
Supplemental Figure 1. Decreased number of myeloid-derived suppressor cells (MDSCs) in the peripheral blood of aplastic anemia (AA) patients.

A, The representative cytograms of MDSC subsets CD33⁺CD11b⁺HLA-DR⁻CD15⁺ polymorphonuclear (PMN)-MDSCs and early-stage MDSCs (eMDSCs) within the gate of peripheral blood mononuclear cells (PBMNCs). B, C, The percentage of PMN-MDSCs (B) and eMDSCs (C) in PBMNCs from non-severe AA (NSAA) patients (n=11), severe AA...
(SAA) patients (n=10) and healthy donors (HDs) (n=23). D, E, The percentage of MDSCs (D) and M-MDSCs (E) were elevated in AA patients with partial response (PR) (n=9) and complete response (CR) (n=13). F, G, The level of Arg-1 (F) and iNOS (G) were increased in AA patients with PR (n=6) and CR (n=6). NS, not significant; Lin, lineage-specific markers (Lin); HLA-DR, human leukocyte antigen-D-related; Arg-1, arginase-1; iNOS, inducible nitric-oxide synthase; *P<0.05, ** P<0.01, *** P<0.001.
Supplemental Figure 2. Relationship between myeloid-derived suppressor cells (MDSCs) and clinical characteristics of aplastic anemia (AA).

A, Relationship between MDSC and age (n=15). B, C, Percentages of monocytic (M)-MDSC and eMDSC were positively correlated with WT1 levels (n=15). D-E, Plasma levels of tumor necrosis factor (TNF)-α (D), IL-10 (E) and interferon (IFN)-γ (F) of AA (n=15) and healthy donors (HD) (n=17) determined by cytometric bead array.
Supplemental Figure 3. The gene expression pattern of myeloid-derived suppressor cells (MDSCs) from aplastic anemia (AA) patients and healthy donors (HDs).

A, Correlation analysis between samples. B, A clear clustering between AA and HD MDSCs was observed by principal component analysis. C-F, Gene set enrichment analysis reveals pyrimidine metabolism (C), glycolysis (D), transforming growth factor (TGF)-β (E), reactive oxygen species (ROS) pathways in AA MDSCs compared with HD MDSCs. ES, enrichment score.
Supplemental Figure 4

Supplemental Figure 4. The apoptotic rates of MDSCs from patients with AA and HD were detected by flow cytometry. Peripheral blood mononuclear cells were stained with CD14, HLA-DR, 7-AAD and Annexin V. Compared with HD, significantly increased late apoptotic cells (7-AAD+ Annexin+) were detected in MDSCs (CD14+ HLA-DR+) from AA patients (HD: n=5; AA: n=5). AA, aplastic anemia; HD, healthy donor; *P<0.05.
Supplemental Figure 4. The expression of CD86 from patients with AA and HD was detected by flow cytometry (HD: n=5; AA: n=4).

Peripheral blood mononuclear cells were treated with rapamycin or control for 6 days before lipopolysaccharide (LPS; 1µg/ml) stimulation for 24 hours. All quantitative data represent mean±SEM. NS, not significant; AA, aplastic anemia; HD, healthy donor.