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Interleukin-18 plays a dispensable role in murine and likely also human bone marrow failure

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Interleukin-18 (IL-18), also known as interferon-gamma (IFN-γ)-inducing factor, is involved in Th1 responses and regulation of immunity. Accumulating evidence implicates IL-18 in autoimmune diseases, but little is known of its role in acquired aplastic anemia (AA), the immune-mediated destruction of bone marrow (BM) hematopoietic stem and progenitor cells (HSPCs). IL-18 protein levels were significantly elevated in sera of severe AA (SAA) patients, including both responders and nonresponders assayed before treatment, and decreased after treatment. IL-18 receptor (IL-18R) was expressed on HSPCs. Co-culture of human BM CD34$^+$ cells from healthy donors with IL-18 upregulated genes in the helper T-cell and Notch signaling pathways and downregulated genes in the cell cycle regulation, telomerase, and IL-6 signaling pathways. Plasma IL-18 levels were also elevated in murine models of immune-mediated BM failure. However, deletion of IL-18 in donor lymph node cells or deletions of either IL-18 or IL-18R in recipients did not attenuate elevations of circulating IFN-γ, tumor necrosis factor-alpha, or IL-6, nor did they alleviate BM failure. In summary, our findings suggest that, although increased circulating IL-18 is a feature of SAA, it may reflect an aberrant immune response but be dispensable to the pathogenesis of AA. Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Acquired aplastic anemia (AA) is a bone marrow (BM) failure syndrome characterized by peripheral blood (PB) pancytopenia and BM hypoplasia [1,2]. Success of immunosuppressive therapy (IST), among other clinical and laboratory clues, is compelling evidence of the immune pathophysiology of AA [3]. In most cases, AA is an immune-mediated disorder with active destruction of hematopoietic cells by effector T lymphocytes. Increased production of interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) by patients' T cells suggests important roles for a type 1 immune response in BM hematopoietic cell destruction [4–6]. Th17 response [7] and impairment of regulatory T cells [8] may also contribute to the pathogenesis of AA. Production of type 1 cytokines by activated T cells or BM cells may contribute to hematopoietic failure development [6–8] because IFN-γ suppresses hematopoiesis [9,10] and facilitates destruction of hematopoietic cells by augmenting apoptosis in the presence of activated cytotoxic T cells (CTLs) in murine BM failure models [11].

IL-18, initially identified as an IFN-γ-inducing factor in T and natural killer (NK) cells, is a member of the IL-1 family [12,13] and is constitutively secreted by several types of cells, such as macrophages and dendritic cells. Biological activity of IL-18 is mainly regulated by its enzymatic processing rather than at a transcriptional level [14]. IL-18 receptor (IL-18R) is expressed mostly on CTLs. Binding of IL-18 to the IL-18Rα recruits the IL-18Rβ chain and initiates downstream signaling transduction through myeloid differentiation primary response 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb), and activator protein 1 (AP-1) [15–17]. IL-18 cooperates with IL-12 for NK cell activation and induction of IFN-γ production and other type 1 cytokines in response to pathogen products, thus promoting Th1 polarization and CTL

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responses [18,19]. IL-18 binding protein (IL-18BP), a natural inhibitor of IL-18, has high affinity to mature IL-18 and blocks interactions between IL-18 and IL-18Rα, and subsequent signaling by preventing receptor dimerization [20,21]. Exaggerated IFN-γ production triggered by IL-18 leads to IL-18BP secretion as a negative-feedback loop to subdue the type 1 immune responses [22,23].

IL-18 has been implicated in the pathogenesis of many inflammatory and autoimmune diseases, including type 1 diabetes, rheumatic arthritis, allergy, asthma, Crohn's disease, multiple sclerosis, and myasthenia gravis [24–30]. Elevated IL-18 and IL-18BP levels are found in immune thrombocytopenia purpura patients [31]. IFN-γ and IL-18 plasma levels are significantly increased in patients with active AA compared with healthy controls and AA patients in remission, and the levels of IL-18 were also found to be correlated with disease severity [32]. However, the roles of IL-18 in the pathogenesis of AA remain largely unknown.

We have previously developed murine models for immune-mediated BM failure by infusion of allogeneic lymph node (LN) cells into sublethally irradiated recipients [33,34] and successfully used these models to study AA pathophysiology [4,11] and to test new treatments [35,36]. Our current study was aimed at defining the roles of IL-18 in immune-mediated BM destruction and in the development of clinical pancytopenia. In addition to the analyses of clinical samples collected from AA patients, we also utilized our murine models with targeted germline deletions of IL-18 and IL-18R, respectively, to assess the roles of the IL-18/IL-18R signaling pathway in the development of immune-mediated BM failure.

Methods

Human samples

Serum samples were obtained after informed consent. In Cohort 1, 88 patients with severe AA (SAA) and 20 age- and sex-matched healthy subjects were included: IL-18 levels at baseline (88 SAA patients) and after treatment (23 SAA patients), as well as IL-18BP levels prior to treatment (27 SAA patients) were analyzed. In Cohort 2, another 39 SAA patients and 31 patients with myelodysplastic syndrome (MDS) were included and only baseline IL-18 levels were analyzed. Standard criteria were used for the diagnosis of SAA, MDS, and evaluation of disease severity [37,38]. SAA patients were enrolled in clinical research protocols (www.clinicaltrials.gov identifiers NCT00071045, NCT00260689, NCT00922883, NCT01623167, NCT00001397, and NCT00001620). MDS patients were enrolled in clinical research protocols (www.clinicaltrials.gov identifiers NCT00001397, NCT00001620, NCT00217594, and NCT00961064). BM samples of healthy controls were obtained from donors of the National Institutes of Health (NIH) Clinical Center. All human subjects were enrolled in clinical protocols approved by the NIH's National Heart, Lung, and Blood Institute (NHLBI) Institutional Review Board. Clinical characteristics of patients in this study are summarized in Table 1.

Assays of IL-18 and IL-18BP levels

Sera from both patients and controls and plasma from mice were stored at −80°C until analysis. IL-18 levels in human serumsera and mouse plasma were measured by ELISA assay (MBL, Woburn, MA) according to the manufacturer's

Table 1. Patient demographics

Characteristics	Cohort 1 SAA	Cohort 2 SAA	MDS
No.	88	39	31
Median Age, Years (Range)	33 (2–75)	31 (6–82)	60 (23–86)
Sex, Male/Female	48/40	20/19	17/14
Disease Status			RCUD (7), RCMD (15) RARS (4), RAEB-1 (5)
Treatment			
IST	41	15	
IST + Eltrombopag	47	24	
Time Analysis			
Samples at Diagnosis	88	39	31
Samples 6 Months after Treatment	23	0	0
Responses to Treatment			
Responders	46	29	
Complete Response	21	15	
Partial Response	25	14	
Nonresponders	37	10	
Blood Counts, Median (Range)			
ANC (K/μL)	0.43 (0.01–9.23)	0.37 (0.01–3.15)	1.18 (0.13–4.19)
HGB (g/dL)	8.7 (5.1–13.7)	8.2 (6.4–11.3)	9.7 (4.9–12.7)
ARC (K/μL)	24.95 (0–95.7)	25.7 (5–78.7)	59.8 (2.3–115.7)
PLT (K/μL)	20 (3–229)	25 (2–377)	34 (9–240)

ANC = absolute neutrophil count; HGB = hemoglobin; ARC = absolute reticulocyte count; PLT = platelet count; RCUD = refractory cytopenia with unilineage dysplasia; RCMD = refractory cytopenia with multilineage dysplasia; RARS = refractory anemia with ringed sideroblasts; RAEB-1 = refractory anemia with excess blasts-1

instructions. Samples and standards were assayed in duplicate. Absorbance was read at 450 nm and 620 nm (for background subtraction) using the VICTOR3 1420 Multilabel Plates Counter (PerkinElmer, Waltham, MA). Human IL-18BP was measured with the magnetic Luminex kit (R&D Systems, Minneapolis, MN).

Co-culture of human CD34$^+$ cells with IL-18

BM mononuclear cells (MNCs) from three healthy donors were stained with Pacific blue-labeled anti-CD3 (Biolegend, San Diego, CA), anti-CD14, anti-CD19 (Invitrogen, Carlsbad, CA), and anti-CD34-PE (BD Biosciences, Franklin Lakes, NJ). Lineage-negative CD34$^+$ cells were sorted using the FACSAria II Cell Sorter (BD Biosciences). Sorted CD34$^+$ cells were cultured in StemSpan SFEM II serum-free medium plus StemSpan CD34$^+$ Expansion Supplement (STEMCELL Technologies, Vancouver, BC, Canada) in the presence or absence of 20 ng/mL recombinant human IL-18 (MBL) for 48 hours, followed by RNA extraction.

PCR array

Total RNA was isolated from human CD34$^+$ cells using the RNeasy kit (Qiagen, Valencia, CA), digested with RNase-free DNase I (Qiagen), and assessed by using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). First-strand complementary DNA was synthesized by using the RT^2 First-Strand kit (Qiagen). Quantitative analysis of messenger RNA expression of hematopoiesis-related genes was performed by using the human polymerase chain reaction (PCR) array PAHS-054Z (Qiagen) and each sample was run in duplicate.

Animals and induction of BM failure

Congenic C.B10-H2b/LilMcd (C.B10), inbred FVB/N (FVB), inbred C57BL/6 (B6), and induced mutant B6.129P2-Il18^{tm1Aki}/J (IL-18$^{-/-}$) and B6.129P2-Il18r1^{tm1Aki}/J (IL18R$^{-/-}$) mice were all from The Jackson Laboratory (Bar Harbor, ME) and were bred and maintained in the NIH Animal Facilities under standard care and nutrition. The genotypes of IL-18$^{-/-}$ and IL18R$^{-/-}$ mice were confirmed by PCR analyses according to producer's protocols. All animal studies were approved by the Animal Care and Use Committee at the NHLBI.

Immune-mediated BM failure was induced as described previously [33,35]. In one model, LN cells from IL-18$^{-/-}$ or wild-type (WT) B6 donor mice were homogenized, washed, filtered, and intravenously injected into sex-matched C.B10 recipients pre-irradiated with 5 Gy of total body irradiation (TBI) 4–6 hours earlier at 5×10^6 cells per recipient [33]. Recipient animals were bled and euthanized 12–18 days later to obtain tissues for histological and cytological assessments. In the other models, the same number of LN cells from FVB donors were infused into IL-18$^{-/-}$, IL-18R$^{-/-}$, or WT B6 recipients pre-irradiated with 6.5 Gy of TBI 4–6 hours earlier [35]. The recipient animals were bled and analyzed 9 days later. In all experiments, mice were used at 8–12 weeks of age.

Blood counts, cell staining, and flow cytometry

Blood was collected from the retro-orbital sinus into Eppendorf tubes with EDTA. Complete blood count was performed using a HemaVet 950 analyzer (Drew Scientific, Waterbury, CT). After mice were euthanized by CO_2, BM cells were extracted from tibiae and femurs, filtered through a 95 μM nylon mesh, counted with a Vi-Cell counter (Beckman Coulter, Miami, FL), and stained with antibody mixtures on ice for 30 minutes in RPMI 1640 (Life Technologies, Carlsbad, CA). Samples were subsequently acquired using a BD LSR Fortessa cytometer (BD Biosciences) and post-acquisition analysis was performed using FlowJo software (version 7.6.4, BD Biosciences).

Human monoclonal antibodies used for flow cytometry analyses were: CD34 (clone 8G12) from BD Biosciences; and CD38 (clone HIT2), CD45RA (clone HI100), CD90 (Thy1, clone 5E10), and CD18Rα (CD218a, clone H44) from BioLegend. Monoclonal antibodies for murine CD3ε (clone 145-2C11), CD4 (clone GK 1.5), CD8α (clone 53-6.7), CD45RA (clone MB4B4), CD11b (clone M1/70), granulocytes (Gr1/Ly6-G, clone RB6-8C5), erythroid cells (clone Ter119), stem cell antigen 1 (Sca-1, clone D7), CD117 (c-Kit, clone 2B8), CD48 (clone HM48-1), CD150 (SLAM, clone TC15-12F12.2), and IL18Rα (CD218a, clone BG/IL18RA) were also from BioLegend. Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), APC-cyanin 7 (APC-Cy7), PE-Cy7, brilliant violet (BV) 421, and BV711. IFN-γ, TNF-α, and IL-6 protein levels in mouse plasma were measured using the LEGENDplex Mouse Th1 Panel (5-plex) (BioLegend).

Data analysis and statistics

Data analysis was performed using Prism software (version 7.02; GraphPad, La Jolla, CA). Results are shown as median $\pm$ interquartile range unless stated otherwise. Statistical analysis was performed using two-sided unpaired Mann–Whitney test or t test for two groups when applicable or one-way analysis of variance for comparisons between three or more groups. Correlation analysis was determined using Pearson's correlation test for normally distributed data and Spearman's correlation test for data without normal distribution. PCR array results were analyzed using the Qiagen online tool (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/). Subsequently, scatter plots, volcano plots, and heat maps containing differentially expressed genes were generated. Differentially expressed genes, identified using a threshold $p < 0.05$ and a fold change > 2, were further subjected to pathway analysis using Ingenuity Pathway Analysis software (www.ingenuity.com, version 33559992, Qiagen Bioinformatics). $p < 0.05$ was considered statistically significant.

Results

Serum IL-18 and IL-18BP levels are elevated in treatment-naïve SAA patients

Serum levels of IL-18 in SAA patients ($n = 88$) and healthy controls ($n = 20$) were measured using ELISA. Among these samples, IL-18BP levels were also measured in 27 SAA patients and nine healthy controls. At diagnosis, IL-18 serum levels were significantly increased in SAA patients compared with controls

(475.4 ± 44.15 vs. 192 ± 23.47 pg/mL, $p = 0.0001$, Figure 1A). There were no correlations between serum IL-18 levels and blood counts in patients before treatment (data not shown). We also measured levels of IL-18BP (a natural inhibitor of IL-18) in 27 SAA patients and nine healthy controls and they were significantly higher in SAA patients at diagnosis than in controls ($p = 0.0077$, Figure 1B). To investigate whether the increased IL-18 levels in AA patients were also seen in MDS, a similar BM failure disease, we measured circulating IL-18 levels in another cohort of 31 MDS patients, 39 SAA patients, and eight healthy donors. Both SAA and MDS patients showed higher IL-18 levels than did heathy donors, but no difference was observed between SAA and MDS patients (Figure 1C). High IL-18 levels might reflect an inflammation state in both diseases.

Dynamic changes of IL-18 levels with treatment

Twenty-three SAA patients (16 responders and seven nonresponders) in the first cohort were studied for IL-18 levels before and 6 months after treatment. Overall, high serum IL-18 protein levels significantly decreased after treatment ($p = 0.0181$, Figure 1D) to levels similar to those in healthy controls ($p = 0.7426$). When patients were divided based on their response to treatment at the 6-month time point, both responders ($p = 0.0486$) and nonresponders ($p < 0.0001$) at diagnosis showed significantly higher IL-18 serum levels than did controls and nonresponders displayed higher levels of IL-18 than did responders ($p = 0.0052$, Figure 1E). The high levels of IL-18 in both responders and nonresponders before treatment tended to decrease after treatment, but did not reach a significant difference ($p = 0.0696$ and $p = 0.1354$, respectively; Figure 1E). Both responders and nonresponders exhibited IL-18 levels identical to controls after treatment ($p = 0.4132$ and $p = 0.1398$, respectively).

IL-18/IL-18R signaling regulates gene expression in human HSPCs

To look for a possible direct effect of IL-18 on human HSPCs, we first confirmed the existence of IL-18R on human BM-MNCs by flow cytometry. IL18-R was expressed on 24.7 ± 6.25% of $CD34^+$ cells, with 36.3 ± 7.6% of $CD34^+CD38^-$ cells and 14.10 ± 3.0% of $CD34^+CD38^+$ cells. IL-18R was further measured on hematopoietic cell subpopulations [39], including hematopoietic stem cells (HSCs, 64.63 ± 3.12%), multipotent progenitors (MPPs, 33.13 ± 2.82%), granulocyte–monocyte progenitors (GMPs, 6.04 ± 1.78%), common myeloid

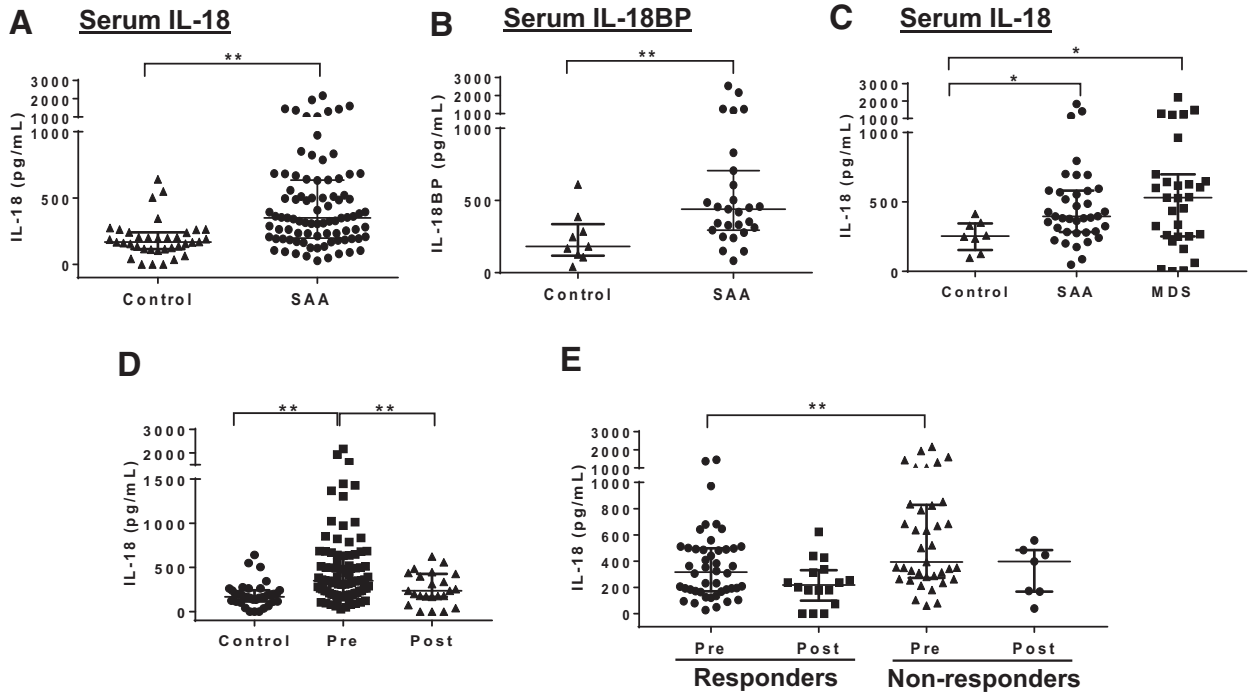

Figure 1. Serum IL-18 and IL-18BP levels in SAA patients. Serum IL-18 **(A)** and IL-18BP **(B)** levels were measured in SAA patients ($n = 88$ and $n = 27$, respectively) and healthy controls ($n = 20$ and $n = 9$, respectively). **(C)** Serum IL-18 protein levels in another cohort of SAA ($n = 39$) and MDS ($n = 33$) patients compared with healthy controls ($n = 8$). **(D)** Serum IL-18 protein levels in SAA patients before ($n = 88$) and after ($n = 23$) therapy and in healthy controls ($n = 20$). **(E)** Serum IL-18 protein levels in responders and nonresponders before ($n = 46$ and $n = 37$, respectively) and after ($n = 16$ and $n = 7$, respectively) therapy. *$p < 0.05$; **$p < 0.01$. Pre = pre-treatment; Post = post-treatment.

progenitors (CMPs), and megakaryocyte-erythroid progenitors (MEPs, 19.16 ± 6.41%) (Figures 2A and 2B).

Human BM CD34$^+$ cells were sorted and co-cultured with IL-18 for 48 hours *in vitro*. PCR-based transcriptome assay focusing on genes in hematopoiesis showed that 20 genes were upregulated and 19 downregulated relative to control CD34$^+$ cells (Figures 3A–3C). Pathway analysis revealed that upregulated genes were mainly involved in the Th1 and Th2 pathways (*CCR1*, *CD3D*, *CD8A*, *DLL1*, *IL-12B*, *JAG2*, and *NOTCH1*), Notch 1 signaling (*DLL1*, *JAG2*, and *NOTCH1*), and hematopoiesis from pluripotent stem cells (*CD3D*, *CD8A*, *CSF1*, *IL-11*, *IL-12B*, and *IL-1A*). Downregulated genes were involved in cell cycle regulation, telomerase (*HDAC4*, *HDAC7*, and *HDAC9*) and IL-6 signaling (*CD14*, *IL-6ST*, and *VEGFA*) (Figure 3D).

Knockout of IL-18 or IL-18R does not alleviate murine immune-mediated BM failure

Next, we investigated whether disruption of IL-18 signaling could alleviate murine immune-mediated BM failure (Figure 4A). When LN cells from IL-18$^{-/-}$ or

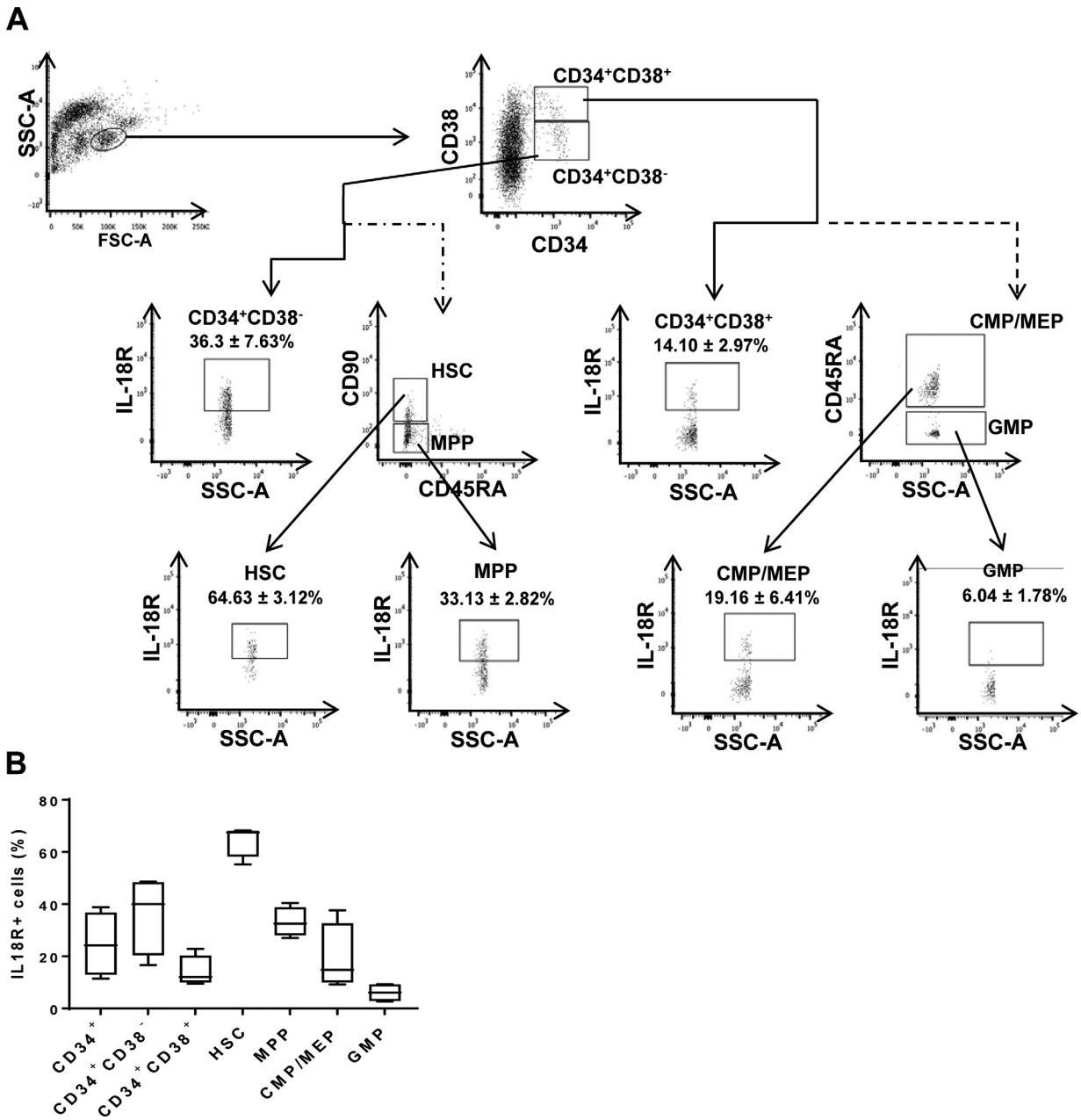

Figure 2. Expression of the IL-18 receptor on human BM HSPCs. **(A)** Gating strategy and IL-18R expression on HSCs, MPPs, GMPs, and CMPs/MEPs. **(B)** Bar chart of IL-18R expression in HSCs, MPPs, CMP/MEPs, and GMPs ($n = 4$).

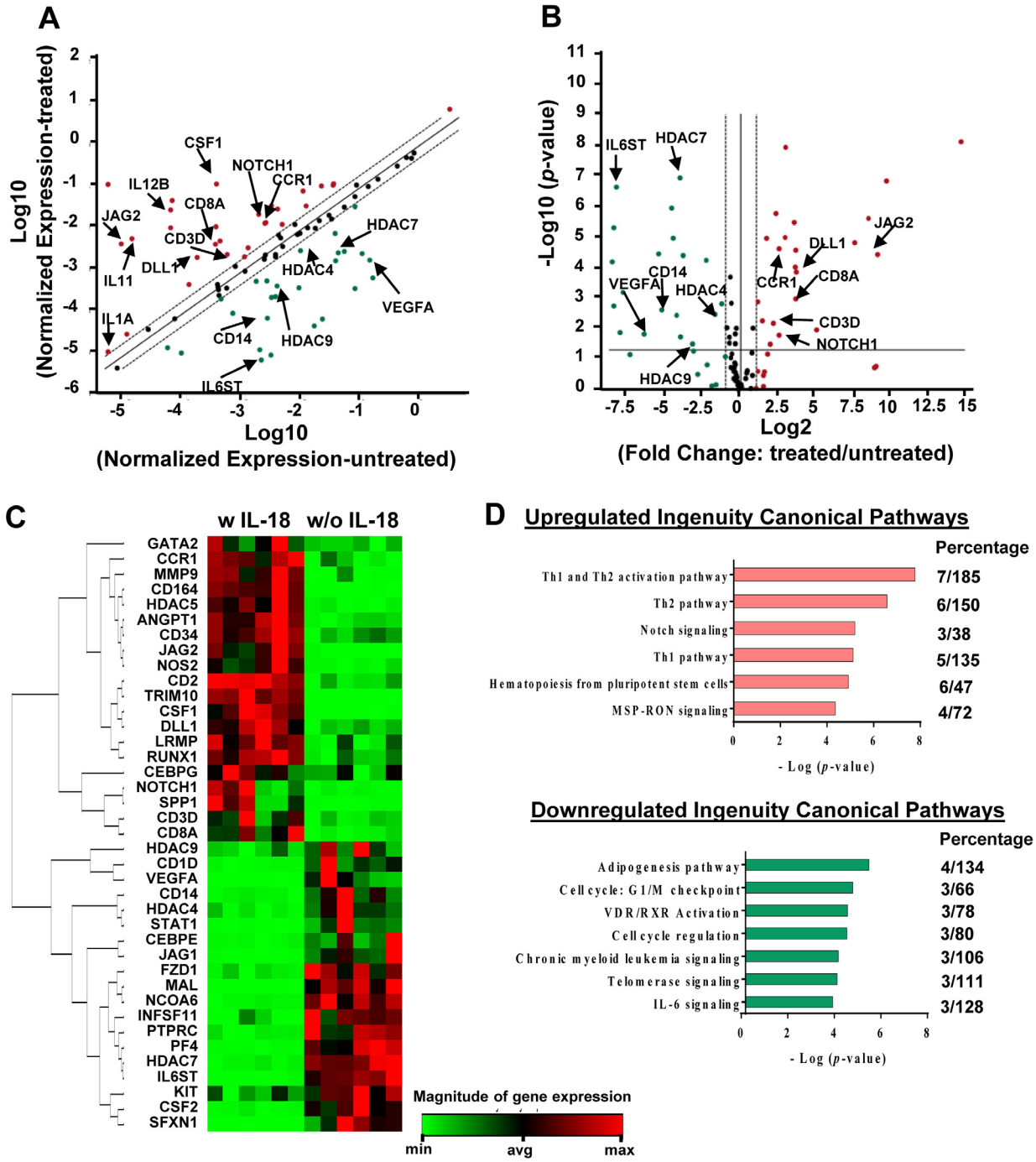

Figure 3. Direct effects of IL-18 on gene expression of human CD34$^+$ cells. **(A)** Scatter plot of gene expression in IL-18-treated CD34$^+$ cells compared with untreated CD34$^+$ cells. *x* and *y* axes indicate Log10 normalized expression in untreated cells and Log10 normalized expression in treated cells, respectively. Upregulated and downregulated genes are labeled in red and green, respectively. **(B)** Boxplot of gene expression in IL-18-treated CD34$^+$ cells compared with untreated CD34$^+$ cells. *x* and *y* axes indicate Log2 fold change in treated/untreated cells and Log10 *p*-value, respectively. **(C)** Heat map of differentially expressed genes in cells treated with and without IL-18. **(D)** Differentially expressed genes were subjected to Ingenuity Pathway Analysis. *x* and *y* axes indicate the log *p*-value and aberrant pathways, respectively. Percentage indicates the number of differentially expressed genes versus the total number of genes in individual pathways. Differentially expressed genes were defined as $p < 0.05$ and fold change > 2.0.

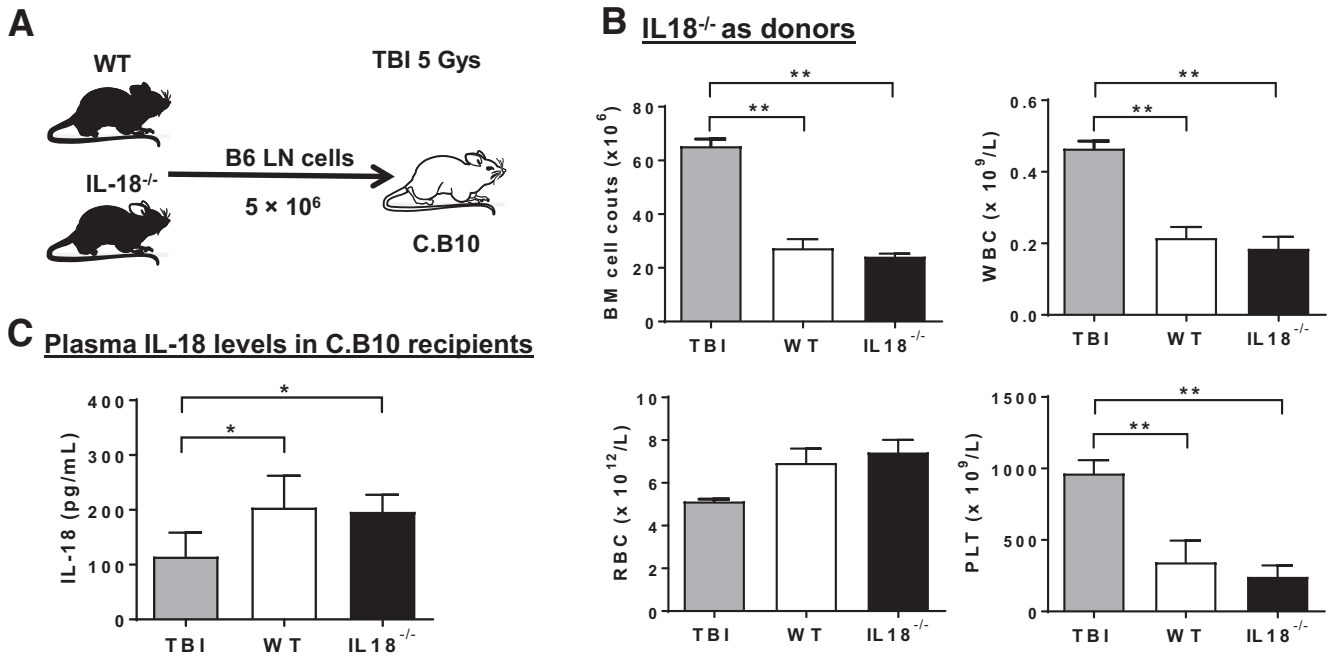

Figure 4. Infusion of LN cells from IL-18$^{-/-}$ donors into recipient mice with induced BM failure. **(A)** Experimental schematic of mouse experiments with B6 (donors) and C.B10 (recipients) mice. LN cells from IL-18$^{-/-}$ or WT B6 mice were infused into C.B10 recipient mice ($n = 4$, respectively) to induce BM failure. C.B10 mice receiving only TBI were used as controls. **(B)** BM cell, WBC, RBC, and PLT counts in C.B10 recipients after being infused with LN cells from IL-18$^{-/-}$ or WT B6 donor mice. **(C)** Plasma IL-18 levels in C.B10 recipients with BM failure induced by injection of LN cells from IL-18$^{-/-}$ or WT B6 donor mice compared with TBI controls. *$p < 0.05$; **$p < 0.01$.

wild-type B6 mice were infused into C.B10 recipient mice, both types of LN cells induced a similar severity of BM failure, as evidenced by similar decreases in BM cell counts, white blood cells (WBCs), red blood cells (RBCs), and platelets (PLTs) in C.B10 recipient mice on day 14 (Figure 4B), indicating that IL-18 in donor T cells was dispensable in the induction of murine BM failure. We measured IL-18 levels in C.B10 recipient mice by ELISA and plasma IL-18 protein levels were significantly increased in recipients with BM failure induced by injection of LN cells from wild-type B6 donor mice (Figure 4C) compared with TBI controls, similar to the finding from SAA patients (Figure 1A). However, recipients that received LN cells from IL-18$^{-/-}$ donor mice exhibited similarly high plasma IL-18 levels (Figure 4C).

Next, we tested the susceptibility of IL-18$^{-/-}$ and IL-18R$^{-/-}$ recipient mice to immune-mediated BM destruction after receiving TBI and lymphocytes injection from FVB donors. Wild-type B6 recipients that had received the same FVB donor LN infusion were used as controls (Figure 5A). All three genotypes developed BM failure. The severity of BM failure, quantified by decrease of total BM cell counts and peripheral WBC, RBC, and PLT counts, also was not different among IL-18$^{-/-}$ (Figure 5B), IL-18R$^{-/-}$ recipients (Figure 5E) and control WT B6 mice after LN infusion. IL-18 levels were not detectable in IL-18$^{-/-}$ mice with TBI only or BM failure (Figure 5C), confirming an overall deficiency of IL-18 production in IL-18$^{-/-}$ mice, whereas wild-type and IL-18R$^{-/-}$ mice with BM failure showed more elevated IL-18 levels than did TBI controls (Figure 5F). We also measured IL-18-related inflammatory cytokines in the plasma of recipients and found that IFN-γ, TNF-α, and IL-6 were significantly elevated with BM failure and the levels of these cytokines did not differ between WT B6 and IL-18$^{-/-}$ (Figure 5D) or IL-18R$^{-/-}$ recipients (Figure 5G).

Discussion

In the current study, we demonstrated increased circulating IL-18 levels in both SAA and MDS patients as well as murine models of immune-mediated BM failure. Furthermore, IL-18 altered the expression of genes critical to hematopoiesis in human CD34$^+$ cells, possibly through the IL-18R signaling pathway because IL-18R was expressed on human CD34$^+$ HSPCs.

Our observation of increased IL-18 levels in SAA patients was consistent with a previous report [32] in which serum IL-18 levels in AA patients in remission decreased to similar levels as in healthy controls. In our study, we observed an overall decrease in IL-18 levels after treatment. However, the trend of decline was observed in both responders and nonresponders, indicating that serum IL-18 levels decreased after IST or IST + eltrombopag, but was not correlated to remission or hematopoietic recovery. The fact that serum

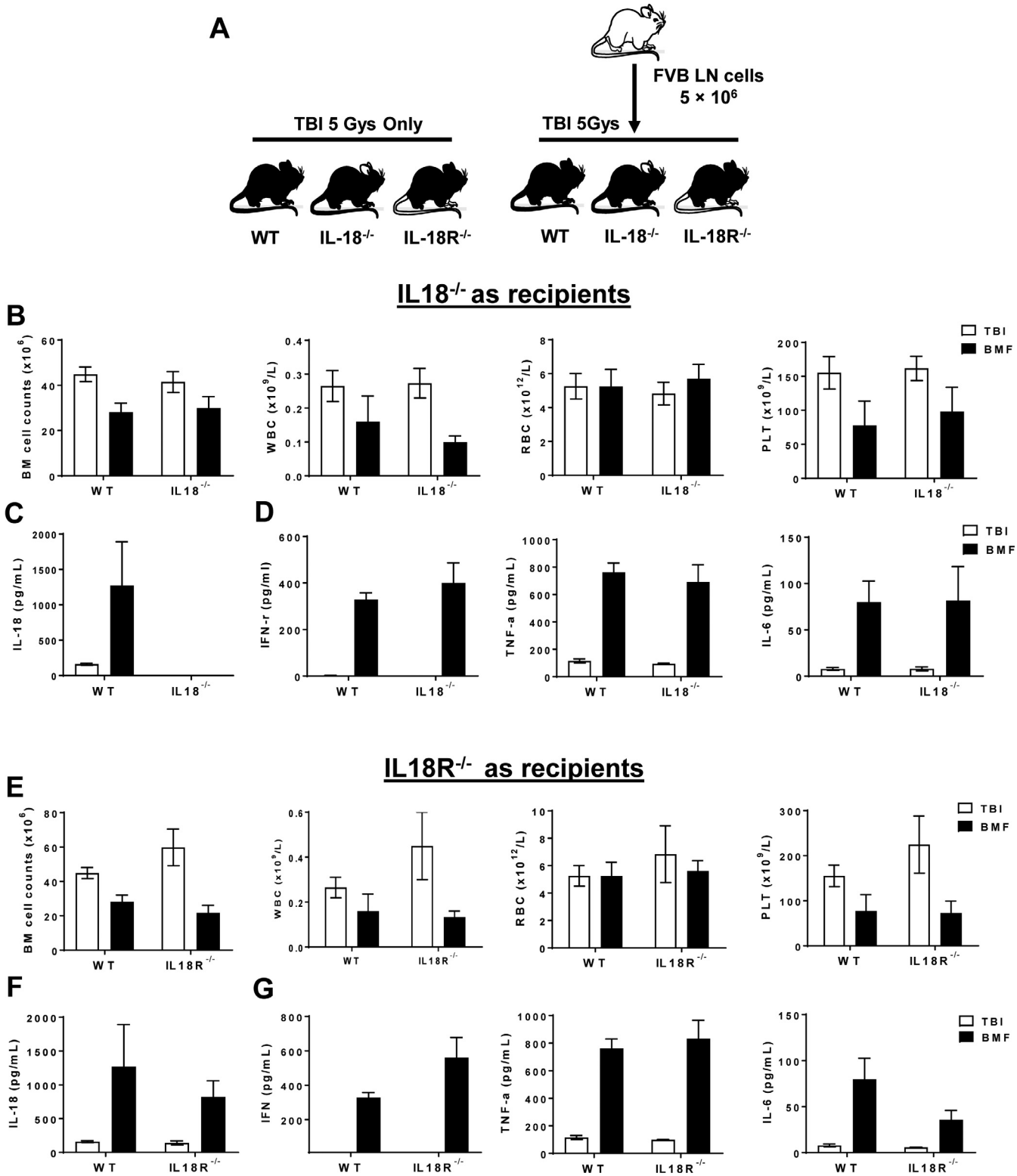

Figure 5. BM failure in IL-18$^{-/-}$ and IL-18R$^{-/-}$ recipient mice. **(A)** Experimental schematic of mouse experiments with FVB (donors) and B6 mice (recipients). LN cells from FVB donor mice were infused into IL-18$^{-/-}$ ($n = 5$), IL-18R$^{-/-}$ ($n = 5$) mice, or WT B6 ($n = 6$) recipient mice after TBI in order to induce BM failure. Mice receiving only TBI were used as controls. **(B)** BM cell, WBC, RBC, and PLT counts in WT B6 and IL-18$^{-/-}$ mice. **(C,D)** IL-18 **(C)** and IFN-γ, TNF-α, and IL-6 **(D)** protein levels in plasma of WT B6 and IL-18$^{-/-}$ mice. **(E)** BM cell, WBC, RBC, and PLT counts in WT B6 and IL-18R$^{-/-}$ mice. **(F,G)** IL-18 **(F)** and IFN-γ, TNF-α, and IL-6 **(G)** protein levels in plasma of WT B6 and IL-18R$^{-/-}$ mice. *$p < 0.05$; **$p < 0.01$.

IL-18 levels showed no correction with blood counts at a baseline further added complexity to the picture: high serum IL-18 levels might only be a reliable marker of AA at disease onset, and the role of IL-18 in disease progression and remission was very much unclear.

Along with increased levels of IL-18 in AA patients before treatment, we also observed higher levels of IL-18BP in patients than in controls, possibly reflecting negative feedback to the type 1 immune responses [22,23]. As a natural inhibitor of IL-18's biologic activity, IL-18BP increases with IL-18 in many pathophysiological settings [31]. In AA, it is possible that the biologic effect of elevated IL-18 was offset by the increased IL-18BP.

IL-18 has been extensively studied in immunity, but its regulatory effect on HSPCs is largely unknown. The IL-18 gene was found to be expressed in proximal rather than distal osteolineage cells in murine BM niche [40]. IL-18R1 is expressed on short-term progenitors (ST-HSCs), but not long-term (LT)-HSCs. Increased proliferation in ST-HSCs and MPPs, but not in LT-HSCs, was observed in IL-$18^{-/-}$ mice. $Lin^{-}Scal\text{-}1^{+}kit^{+}$ (LSK) cells, $Lin^{-}Scal\text{-}1^{-}kit^{+}$ myeloid progenitors, and common lymphoid progenitors (CLPs) were better preserved in IL-$18^{-/-}$ mice with fluorouracil (5-FU) treatment compared with 5-FU-treated WT controls [40]. We observed IL-18R expression on human HSPCs in our current study. Together with the high serum IL-18 levels in SAA patients, our data suggest a direct effect of IL-18 on HSPCs, resulting in suppression of hematopoiesis in AA patients. Indeed, results from our cell culture study *in vitro* affirmed that IL-18 upregulated the expression of genes associated with the Th1 and Th2 immune pathways and the NOTCH signaling pathway, with downregulation of genes associated with cell cycle, telomerase, and IL-6 signaling. Notch signaling is critical in regulating hematopoiesis. The role of Notch signaling in regulating the proliferation, self-renewal, and differentiation of HSCs is still controversial [41]. Activation of the Notch pathway enhances HSC proliferation and self-renewal while inhibiting especially myeloid differentiation. In contrast, another model proposed that Notch signaling inhibits HSC self-renewal, but promotes differentiation. Activation of the Notch pathway directs lymphoid progenitor cells to differentiate toward the T-cell lineage at the expense of B-cell lineage. Telomerase activity may affect telomere length, which is critical to cell replication. Cells will eventually enter either senescence or apoptosis if fail to restore telomere length as they replicate [42]. Impairment of telomerase complex functions usually leads to telomere length shortening, which is observed in patients with AA and other BM failure syndromes [42]. As a pleiotropic growth factor with regulatory roles in hematopoiesis and in immune response, IL-6 has been shown to promote HSPC proliferation and differentiation *in vitro* in combination with other cytokines such as IL-3, granulocyte-macrophage colony-stimulating factor, and stem cell factor [43]. In summary, consistent with the mouse study [40], our results suggest direct regulatory roles of IL-18 on human HSPCs. IL-18 may favor helper T-cell signaling and might suppress progenitor cell proliferation and/or myeloid differentiation. These features were consistent with T-cell activation and hematopoiesis suppression in AA.

Because human BM failure has been modeled in mice by infusion of LN cells into recipients mismatched at major histocompatibility complex or minor histocompatibility antigen loci, we explored effects of the deficient IL-18/IL-18R signaling pathway in both B6 to CB10 ($IL18^{-/-}$ as donors) and FVB to B6 (IL-$18^{-/-}$ and IL-$18R^{-/-}$ as recipients) models. Both models mimic human AA in hallmark features including severe pancytopenia and BM hypocellularity; oligo T-cell expansion, T-cell activation, and Fas–Fas ligand expression; altered plasma cytokines including elevated Th1 cytokines; and increased apoptosis and BM cell destruction [33–35]. Moreover, increased levels of plasma IL-18 together with IFN–γ and TNF–α levels were also observed in both murine models of immune-mediated BM failure used in the current work. However, the lack of IL-18 in donor LN cells and the lack of IL-18 or IL-18R in recipients did not attenuate BM failure. Furthermore, deficiency of IL-18 and IL-18R did not suppress the elevation of inflammatory cytokines such as IFN-γ, TNF-α, and IL-6 following allogeneic LN cell infusion. Our observations indicated that IL-18/IL-18R signaling played a minor role in the development of immune-mediated BM failure such that disruption of this signaling pathway did not affect the overall destruction of BM cells by allogeneic LN cells. It had been shown that murine BM-derived macrophages secrete IFN-γ upon combined IL-12 and IL-18 stimulation, which defines a new pathway of autocrine macrophage activation [44]. IL-12/IL-18-stimulated IFN-γ production requires STAT4 signaling and is inhibited by IL-4 [45]. We speculate that the role of IL-18 in immune-mediated BM failure lies in its stimulation of IFN-γ secretion by various cell components. Because the IFN-γ-stimulatory effect can be mediated by many factors other than IL-18, disruption of the IL-18/IL-18R signaling pathway would not affect IFN-γ concentration to alter the level of BM destruction. However, IL-18BP was increased in AA in our and other studies, which might be another aspect of IL-18/IL-18R regulation as revealed by two recent reports in macrophage activation syndrome [46,47] and therefore may be worth further study in BM failure diseases.

Our current work demonstrated IL-18 as a marker of SAA onset. High levels of IL-18 more likely reflected aberrant immune responses and exaggeration of IFN-γ signaling and might exhibit fine modifications of

hematopoiesis. However, deficiency in IL-18 or IL-18R did not alleviate BM failure as observed in our murine models, so high levels of IL-18 might play dispensable roles in the regulation of the immune response or hematopoiesis in AA.

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ZW, XF, JC, WS, ZL, and NT performed experiments. ZW and XF analyzed data and wrote the manuscript. VG performed experiments, analyzed data, and edited the manuscript. KK performed cell sorting. SK analyzed data and edited manuscript. XF and NSY conceived, designed, and supervised the experiments; analyzed results; and edited the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interests.

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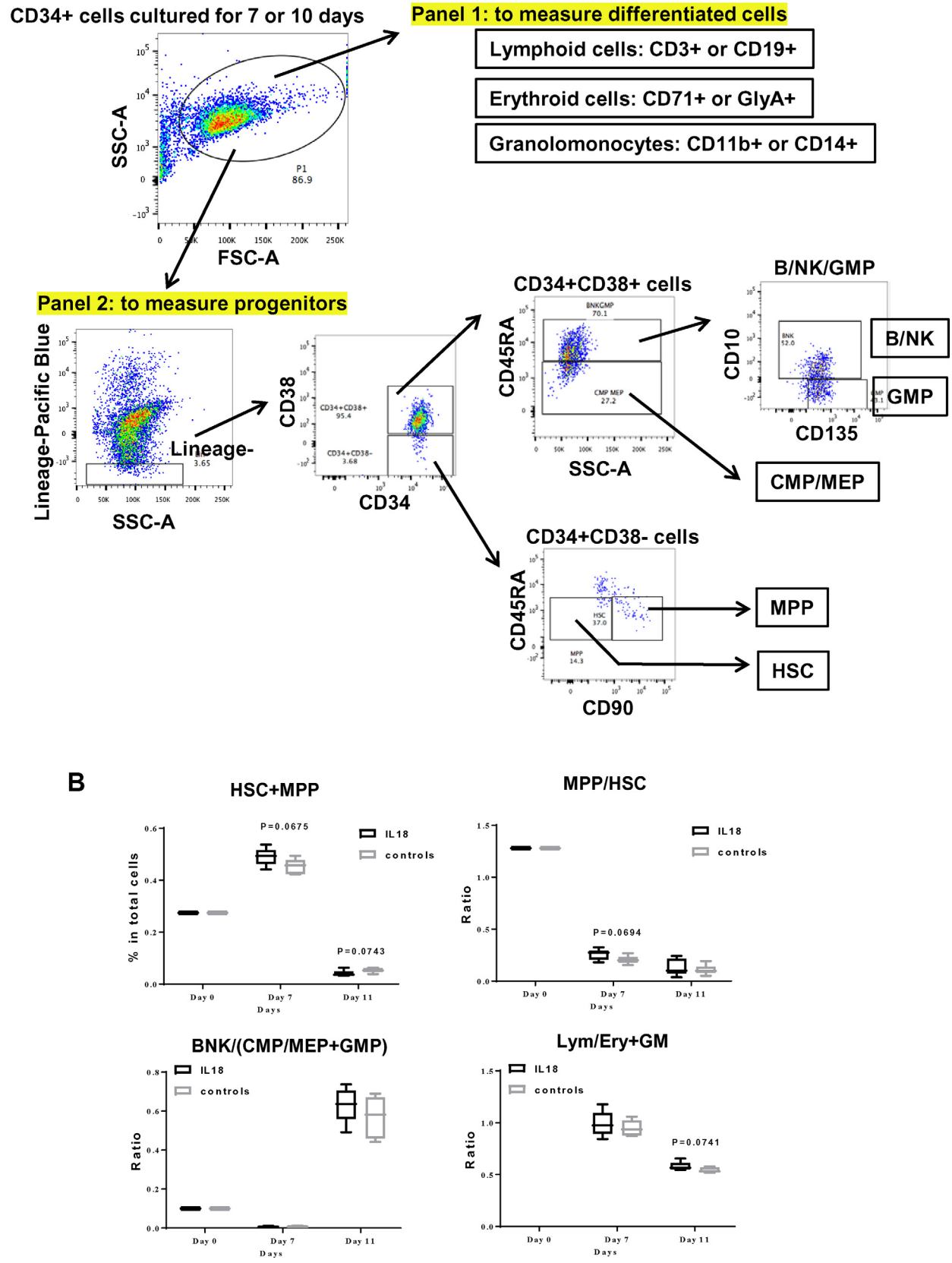
Supplemental Methods

Co-culture of human CD34+ cells with IL18

Lineage-CD34+ cells from 2 independent healthy donors were sorted after BM-MNCs separation from whole BM. Sorted CD34+ cells were cultured in StemSpan SPEM II serum-free medium plus StemSpan CD34+ Expansion Supplement (STEMCELL Technologies Inc., Vancouver, BC, Canada) in the presence or absence of 20 ng/ml recombinant human IL-18 (MBL), followed by flow cytometry analysis of cell subpopulations on day 7 and day 11. From lineage- cells, gating strategy of HSC, MPP, CMP/MEP, B/NK progenitors and GMP were shown in supplemental Figure 1A. For differentiated cells, erythroid cells were defined as CD71+ or GlyA+; lymphoid cells were defined as CD3+ or CD19+; granulomonocytes were defined as CD11b+ or CD14+. Experiments were done in triplicate. In progenitor cells, the percentage of multipotent-progenitor cells (HSC and MPP), and the ratio of lymphoid progenitors (B/NK progenitors) vs. myeloid progenitors (CMP/MEP+GMP) were calculated. In differentiated cells, the ratio of lymphoid cells (T and B cells) vs. myeloid cells (erythroid cells and granulomonocytes) were calculated.

Supplemental Figure 1.



Supplemental Figure 1. (**A**) Gating strategy of progenitor cell population (HSC, MPP, B/NK progenitors, GMP, and CMP/MEP) and differentiated cell population. (**B**) Percentage of HSC and MPP cells, ratio of MPP vs. HSC, ratio of B/NK progenitor cells vs. the sum of CMP/MEP and GMP, and ratio of lymphoid cells vs. myeloid cells in cultured human CD34+ cells at baseline, day 7 and day 11 (n=2, in triplicate). Abbreviations: HSC, hematopoietic stem cells; MPP, multi-potent progenitors; B/NK, B cell and NK cell progenitors; CMP, common myeloid progenitors; MEP, megakaryocyte-erythrocyte progenitors; GMP, granulocyte-monocyte progenitors; Lym, lymphoid cells including T cells and B cells; Ery, erythroid cells; GM, granulomonocytes.