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Original Research

Elucidating Mechanisms Underlying Fibrocyte-Mediated Myelofibrosis Induction in Myelodysplastic Syndromes

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Abstract

Background: The mechanism underlying myelofibrosis, which is associated with poor prognosis in myelodysplastic syndromes (MDS), remains unclear. **Methods:** We investigated the mechanisms underlying myelofibrosis using peripheral blood mononuclear cells (PBMCs) from healthy donors, MDS92 cell culture supernatants and *Polg*$^{A/+}$ and *Polg*$^{A/A}$ mice treated with high-dose romiplostim as an MDS model. We used the LEGENDplex™ immunoassay to analyze cytokine levels in MDS92 culture supernatants and serum samples from 12 patients with MDS or acute myeloid leukemia with myelodysplasia-related cytogenetic abnormalities. **Results:** The MDS92 cell culture supernatants inhibited monocyte differentiation into fibrocytes and contained significantly lower levels of CXCL1 (C-X-C motif chemokine ligand 1), IL-6 (Interleukin-6), and CCL22 (C-C motif chemokine ligand 22) than PBMC culture supernatants from healthy donors. Among serum samples from patients with MDS, IL-18 level was positively correlated with myelofibrosis grade and splenomegaly. In *Polg*$^{A/A}$ mice, the F4/80^{high} CD11b$^{low/int}$ cell fraction of the spleen was significantly increased following romiplostim administration, compared with *Polg*$^{A/+}$ mice; however, worsening of splenomegaly was not observed. Furthermore, reticulin fibers were observed mainly in the subcortical region in all *Polg*$^{A/+}$ mice but were rare in the *Polg*$^{A/A}$ mice. **Conclusions:** These findings suggest an association between altered cytokine signaling fibrocyte differentiation, and myelofibrosis in MDS. Further studies are required to clarify how cytokine levels and cellular responsiveness contribute to fibrocyte-mediated myelofibrosis.

Keywords: myelodysplastic syndrome; myelofibrosis; splenomegaly; fibrocyte; DNA polymerase gamma; fibroblast

1. Introduction

Myelodysplastic syndromes (MDS) are hematologic disorders caused by genetic abnormalities in hematopoietic stem cells of the bone marrow. Approximately 15% of patients have moderate to severe (grade 2–3) myelofibrosis [1,2,3,4], whereas mild (grade 0–1) myelofibrosis is observed in up to 50% of patients [4]. Furthermore, myelofibrosis is a poor prognostic factor in patients with MDS [3,5,6] and is associated with delayed engraftment during hematopoietic stem cell transplantation [7,8].

Although the precise pathophysiology underlying myelofibrosis remains unclear, increased production of cytokines, such as transforming growth factor-β, by megakaryocytes and platelets is considered to contribute to disease onset [9]. Other cytokines implicated in the pathophysiology of myelofibrosis include platelet-derived growth factor, calmodulin, and basic fibroblast growth factor [10]. Spliceosome and RAS pathway mutations, which are implicated in myelofibrosis observed in MDS [11], are thought to represent reactive processes, and fibroblasts within the bone marrow underlying myelofibrosis in patients with MDS are not monoclonal [9]. Furthermore, the *JAK2* V617F mutation is detected at a significantly higher frequency in patients with MDS and myelofibrosis than in those without myelofibrosis [3,12]; however, its direct contribution to the pathogenesis of myelofibrosis is unclear.

Fibrocytes, which are spindle-shaped monocyte-derived cells that express stromal cell markers, such as collagen, as well as hematopoietic cell markers, such as CD34, are considered to be involved in the pathogenesis of fibrosis in various organs. For example, five studies have reported fibrocytes as major players in promoting the progression of myelofibrosis and splenomegaly in myeloproliferative neoplasms (MPNs) [13,14,15,16,17]. In a previous study investigating the role of fibrocytes in MPN-related myelofibrosis, we demonstrated that fibrocyte differentiation was dependent on thrombopoietin signaling in mouse fibrocyte cell lines and in a mouse model of myelofibrosis induced by the thrombopoietin receptor agonist romiplostim [14]. Additionally, we showed that fibrocytes con-

tributed to MPN-related myelofibrosis via the thrombopoietin signaling pathway and that signaling lymphocytic activation molecule F7 (SLAMF7), a fibrocyte surface antigen, could be utilized as a molecular therapeutic target [15]. Furthermore, we investigated the role of fibrocytes in myelofibrosis and developed a method to identify fibrocyte precursors in peripheral blood (PB) using flow cytometry [14,15], allowing us to confirm an increase in the fraction of cells considered to be fibrocyte precursors in PB samples of patients with various hematologic diseases, including MDS, who presented with secondary myelofibrosis [14]. Therefore, in the present study, we aimed to elucidate the role of fibrocytes in myelofibrosis observed in patients with MDS.

2. Materials and Methods

2.1 Human Participants

This cross-sectional study included 12 participants with newly diagnosed MDS or acute myeloid leukemia (AML) with myelodysplasia-related cytogenetic abnormalities, as defined by the 2016 WHO classification and diagnostic criteria [18], between 2021 and 2023. All participants provided written informed consent and underwent bone marrow puncture and bone marrow needle biopsy. Additionally, PB mononuclear cells (PBMCs) from two healthy donors who provided written consent were used in cell culture experiments.

Clinical information was collected on the degree of bone marrow fibrosis, disease type, whether the disease was primary or relapsed, complete blood count, and splenomegaly. PBMCs and serum samples obtained at the time of bone marrow examination were stored for analysis. Bone marrow fibrosis was assessed by multiple clinical pathologists for each case, who evaluated bone marrow needle biopsy specimens according to the European consensus criteria [19] using a 4-point scale ranging from 0 to 3. Complete blood counts were performed with an automated multiparameter hematology analyzer (XE-5000; Sysmex, Hyogo, Japan). Splenomegaly was defined as the presence of a palpable spleen under the left costal arch on physical examination or a spleen with a long diameter of $\geq$10 cm on computed tomography images. Table 1 shows the patient characteristics.

2.2 Collection of PBMCs and Serum

PBMCs were isolated from heparinized PB samples collected from patients and healthy donors using density gradient centrifugation with Pancoll® (cat. no: P04-60500; PAN-Biotech, Aidenbach, Germany). Briefly, Pancoll was injected into the lower layer of the PB samples, which were centrifuged at 800 × *g* for 20 min. The middle layer was collected in a separate test tube and washed twice with phosphate-buffered saline supplemented with 2% fetal bovine serum (FBS). The resulting cell pellets were suspended in the cell preservation solution CELLBANKER-1® (cat. no: CB011; ZENOACH, Fukushima, Japan) and stored at −80 °C. Serum was collected by centrifuging PB samples at 1500 × *g* for 10 min and stored at −20 °C.

2.3 Culture and Evaluation of Human Fibrocytes

For human fibrocyte cultures, PBMCs from healthy donors at a final concentration of 5×10^5 cells/mL were suspended in RPMI-1640 medium (cat. no: R8758; Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% FBS, 1% penicillin/streptomycin, and 20 ng/mL Interleukin-3 (IL-3) (cat. no: AF-200-03; Peprotech, Cranbury, NJ, USA), plated at 3 mL/well in 6-well plates, and incubated at 37 °C with 5% CO_2 for 9 days. The medium was changed once on day 4. Experiments were performed with 3 wells/condition. On day 9 of culture, five fields of view at 200× were evaluated with an optical microscope to determine the percentage of differentiated fibrocytes, defined as adherent cells with elongated, spindle-shaped, and oval nuclei, among the total cells in each well, as previously described [14,15]. Cells that were detached or floating during measurement were considered lymphocytes or dead cells and were excluded from the count. This culture system was designated as the control.

2.4 Evaluation of the Fibrocyte-Inducing Activity of MDS92 Cell Culture Supernatants

MDS92 cells, established from tumor cells of patients with MDS [20], were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 5 µg/mL IL-3 at 37 °C with 5% CO_2. The MDS92 cell line was validated by STR profiling and tested negative for mycoplasma by the RIKEN BRC Cell Bank. For experiments, MDS92 cells were plated at a concentration of 5×10^5 cells/mL, cultured for 24 h, then detached and centrifuged at 400 × *g* for 10 min to collect culture supernatants. The culture supernatants were stored at −20 °C until use. After thawing the MDS92 culture supernatants, PBMCs from two healthy donors were suspended at a final concentration of 5×10^5 cells/mL and cultured in 6-well plates for 9 days under the same conditions as the control group described above. The number of adherent cells, the number of fibrocytes, and the percentage of fibrocytes among adherent cells were compared between the control and MDS92 cell culture supernatant groups.

In addition, RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 5 µg/mL IL-3 was mixed with MDS92 culture supernatants at ratios of 1:3, 1:1, and 3:1 and added to PBMCs isolated from two healthy donors. The PBMCs were suspended at a final concentration of 5×10^5 cells/mL and cultured in 6-well plates for 9 days under the same conditions as the control group described above. The number of adherent cells, the number of fibrocytes, and the proportion of fibrocytes among adherent cells were assessed after 9 days of culture.

Table 1. Characteristics of patients with myelodysplastic syndromes presenting with grade 0–3 myelofibrosis.

Variables	Number of patients (n = 12)
Female, n	4
Age (years), mean ± SD	61.8 ± 15.9
Disease, n	
MDS-MLD	2
MDS-RS	2
MDS-EB	2
MDS-U	1
MDS/MPN	3
AML-MRC	2
Myelofibrosis grade, n	
Grade 0	3
Grade 1	6
Grade 2	3
Splenomegaly, n	5
White blood cell count (10^9/L), mean ± SD	5.9 ± 6.7
Hemoglobin (g/dL), mean ± SD	7.9 ± 1.7
Platelet count (10^9/L), mean ± SD	12.3 ± 10.7
C-reactive protein (mg/dL), mean ± SD	2.7 ± 4.1

MDS-MLD, myelodysplastic syndrome with multilineage dysplasia; MDS-RS, myelodysplastic syndrome with ring sideroblasts; MDS-EB, myelodysplastic syndrome with excess blasts; MDS-U, myelodysplastic syndrome with unclassifiable; MPN, myeloproliferative neoplasm; AML-MRC, acute myeloid leukemia with myelodysplasia-related changes; SD, standard deviation.

2.5 Measurement of Cytokines in Culture Supernatants and Patient Serum Samples

Cytokines secreted by MDS92 cells were measured in culture supernatants using a multiplex bead assay for human cytokines and chemokines (LEGENDplex™; BioLegend, San Diego, CA, USA); the assay included measurements for TNF-α, interferon-γ, CCL2, CCL4, CCL22, CXCL1, IL-1RA, IL-4, IL-6, IL-7, IL-12p70, IL-13, IL-18, and macrophage-colony stimulating factor. Samples were processed according to the manufacturer's instructions, and data were acquired on a BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using the dedicated data analysis software suite for LEGENDplex™. Additionally, serum samples from 12 patients diagnosed with MDS or AML with myelodysplasia-related cytogenetic abnormalities were used to measure the levels of 14 cytokines using LEGENDplex™, as described above. In all LEGENDplex™ experiments, each sample was measured in duplicate.

2.6 Induction of Myelofibrosis by High-Dose Romiplostim in Polg Mice

High-dose romiplostim induces myelofibrosis in 40-week-old female heterozygous *Polg*$^{A/+}$ and homozygous *Polg*$^{A/A}$ mice in a model of MDS, as previously reported [14,15]. In the present study, four *Polg*$^{A/+}$ and four *Polg*$^{A/A}$ mice (for a total of eight mice) were administered romiplostim subcutaneously at 1000 µg/kg on days 1 and 8 and sacrificed on day 10. This sample size was predetermined to detect a statistically significant difference in the presence or absence of bone marrow fibrosis and splenomegaly in a nonparametric analysis of two unpaired groups. These individuals were generated by breeding a pair of *Polg*$^{A/+}$ mice—purchased from the Jackson Laboratory (Bar Harbor, ME, USA) in December 2022—through May 2024 at the Animal Experimental Facility of National Defense Medical College. This experiment was conducted as an open-label study without randomization at the Animal Experimental Facility of National Defense Medical College; to minimize confounding factors, the mice were administered the same batch of the drug on the same day and at the same time. In addition, a humane endpoint was established in advance, and it was decided that euthanasia would be performed promptly if any of the following conditions were observed: difficulty eating or drinking due to growth retardation; signs of distress; long-term physical abnormalities with no signs of recovery; or rapid weight loss. However, no individuals met these criteria. Euthanasia was performed by anesthetizing the mice through intraperitoneal administration of medetomidine hydrochloride (0.3 mg/kg, 1 mg/mL), midazolam (4 mg/kg, 5 mg/mL), and butorphanol tartrate (5 mg/kg, 5 mg/mL), followed by exsanguination through blood collection from the inferior vena cava. The study was carried out in accordance with the ARRIVE guidelines.

The ratios of fibrocyte progenitor cells in PB and spleen, as well as the degrees of splenomegaly and myelofibrosis, were compared between *Polg*$^{A/+}$ and *Polg*$^{A/A}$ mice. Briefly, following hematoxylin/eosin, silver, and Masson's trichrome staining, tissue specimens from the femurs of mice were independently evaluated by a hematologist and a pathologist to determine the grade of myelofibrosis in humanized bone marrow regions according to the World Health Organization Classification of Tumors, Fifth Edition [21]. Additionally, cells from the PB and spleen of *Polg* mice were resuspended in 10 µL FACS buffer (phosphate-buffered saline with 2% FBS and 0.05% NaN_3) containing 0.5 µL anti-mouse CD16/CD32 (cat. no: 14-0161-81; eBioscience, San Diego, CA, USA) for 10 min on ice to block Fc receptors. Next, 2 µL FITC-conjugated anti-F4/80 antibody (cat. no: 123108; BioLegend), 0.4 µL PE-conjugated anti-CD11b antibody (cat. no: 101207; BioLegend), 0.4 µL V450-conjugated anti-CD14 antibody (cat. no: 560639; BD Biosciences), 0.4 µL APC-conjugated anti-SLAMF7 antibody (cat. no: bs-2544R-A647; Bioss, Woburn, MA, USA), and 6.8 µL FACS buffer were added to each sample and incubated at 4 °C in the dark for 30 min. Next, the cells were washed twice with 500 µL FACS buffer and analyzed using BD FACS Canto™ II. Macrophages were defined as F4/80^{high}/CD11b^{high} cells, and fibrocyte precursors were defined as F4/80^{high}/CD11b^{high}/SLAMF7^{high}/CD14^{low} cells, based on previous studies [14,15]. For F4/80^{high}/CD11b^{high} cells in the spleen, a gate was set on the monocyte population using FSC and SSC; doublets were removed by plotting against FSC-H and FSC-W; the plot was then plotted against F4/80 and CD11b, and the target fraction was defined as shown in Fig. 5C. For F4/80^{high}/CD11b^{high}/SLAMF7^{high}/CD14^{low} cells in peripheral blood, a gate was set on the monocyte population using FSC and SSC; doublets were removed by plotting against FSC-H and FSC-W; the plot was then plotted against F4/80 and CD11b, and a gate was set on the F4/80^{high}/ CD11b^{high}, and defined the target fraction as shown in Fig. 5C.

2.7 Statistical Analysis

Descriptive statistics were reported as means ± standard deviation of data obtained from three independent experiments, unless specified otherwise, and statistical significance was determined using the unpaired Student's *t*-test or one-way analysis of variance with Dunnett's correction. All *p*-values were calculated using two-tailed tests, and a *p*-value of <0.05 was considered statistically significant. GraphPad Prism 11.0.2 (GraphPad Software, La Jolla, CA, USA) and JMP version 17.0.0 (SAS Institute, Cary, NC, USA) were used for data visualization and statistical analysis.

3. Results

3.1 MDS92 Culture Supernatants Inhibit the Differentiation of PBMCs Derived From Healthy Donors Into Fibrocytes

In experiments evaluating the ability of MDS92 cell culture supernatants to induce the differentiation of human PBMCs isolated from healthy controls, the mean number of fibrocytes were lower in the MDS92 culture supernatant group than in the control group (13.8 ± 3.3 vs 101.0 ± 11.4, $p < 0.01$, Cohen's d –13.04, 95% confidence interval –98.34 to –75.99) (Fig. 1A). On the other hand, no significant difference was observed in the mean number of adherent cells between the two groups (496.2 ± 43.3 vs. 525.7 ± 63.0, Cohen's d –0.5934, 95% confidence interval –112.6 to 53.63) (Fig. 1B). Therefore, the mean frequency of fibrocytes among adherent cells, i.e., the fibrocyte proportion, was lower in the MDS92 culture supernatant group than in the control group (2.8 ± 0.6% vs. 19.4 ± 3.6%, $p < 0.01$, Cohen's d –8.311, 95% confidence interval –20.00 to –13.30) (Fig. 1C).

Furthermore, among PBMCs cultured with varying ratios of RPMI-1640 medium and the MDS92 culture supernatants, the mean number of fibrocytes decreased in parallel with increasing MDS92 culture supernatant ratios (42.0 ± 12.7, 31.0 ± 2.8, 24.0 ± 4.2, 16.0 ± 8.5, and 12.5 ± 6.4 in cultures exposed to MDS92 culture ratios of 0:1, 1:3, 1:1, 3:1, and 1:0, respectively) (Fig. 1D). In contrast, the mean number of adherent cells increased in the group to which MDS92 culture supernatant was added (229.0 ± 32.5, 664.5 ± 47.4, 580.5 ± 72.8, 574.5 ± 109.6, and 457.0 ± 24.0 in cultures exposed to MDS92 culture ratios of 0:1, 1:3, 1:1, 3:1, and 1:0, respectively) (Fig. 1E). Therefore, the mean fibrocyte proportion decreased in parallel with increasing MDS92 culture supernatant ratios (18.1 ± 3.0%, 4.7 ± 0.7%, 4.2 ± 0.2%, 2.7 ± 1.0%, and 2.7 ± 1.3% in cultures exposed to MDS92 culture ratios of 0:1, 1:3, 1:1, 3:1, and 1:0, respectively) (Fig. 1F).

3.2 Significantly Lower Levels of CXCL1, IL-6, and CCL22 in MDS92 Culture Supernatants Compared With the PBMC Culture Supernatants of Healthy Donors are Correlated With the Proportion of Fibrocytes

The mean levels of CXCL1, IL-6, and CCL22 were significantly lower in the MDS92 culture supernatants than in the supernatants of PBMCs from healthy donors (5.3 ± 1.2 vs. 149.9 ± 76.1 pg/mL, $p < 0.01$, Cohen's d –4.653, 95% confidence interval –206.8 to –82.55; 1.7 ± 0.1 vs. 9.0 ± 6.1 pg/mL, $p < 0.05$, Cohen's d –2.934, 95% confidence interval –12.34 to –2.342; and 34.0 ± 43.2 vs. 1024.0 ± 1203.0 pg/mL, $p < 0.05$, Cohen's d –2.011, 95% confidence interval –1975 to –6.274 respectively; Fig. 2A). The levels of the remaining 11 cytokines were not significantly different between the two groups.

The CXCL1 and IL-6 levels in MDS92 culture supernatants were significantly correlated with the proportion of

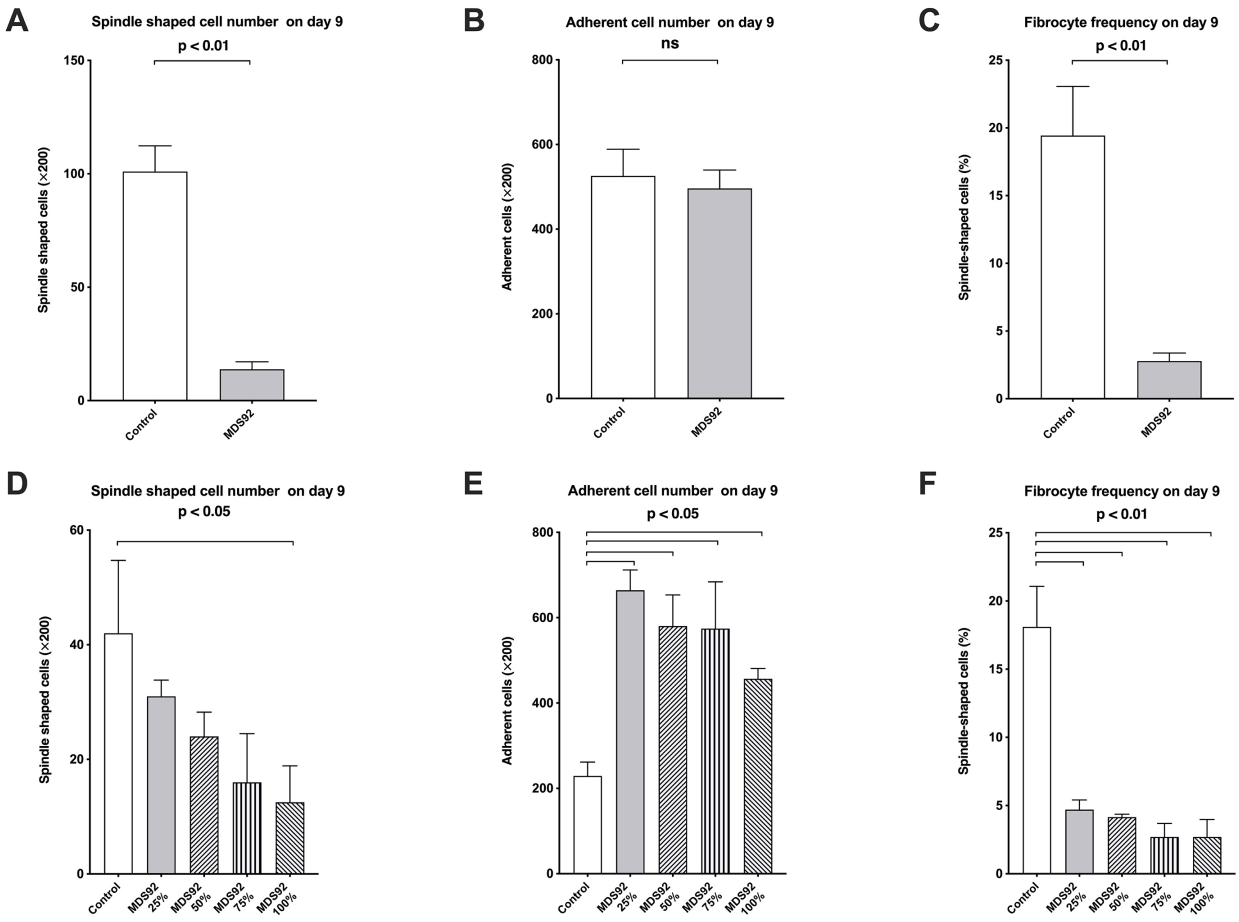

Fig. 1. MDS92 cell culture supernatants inhibit the differentiation of peripheral blood mononuclear cells derived from healthy subjects into fibrocytes. (A) The mean number of fibrocytes is lower in peripheral blood mononuclear cell (PBMC) cultures in the MDS92 culture supernatant group than in the control group ($p < 0.01$). (B) The mean total number of adherent cells, including macrophages, is not significantly different between the MDS92 culture supernatant and control groups. (C) The mean proportion of fibrocytes is significantly lower in the MDS92 culture supernatant group than in the control group ($p < 0.01$). (D) In culture experiments using different ratios of RPMI-1640 medium and MDS92 culture supernatants, fibrocyte numbers either remained unchanged or were significantly reduced in cultures exposed to medium containing MDS92 supernatants compared with RPMI-1640 alone ($p < 0.05$). (E) In culture experiments using different ratios of the RPMI-1640 medium and MDS92 culture supernatants, the mean number of adherent cells increased in the group to which MDS92 culture supernatant was added ($p < 0.05$). (F) In culture experiments using different ratios of RPMI-1640 medium and MDS92 culture supernatants, the proportion of fibrocytes is significantly lower in cultures exposed to medium containing MDS92 culture supernatants compared with RPMI-1640 medium ($p < 0.01$). ns, not significant.

fibrocytes ($p < 0.01$, $R^2 = 0.801$ and $p < 0.05$, $R^2 = 0.629$, respectively) (Fig. 2B). No significant correlations were observed for the remaining 12 cytokines.

3.3 Increased Serum Levels of IL-18 in Patients With MDS Correlate With the Severity of Myelofibrosis and Splenomegaly

By simple linear regression analysis, myelofibrosis grade was significantly correlated with serum CXCL1, IL-18, and IL-6 levels in patients with MDS ($p < 0.01$, $R^2 = 0.502$; $p < 0.01$, $R^2 = 0.571$; and $p < 0.05$, $R^2 = 0.389$, respectively) (Fig. 3A). No significant correlations were observed for the remaining 11 cytokines.

The mean serum CXCL1 level tended to be higher in patients with MDS and splenomegaly than in those with MDS in the absence of splenomegaly (100.6 ± 75.6 vs. 33.2 ± 27.5 pg/mL, $p = 0.0523$, Cohen's d 1.289, 95% confidence interval −0.8179 to 135.7), whereas the mean serum IL-18 level was higher in patients with MDS and splenomegaly than in those with MDS in the absence of splenomegaly (138.5 ± 70.9 vs. 72.5 ± 19.4 pg/mL, $p < 0.05$, Cohen's d 1.397, 95% confidence interval 4.364 to 127.8) (Fig. 3B). No significant increases were observed for the remaining 12 cytokines.

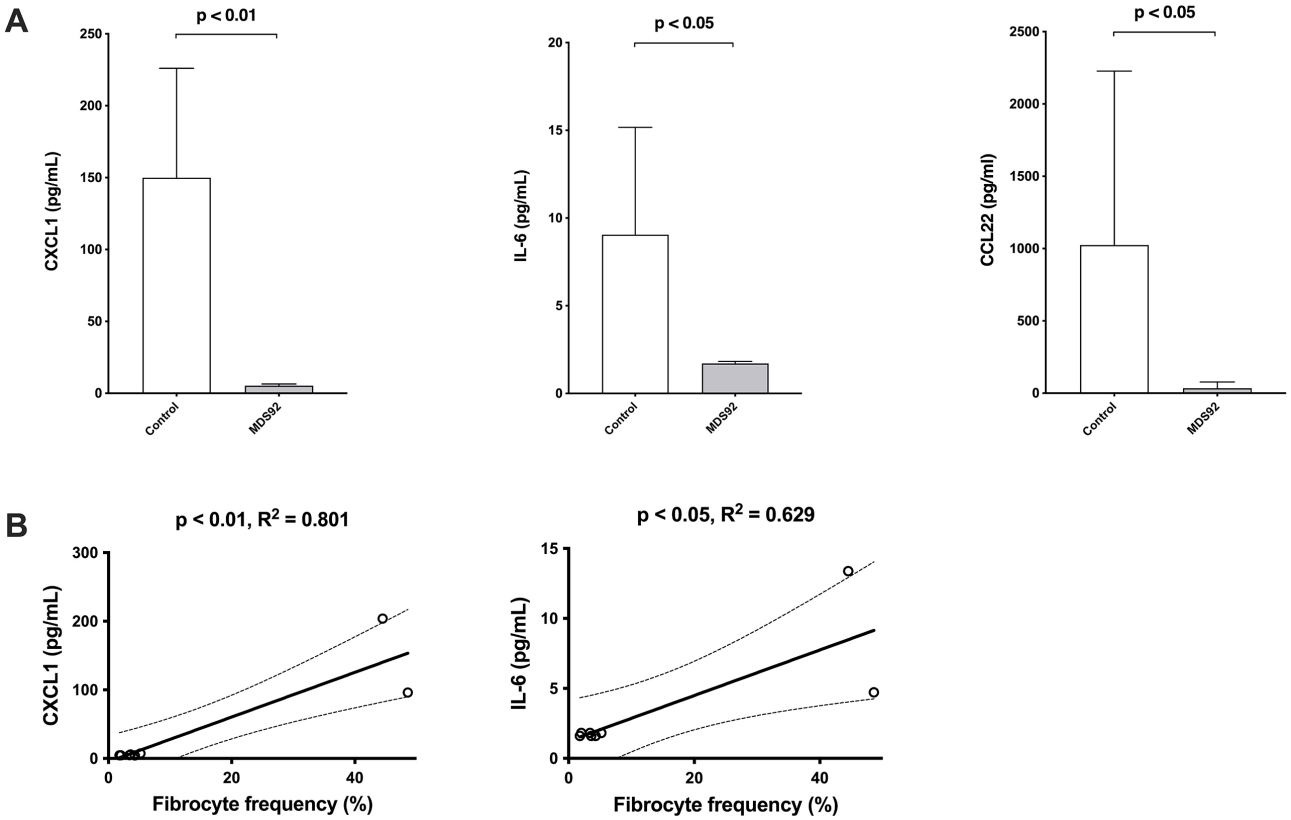

Fig. 2. Comprehensive analysis of cytokines in culture supernatants. (A) MDS92 cell culture supernatants exhibit significantly lower levels of CXCL1, IL-6, and CCL22 compared with the PBMCs from healthy donors. (B) The levels of CXCL1 and IL-6 in culture supernatants are significantly correlated with the proportion of fibrocytes. The dotted lines indicate the 95% confidence interval.

3.4 Increased Reticular Fiber Density Immediately Beneath the Femoral Cortical Bone is Observed in Polg$^{A/+}$ Mice but is Absent in Polg$^{A/A}$ Mice

Finally, we compared the degree of femoral fibrosis between the *Polg*$^{A/+}$ and *Polg*$^{A/A}$ mice administered high-dose romiplostim. The reticular fiber density immediately beneath the cortical bone was increased in all four *Polg*$^{A/+}$ mice, which were graded to harbor grade 0 myelofibrosis in hematopoietic niches. In contrast, no increased reticular fiber density was observed directly beneath the cortical bone of any of the four *Polg*$^{A/A}$ mice that were evaluated, although these mice harbored grade 0 myelofibrosis in hematopoietic niches. These results indicated significantly reduced myelofibrosis in the *Polg*$^{A/A}$ mice (n = 4) compared with the *Polg*$^{A/+}$ mice (n = 4) ($p < 0.05$, Mann–Whitney test) (Fig. 4A,B).

3.5 Splenomegaly is not Worse Despite an Increase in Splenic Fibrocyte Precursor Number in Polg$^{A/A}$ Mice Compared With Polg$^{A/+}$ Mice

Flow cytometry analysis of spleen samples after romiplostim administration revealed that the mean proportion and mean absolute number of F4/80^{high+}/CD11b$^{low/int}$ cells were significantly higher in the spleen of the *Polg*$^{A/A}$ mice (n = 4) than in the *Polg*$^{A/+}$ mice (n = 4) (14.1 ± 4.1% vs. 4.1 ± 0.7% and 232,885 ± 46,575/spleen vs. 103,680 ± 23,861/spleen, $p < 0.01$, Cohen's d 3.434 and 3.492, 95% confidence interval 4.985 to 15.11 and 65,179 to 193,231). However, the progression of splenomegaly was not worse in the *Polg*$^{A/A}$ than in the *Polg*$^{A/+}$ mice (mean spleen weight, 173.0 ± 21.5 mg and 188.5 ± 23.3 mg, respectively, Cohen's d −0.6919, 95% confidence interval −54.26 to 23.26) (Fig. 5A,C).

Although there was no difference in the mean proportion of F4/80^{high}/CD11b^{high}/SLAMF7^{high}/CD14^{low} cells, i.e., fibrocyte precursors, in PB samples between the *Polg*$^{A/A}$ (n = 4) and *Polg*$^{A/+}$ mice (n = 4) (9.1 ± 4.9% vs 12.7 ± 6.2%, respectively, Cohen's d −0.6449, 95% confidence interval −13.32 to 6.088), the mean absolute number of fibrocyte precursors was significantly lower in the *Polg*$^{A/A}$ mice than in the *Polg*$^{A/+}$ mice (106.3 ± 64.1/body vs 277.5 ± 29.0/body, $p < 0.01$, Cohen's d −3.443, 95% confidence interval −257.3 to −85.19) (Fig. 5B,C).

4. Discussion

In the present study, which aimed to elucidate the role of fibrocytes in myelofibrosis observed in patients with MDS, we found that MDS92 culture supernatants promoted

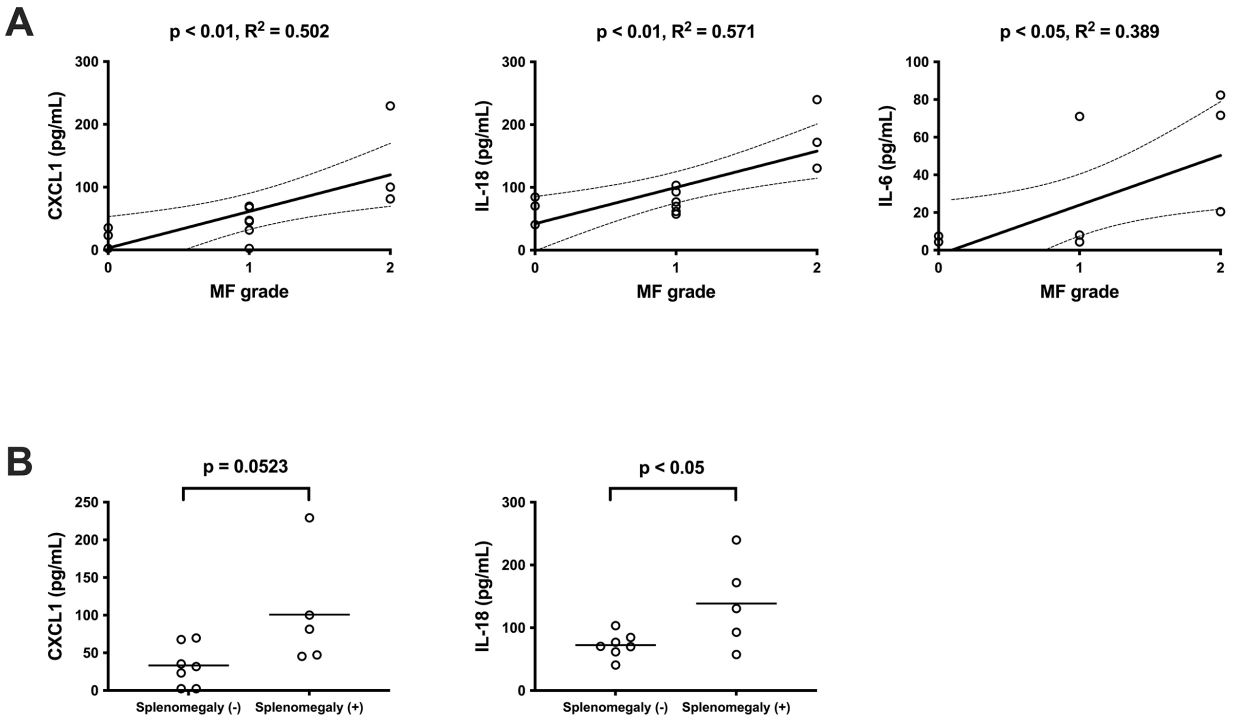

Fig. 3. Comprehensive analysis of cytokines in serum samples of patients with myelodysplastic syndromes. (A) In serum samples from patients with myelodysplastic syndromes (MDS), myelofibrosis grade is significantly correlated with the levels of CXCL1, IL-18, and IL-6 (simple linear regression). The dotted lines indicate the 95% confidence interval. (B) The level of CXCL1 tends to be higher, and the level of IL-18 is significantly higher in the serum samples from patients with MDS and splenomegaly than in those from patients with MDS without splenomegaly. MF, myelofibrosis.

monocyte differentiation into macrophages but not into fibrocytes. We also found that the levels of several cytokines, including CXCL1, IL-6, and CCL22, were lower in MDS92 culture supernatants than in control supernatants. Regarding the relationship between fibrocytes and cytokines, the addition of IL-1β to fibrocytes isolated from human PB has been shown to induce the secretion of chemokines (CCL3, CCL4, CCL2, IL-8, and CXCL1), hematopoietic growth factors (IL-6, IL-10, and Macrophage colony-stimulating factor (M-CSF)), and the fibrosis-inducing cytokine TNF-α [22]. Moreover, IL-18/IL-18 receptor 1 has been demonstrated to promote the differentiation of fibroblast-like cells circulating in the blood of elderly individuals [23]. Furthermore, in an analysis of patients with MDS and chronic myelomonocytic leukemia, both plasma IL-1β and IL-18 were elevated; in particular, IL-18 was significantly elevated in patients with advanced disease and served as a predictor of survival [24]. This finding was consistent with a mouse study showing that, in an age-related MDS model, IL-1β and IL-18 directly impair the function of bone marrow mesenchymal stem cells and reduce their ability to support hematopoietic stem/progenitor cells and erythroid progenitor cells [25]. In addition, the addition of IL-4 and IL-13 to human PBMCs cultured in serum-free medium promotes their differentiation into fibrocytes, a process that is inhibited by the addition of interferon-γ and IL-12 [26]. Therefore, the direct or indirect release of cytokines by tumor cells that promote the differentiation of monocytes into fibrocytes is thought to be closely involved in the development of myelofibrosis in MDS. Furthermore, our findings suggest that MDS92 cells do not secrete cytokines that directly promote monocyte differentiation into fibrocytes.

In patients with MDS complicated by myelofibrosis, plasma concentrations of transforming growth factor-β and thrombopoietin are elevated and correlate with the severity of myelofibrosis [27]. In the present study, the serum levels of CXCL1, IL-18, and IL-6 were elevated in patients with MDS; their levels also correlated with the grade of myelofibrosis. Furthermore, the levels of CXCL1 and IL-18 were elevated in patients with MDS complicated by splenomegaly. We previously reported elevated serum levels of TNF-α (**Supplementary Fig. 1A**) and IL-1RA [15] in patients with *JAK2* V617F-positive MPN accompanied by myelofibrosis as well as elevated serum levels of CXCL1, CCL4, IL-6, CCL2 (**Supplementary Fig. 1B**) and IL-1RA [15] in patients with splenomegaly, suggesting that cytokines involved in fibrocyte differentiation contribute to the development of secondary myelofibrosis in MDS and indicating that a fibrocyte-mediated pathway may induce myelofibrosis in MDS.

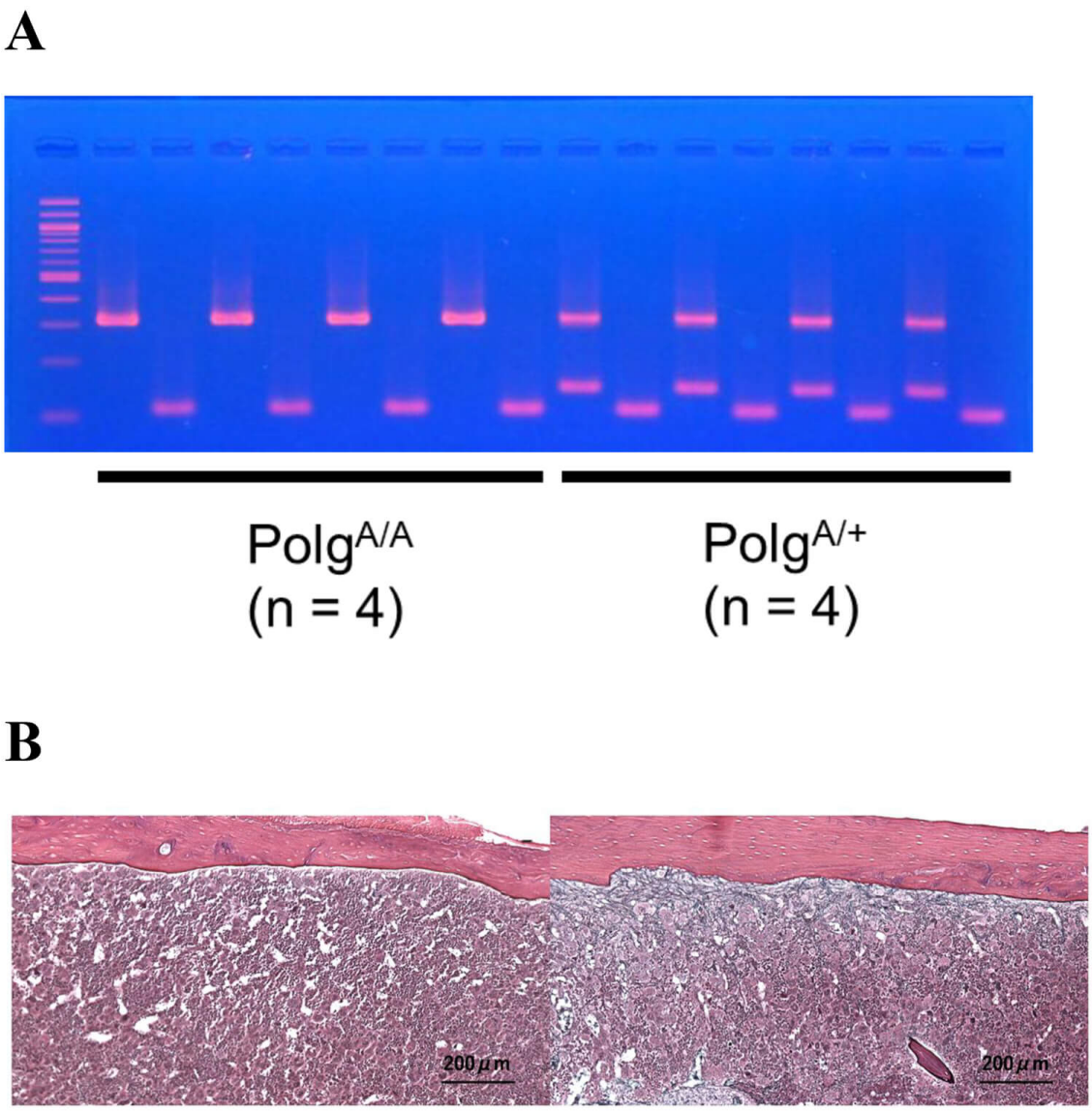

Fig. 4. Comparison of myelofibrosis by silver staining in *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice. (A) Genotyping to differentiate between *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice. We performed PCR on DNA extracted from the ears of *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice using primers published by Jackson Laboratory, followed by electrophoresis of the amplified products on an agarose gel. The primer sequences are as follows: Polg 25251 Common: AGTCCTGCGCCAACACAG, Polg 25252 WT-F: GCTTTGCTTGATCTCTGCTC, Polg oIMR7919 MT-F: ACGAAGTTATTAGGTCCCTCGAC. (B) The increase in reticular fibers immediately beneath the cortical bone observed in all four *Polg*$^{A/+}$ mice (right panel) is absent in all four *Polg*$^{A/A}$ mice (left panel), indicating significantly suppressed myelofibrosis ($p < 0.05$, Mann–Whitney test). All *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice were determined to harbor grade 0 myelofibrosis based on the evaluation of hematopoietic niches. Images were acquired and digitized using a BZ-X810 microscope with BZ-H4A software. The original magnification for (B) was ×100. Scale bar, 200 μm.

Polg$^{A/A}$ mice, which carry a mitochondrial DNA polymerase γ mutation (Polg D257A), exhibit several phenotypes, such as premature aging and MDS-like anemia [28,29]. Although *Npm1* [30,31], *Nup98-Hoxd13* [32], and *Runx1* [33] mice are used to model MDS, these animals die prematurely due to leukemic transformation. Therefore, we considered that it would be challenging to observe the changes of interest over time in our experimental model, which involved the induction of myelofibrosis using a thrombopoietin receptor agonist. We previously demonstrated that the F4/80^{high}/CD11b$^{low/int}$ fraction of the spleen contained fibrocyte precursors and that removing this fraction with clodronate liposomes suppressed several symptoms, such as splenomegaly and myelofibrosis [14]. However, in the present study, although this cell fraction was significantly increased in the *Polg*$^{A/A}$

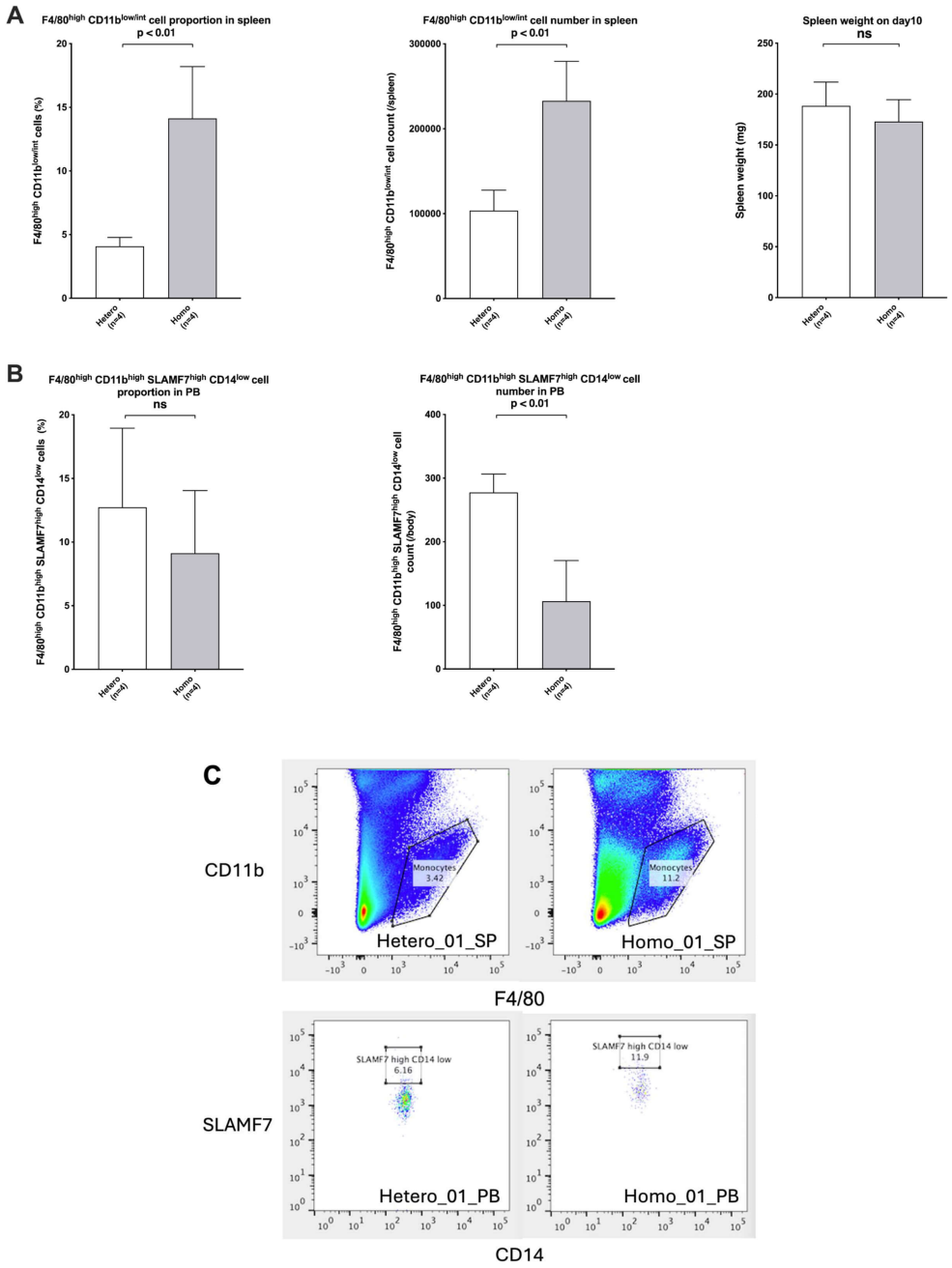

Fig. 5. Flow cytometric analysis of the peripheral blood and spleen samples of *Polg* mice following romiplostim treatment. (A) The percentage and absolute number of F4/80^{high}/CD11b$^{low/int}$ cells in the spleen are higher in the *Polg*$^{A/A}$ mice than in the *Polg*$^{A/+}$ mice, whereas spleen weight is not significantly different between the *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice. (B) Although the proportion of F4/80^{high}/CD11b^{high}/SLAMF7^{high}/CD14^{low} cells in peripheral blood is not different between the *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice, the absolute number of these cells is significantly lower in the *Polg*$^{A/A}$ mice than in the *Polg*$^{A/+}$ mice. (C) Representative flow cytometry plots showing spleen and peripheral blood samples from the *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice (sample #1 for each genotype). PB, peripheral blood; SP, spleen. ns, not significant.

mice compared with the *Polg*$^{A/+}$ mice, the number of F4/80^{high}/CD11b^{high}/SLAMF7^{high}/CD14^{low} cells, the fraction that we believe contains the fibrocyte precursor cells in PB, was actually decreased in *Polg*$^{A/A}$ mice, accompanied by the suppression of myelofibrosis and no worsening of splenomegaly. These results imply two possibilities. Either the mechanism of myelofibrosis induction via the thrombopoietin receptor signaling pathway is not functioning properly in *Polg*$^{A/A}$ mice or the differentiation of monocytes into fibrocytes is inhibited in this mouse line. Reports

suggest that the hypoxic microenvironment in MDS enhances HIF-1α expression, thereby inhibiting NK cell maturation and suppressing their cytotoxic activity [34]. These findings suggest that the function and reactivity of various cells may be impaired in MDS.

Limitation

A major study limitation is the limited number of cases in this single-center study, owing to the low incidence of MDS with myelofibrosis. Furthermore, while this study provides circumstantial evidence suggesting that secondary bone marrow fibrosis in MDS is associated with a fibrocyte-mediated mechanism of bone marrow fibrosis induction, more detailed analyses of phenotypic and functional characteristics are needed to establish a stronger link between these factors. Future investigations based on our findings should include prospective, multicenter studies.

In addition, this study did not investigate the off-target effects of high-dose romiplostim administration in the *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice.

5. Conclusion

Using an MDS cell line and *Polg*$^{A/A}$ and *Polg*$^{A/+}$ mice, we demonstrated that the induction of fibrocyte differentiation and the myelofibrosis phenotype were suppressed in parallel with decreased cytokine levels. Elevated cytokine levels were correlated with myelofibrosis and splenomegaly observed in patients with MDS, suggesting that elevated levels of cytokines that promote fibrocyte differentiation and monocyte responsiveness to these cytokines contribute to the development of myelofibrosis in MDS.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

TM and YO performed the research. NM, YY, EK, TSho, KK, TSo, KT, HO, KS, NT, TK and SKa acquired patient samples and clinical data. TM designed the research study, analyzed the data and wrote the paper. SKo designed the research study and supervised the research study. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal procedures and experiments were approved by the Animal Experiment Ethics Committee of the National Defense Medical College (approval no: 22049) and performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments guidelines and the American Veterinary Medical Association Guidelines for the Euthanasia of Animals. This study involving human participants was approved by the Institutional Review Board of the National Defense Medical College (approval no: 4419) and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL54751.

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