Mobilization of Bone Marrow Cells by CSF3 Protects Mice from Bleomycin-Induced Lung Injury

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Key Words

Lung fibrosis • Bleomycin • Bone marrow stem cells • CSF3

Abstract

Background: Bone marrow-derived cells may play a role in tissue injury and repair. Growth factors facilitate the mobilization of bone marrow-derived cells to the site of injury. **Objectives:** The aim of this study was to determine the effect of the mobilization of autologous bone marrow-derived cells by granulocyte colony-stimulating factor (CSF3) on bleomycin-induced lung injury in mice. **Methods:** The bone marrow from male green fluorescent protein transgenic (C57Bl/6J) mice was transplanted into irradiated female C57Bl/6J mice. Bleomycin lung injury was induced in these bone marrow-reconstituted mice and unreconstituted C57Bl/6J mice, and some mice were treated with recombinant CSF3. Lung histology, survival, cytokine expression and matrix metalloproteinase (MMP) expression were evaluated to determine the effect of CSF3 after bleomycin-induced lung injury. **Results:** Histology and flow cytometry analysis showed successful mobilization of bone marrow-derived cells by CSF3 treatment in the recipient lungs. Importantly, CSF3 attenuated bleomycin-induced lung injury and improved survival. Furthermore, CSF3 administration regulated transforming

growth factor- β , interferon- γ , MMP9 and tissue inhibitors of MMP1 expression during bleomycin injury. **Conclusions:** These data demonstrated that the mobilization of bone marrow-derived cells by CSF3 has a protective effect against bleomycin-induced lung injury and fibrosis.

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Introduction

Interstitial pulmonary fibrosis (IPF) is a progressive and usually fatal lung disease characterized by focal areas of alveolar epithelial cell injury and proliferation of mesenchymal cells in the interstitium resulting in the deposition of extracellular matrix (ECM) and distorted architecture leading to impaired gas exchange [1–3]. Lung function tests show a restrictive ventilatory pattern and reduced diffusion capacity associated with normal expiratory flow [4]. Currently, there is no effective treatment. In patients with end-stage IPF, mechanical ventilation does not appear to have a significant impact on survival

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[5]. The pathogenesis of IPF is largely unknown. Alveolar type II cell apoptosis may be an important early feature in the pathogenesis of pulmonary fibrosis [6, 7]. Cytokines play an important role in the process of pulmonary fibrosis [8–10]. For example, transforming growth factor (TGF)- β 1 increases the synthesis of ECM and decreases its degradation [11–14]. The transcription level of TGF- β 1 has been shown to correlate with the decrease of two lung function parameters, vital capacity and total lung capacity [15].

Pulmonary fibrosis may also be related to an imbalance between Th1 and Th2 cytokines [16]. Interferon (IFN)- γ is a Th1 cytokine and plays an important role in limiting pulmonary fibrosis [17, 18]. IFN- γ can slow or reverse the loss of lung function of patients with IPF [19]. The ECM is considered one of the most important components involved in pulmonary fibrosis [3, 20, 21]. In physiological conditions, the synthesis and degradation of ECM remain in a dynamic balance. Matrix metalloproteinases (MMPs) are a main enzyme system that degrades ECM, while tissue inhibitors of MMPs (TIMPs) negatively regulate MMP activity. An imbalance between MMPs and TIMPs may play an important role in the process of pulmonary fibrosis [22, 23]. MMP9 not only degrades the ECM and basement membrane but also regulates other cytokines in the process of pulmonary fibrosis [22, 23]. On the other hand, TIMP1 specifically inhibits the activity of MMP9 [24].

Bone marrow-derived cells have been suggested to play a role in lung injury and repair [25]. Hematopoietic stem cells play a role in tissue repair [26, 27], although different mechanisms underlying tissue repair have been proposed. Mesenchymal stem cells have strong proliferation ability and differentiation potential [28–30]. They can differentiate into various types of tissue cells and repair injured tissue [28]. This provides a theoretical basis for the treatment of incurable diseases. Mesenchymal stem cells can differentiate into alveolar type I epithelial cells [31, 32]. Moreover, mesenchymal stem cells have been shown to decrease the degree and duration of bleomycin-induced inflammation [33]. However, transplantation of bone marrow stem cells has disadvantages, including in vitro manipulation and highly technical requirements, thus limiting its application in clinical therapy. Granulocyte colony-stimulating factor (G-CSF or CSF3) is widely used as a mobilizer of bone marrow stem cells in the clinic [34] and in experimental conditions [33]. Orlic et al. [35] verified that CSF3 could mobilize bone marrow cells to repair the infarcted heart and improve function and survival. We wanted to know if bone marrow stem cells could be

mobilized to injured tissue by CSF3, thus leading to tissue repair. To test this idea, we used a bleomycin-induced lung injury model, followed by CSF3 administration intraperitoneally. Our data suggest that CSF3 has a role in the mobilization of bone marrow-derived cells to the injured lung, leading to the attenuation of lung injury and fibrosis. This could provide an experimental basis for clinical treatment of patients with IPF.

Animals and Methods

Mice

Female C57BL/6J mice and male green fluorescent protein (GFP) transgenic C57BL/6J mice (8 weeks old) were purchased from the Shanghai Animal Experimental Center, Chinese Academy of Sciences. All mouse experiments were approved by the Department of Laboratory Animal Science, School of Medicine, Shanghai Jiao Tong University.

Experiments to Verify if CSF3 Mobilizes Bone Marrow-Derived Cells to the Injured Lung

Bone Marrow Transplantation and Reconstitution

Female C57BL/6J mice were used as recipients, whereas male GFP transgenic mice [36] were used as donors for bone marrow transplantation. Eighty recipient mice were sublethally irradiated at a dose of 850 cGy with a Mevatron MD2 medical linear accelerator (Siemens AG). Myeloablation was confirmed as white blood cells were reduced to zero 5 days after irradiation of the mice. Four hours after irradiation, 2×10^6 nucleated cells in 0.2 ml of bone marrow suspension from the donor mouse were injected into a recipient mouse via the tail vein. Four weeks after bone marrow transplant, the bone marrow-reconstituted mice were randomly grouped and subjected to bleomycin and CSF3 treatment.

Bleomycin Injury Model and CSF3 Treatment

Sixty of the bone marrow-reconstituted mice (with GFP) were randomly divided into 4 groups, as follows. Group 1 (bleomycin + CSF3): under anesthesia with 846 anesthetic mixture (Military Veterinary Institute, China), bleomycin (dose: 3.5 mg/kg [37]; Nippon Kayaku Co. Ltd.) in 60 μ l of PBS was instilled into the mouse trachea with a 26-gauge needle inserted between the cartilaginous rings of the trachea, and recombinant human CSF3 (Qilu Pharmaceutical Co. Ltd.) was administered intraperitoneally at a continuous dose of 50 μ g/kg in 100 μ l of PBS daily for 5 consecutive days [35] starting 1 day after bleomycin. Group 2 (bleomycin + saline): bleomycin was given intratracheally as above, and saline (100 μ l) was administered intraperitoneally daily for 5 consecutive days starting 1 day after bleomycin. Group 3 (saline + CSF3): saline (60 μ l) was given intratracheally, and then CSF3 was administered intraperitoneally at a continuous dose of 50 μ g/kg in 100 μ l of PBS daily for 5 consecutive days starting 1 day after saline. Group 4 (saline): saline (60 μ l) was given intratracheally, and then 100 μ l of saline was administered intraperitoneally daily for 5 consecutive days starting 1 day after the saline given intratracheally. The tracheostomy site was sutured, and the animals were allowed to recover.

Flow Cytometry to Detect GFP-Positive Cells in Lung Tissue

A single-cell suspension from mouse lung was prepared by digestion with collagenase IV [38]. GFP-positive cells in lung tissue were determined using a flow cytometer (Becton, Dickinson and Company), and the flow data were analyzed with WinMDI software (Scripps Institute). GFP-positive cells in lung tissues were detected 3, 7 and 14 days after CSF3 administration.

Fluorescence Microscopy to Observe GFP-Positive Cells in Lung Tissue

To determine if bone marrow cells are mobilized to the lung after CSF3 administration, GFP-positive cells were observed by fluorescent microscopy in cryosections of lung tissues taken from mice 3, 7 and 14 days after CSF3 administration.

Analysis of Nucleated Cells in Peripheral Blood

The eyes of experimental mice were removed to obtain blood (0.7 ml) 3, 7 or 14 days after CSF3 or saline administration. The blood was collected in EDTA to prevent coagulation, and the number of nucleated cells and the percentages of different kinds of nucleated cells were counted by an automated hematology analyzer (Abbott CD-3700).

Experiment to Verify if CSF3 Can Ameliorate Lung Injury and Aid in Its Repair

Bleomycin Injury Model and CSF3 Treatment

The bleomycin injury model as described above was used. Recombinant human CSF3 was administered at the same dose as above but on 10 consecutive days to determine whether this prolonged its effect.

Groups of Animals

All the mice were divided into 4 groups, as follows. Group 1 (bleomycin + CSF3): bleomycin was given intratracheally, and CSF3 was administered intraperitoneally daily for 10 consecutive days starting 1 or 14 days after bleomycin. Group 2 (bleomycin + saline): bleomycin was given intratracheally, and saline was administered intraperitoneally daily for 10 consecutive days starting 1 or 14 days after bleomycin. Group 3 (saline): saline was given intratracheally, and more saline was administered intraperitoneally daily for 10 consecutive days starting 1 or 14 days after the saline given intratracheally. Group 4 (saline + CSF3): saline was given intratracheally, and then CSF3 was administered intraperitoneally daily for 10 consecutive days starting 1 or 14 days after saline.

Lung Tissue Histology and Masson's Staining

Cryosections (10 μ m thick) of mouse lungs were stained with Hoechst 33342 for visualization of the nucleus. Conventional hematoxylin and eosin staining and Masson's trichrome staining were performed with paraffin sections (5 μ m thick). The extent of alveolitis was evaluated as reported by Szapiel et al. [39], and the proportion of fibrosis in the tissue was assessed and expressed as the percentage of fibrotic area.

Collagen Content

Collagen content [38, 40] was measured indirectly with a commercial hydroxyproline detection kit (Nanking JianCheng Biotechnology Research Institute) according to the manufacturer's recommendations.

Assessment of Cytokines in Lung Tissue

Lung homogenates were processed from the left lung. Protein levels of TGF- β 1, IFN- γ , MMP9 and TIMP1 in the lung tissues were measured by respective ELISAs (Research and Development System, USA) according to the manufacturer's recommendations.

Evaluation of Safety of CSF3

Ten mice from the saline + CSF3 group were observed for 3 months for possible tissue injury and tumor formation caused by CSF3 in lung, liver, kidney, heart, pancreas, spleen and stomach.

Evaluation of Survival

Survival was evaluated in the bleomycin + CSF3 and bleomycin + saline groups. In the bleomycin + CSF3 group, 20 mice were given bleomycin intratracheally, and CSF3 was administered intraperitoneally daily for 10 consecutive days from day 1 (10 mice) or day 14 (10 mice) after bleomycin. In the bleomycin + saline group, 20 mice were given bleomycin intratracheally, and saline was administered intraperitoneally daily for 10 consecutive days from day 1 (10 mice) or day 14 (10 mice) after bleomycin. The mice were kept in a clean room with regular observation, and deaths were recorded daily. The death rate and mean survival time were calculated. The survival curve was analyzed using the Kaplan-Meier test.

Statistical Methods

Statistical analysis was performed with SPSS (version 13) statistical software. An independent-samples *t* test was used to assess the differences between treatment groups. The log-rank test was used to compare the differences between survival curves. A *p* value less than 0.05 was considered statistically significant.

Results

Reconstitution of Mouse Bone Marrow

Bone marrow from male GFP transgenic mice (C57Bl/6J) was transplanted into sublethally irradiated female C57Bl/6J mice. Sixty-seven of the 80 bone marrow-reconstituted mice survived. We determined initially whether the autologous reconstitution of bone marrow was successful by assessing the peripheral white blood cells. White blood cell counts showed successful reconstitution (data not shown). With the aid of GFP, we were able to determine that 44.5–61.6% of cells in the bone marrow 28 days after reconstitution were GFP positive, suggesting that bone marrow reconstitution was successful.

CSF3-Induced Mobilization of Bone Marrow Cells to the Lung

GFP-positive cells were readily observed in cryosections of lung tissues of mice 3 and 7 days after CSF3 administration, as shown in figure 1a–d (and data not shown). Furthermore, flow cytometry was performed to

determine the bone marrow source of cells in the lung. As shown in figure 1e, CSF3 administration significantly increased GFP-positive cells in injured lung tissue. However, the mobilization of bone marrow-derived cells by CSF3 was transient, since the GFP-positive cells returned to normal levels 14 days after CSF3 treatment (fig. 1e). These data suggest that CSF3 is able to mobilize bone marrow-derived cells to the injured lung tissue and that the increase may last less than 2 weeks.

The results of table 1 show the effect of CSF3 on peripheral blood cells. These data suggest that GSF3 elevates the number of nucleated cells in peripheral blood, especially 3 and 7 days after GSF3 administration. However, GSF3 did not change the ratio of different kinds of nucleated cells when given as a single injection. These data also show that bleomycin elevates the number of nucleated cells and the percentage of neutrophils in peripheral blood, especially 3 and 7 days after GSF3 administration.

Attenuation of Lung Injury by CSF3

To determine if CSF3 affects bleomycin-induced lung injury, we evaluated lung injury after CSF3 treatment in bone marrow-reconstituted mice. Histological analysis revealed that CSF3 alleviated bleomycin-induced lung injury. Whilst bleomycin gave a typical lung injury response (fig. 2c), CSF3 treatment significantly improved bleomycin-induced lung injury (fig. 2b). In addition, the level of alveolitis in the lungs of animals given CSF3 was much improved, whilst that of animals given bleomycin alone showed a typical patchy injury (data not shown).

Effects of CSF3 on Survival of the Mice after Bleomycin Injury

We used normal (unreconstituted) C57Bl/6J mice to determine the effect of CSF3 on bleomycin-induced lung injury. As shown in figure 3a, the survival rate of animals given CSF3 immediately after bleomycin was significantly higher than that of animals given bleomycin alone.

We then determined the effect of the timing of CSF3 treatment. In addition to administration of CSF3 starting 1 day after bleomycin injury (fig. 3a), we also treated mice with CSF3 for 10 days starting 14 days after bleomycin injury (fig. 3b). Although CSF3 was still able to protect mice from bleomycin injury, the effect of delayed CSF3 treatment on mouse survival was less significant (fig. 3b) when compared with CSF3 treatment started immediately after bleomycin injury.

Fig. 1. Successful mobilization of bone marrow cells to the lung by CSF3. **a–d** Four weeks after bone marrow reconstitution, the mice were given bleomycin intratracheally. One day after bleomycin injury, CSF3 was administered intraperitoneally daily for 5 consecutive days. **a** GFP-positive cells were present in Hoechst 33342-stained cryosections of lung tissues from bone marrow-reconstituted mice 7 days after CSF3 mobilization. **b** Magnification of the image in **a**. **c** Hoechst 33342-stained nuclei. **d** Merged image of **b** and **c**. **e** Four weeks after bone marrow reconstitution, the mice were given bleomycin intratracheally. One day after bleomycin injury, CSF3 or saline was administered intraperitoneally daily for 5 consecutive days. GFP-positive cells were detected 3 days ($n = 5$ mice), 7 days ($n = 5$ mice) and 14 days ($n = 5$ mice) after bleomycin injury. Lung cells were analyzed by flow cytometry, demonstrating GFP-positive cells in lung homogenates ($n = 5$ mice). * $p < 0.05$.

Fig. 2. CSF3 attenuated bleomycin-induced lung injury. Four weeks after bone marrow reconstitution, the mice were given either bleomycin or saline intratracheally. One day after bleomycin injury, CSF3 or saline was administered intraperitoneally daily for 10 consecutive days. Hematoxylin and eosin staining of lung tissues 7 days after bleomycin administration was evaluated in 5 mice in each group. **a** Mice given saline only (n = 5). **b** Bleomycin injury with CSF3 treatment (n = 5). **c** Bleomycin injury with saline control (n = 5). $\times 200$. BLM = Bleomycin.

Table 1. The total number of nucleated cells and the percentage of neutrophils, lymphocytes and monocytes in peripheral blood

Time point	Group	WBC, $\times 10^9/l$	Neutrophils, %	Lymphocytes, %	Monocytes, %
Day 3	BLM + CSF3	$7.79 \pm 1.070^*$	7.04 ± 1.150	90.63 ± 2.026	0.78 ± 0.859
	BLM + saline	$7.57 \pm 0.640^*$	$20.30 \pm 3.111^*$	$77.35 \pm 5.445^*$	1.49 ± 1.718
	saline + CSF3	$8.25 \pm 0.718^*$	6.18 ± 0.461	92.03 ± 1.415	0.51 ± 0.693
	saline	6.42 ± 0.617	7.16 ± 2.104	91.34 ± 2.452	0.62 ± 0.242
Day 7	BLM + CSF3	$8.51 \pm 1.167^*$	$10.63 \pm 2.503^*$	$87.55 \pm 2.579^*$	0.67 ± 0.242
	BLM + saline	7.07 ± 0.519	$11.90 \pm 3.209^*$	$87.05 \pm 3.503^*$	0.68 ± 0.572
	saline + CSF3	$8.60 \pm 1.207^*$	8.24 ± 1.667	90.93 ± 1.678	0.44 ± 0.088
	saline	6.49 ± 0.913	6.45 ± 0.703	92.48 ± 0.763	0.36 ± 0.161
Day 14	BLM + CSF3	$7.88 \pm 1.083^*$	9.123 ± 2.275	90.20 ± 1.250	0.2167 ± 0.1935
	BLM + saline	$7.53 \pm 0.908^*$	8.520 ± 4.820	89.60 ± 6.222	0.873 ± 1.271
	saline + CSF3	6.43 ± 0.903	9.208 ± 2.019	89.65 ± 1.507	0.585 ± 0.765
	saline	6.24 ± 0.834	7.156 ± 2.104	91.34 ± 2.452	0.557 ± 0.716

* $p < 0.05$ compared to the saline control. BLM = Bleomycin.

Effects of Administration of CSF3 on Pulmonary Fibrosis

CSF3 treatment decreased collagen deposition (fig. 4c, E) compared to the saline-treated controls (fig. 4d, e). The percentage of collagen staining in lungs from animals that received CSF3 was lower than in those animals given bleomycin alone (fig. 4e). Furthermore, the hydroxyproline content was significantly lower in CSF3-treated animals than in saline-treated controls (fig. 4f). These results suggest that administration of CSF3 plays a protective role in bleomycin-induced lung fibrosis.

Delayed Administration of CSF3 Is Less Effective

Treating mice with CSF3 for 10 days starting 14 days after bleomycin injury (fig. 5a) still reduced collagen expression but the effect was attenuated when compared with CSF3 treatment started immediately after bleomycin injury (fig. 5).

Effects of Administration of CSF3 on Cytokines during Bleomycin-Induced Lung Injury

Next we wanted to determine if the mobilization of bone marrow-derived cells by CSF3 had any effect on

Fig. 3. CSF3 protected mice from bleomycin-induced lung injury. C57Bl/6J mice were given bleomycin intratracheally. **a** One day after bleomycin injury, CSF3 or saline was administrated intraperitoneally daily for 10 consecutive days. Survival curves were generated ($n = 20$; $p < 0.05$ by the log-rank test). **b** Fourteen days

after bleomycin injury, CSF3 or saline was administrated intraperitoneally daily for 10 consecutive days. Survival curves were generated ($n = 20$; $p > 0.05$ by the log-rank test). The line just above the x-axis indicates the duration of CSF3 treatment. BLM = Bleomycin.

key cytokines in the pathogenesis of lung fibrosis. CSF3 markedly reduced bleomycin-induced TGF- β in lung tissue (fig. 6a). In contrast, there was no significant effect on IFN- γ expression (fig. 6b).

Effects of CSF3 Treatment on MMP9 and TIMP1 during Bleomycin-Induced Lung Injury

CSF3 significantly increased MMP9 (fig. 7a) and decreased TIMP1 (fig. 7b) expression in lung tissue after bleomycin treatment.

Safety of CSF3

We did not observe any severe tissue injury or tumor formation in liver, kidney, heart, intestine, spleen, stomach or lung in the saline + CSF3 group after 3 months of treatment. All mice in the saline + CSF3 group survived. Hematoxylin and eosin staining of liver, kidney, heart, intestine, spleen stomach and lung of mice in the saline + CSF3 group showed normal structure (data not shown). Minor alveolar inflammation was only observed in 2 mice in the saline + CSF3 group (data not shown).

Discussion

In this study, we demonstrated that CSF3 was able to mobilize bone marrow-derived cells into injured lung and that the mobilization by CSF3 attenuated bleomycin-

induced lung injury and fibrosis. We also found that the expression of TGF- β , MMP9 and TIMP1 in injured lung were significantly altered after CSF3 administration. Therefore, these data support a beneficial role for the mobilization of bone marrow-derived cells by CSF3 in lung injury and fibrosis.

Both hematopoietic stem cells [26, 27] and mesenchymal stem cells [28–33] play a role in tissue repair. Pulmonary fibrosis is irreversible and progressive, and there is no effective treatment for the disease. Stem cells provide an excellent prospect as a new therapeutic modality; however, research into the treatment of lung diseases with stem cells is still in its infancy. Several studies have shown that the transplantation of stem cells influenced the outcome of lung injury. For example, transplantation of bone marrow-derived mesenchymal stem cells decreased the degree and duration of bleomycin-induced inflammation [33]. Systemic administration of mesenchymal stem cells to bleomycin-injured mice resulted in the cells homing to the lung, adopting an epithelium-like phenotype and attenuating lung inflammation and fibrosis [40]. However, transplantation of bone marrow stem cells has intrinsic drawbacks, including difficulty in collection, in vitro manipulation and highly technical requirements, thus limiting its use in clinical applications. Therefore, we investigated whether CSF3 is able to mobilize autologous bone marrow-derived stem cells to the site of an injury to repair the injury.

Fig. 4. CSF3 attenuated bleomycin-induced lung fibrosis. **a** C57Bl/6J mice were given either bleomycin or saline intratracheally. One day after bleomycin injury, CSF3 or saline was administered intraperitoneally daily for 10 consecutive days. Lung tissues were harvested 28 days (**b–d**) or 7, 14 and 28 days (**e, f**) after bleomycin injury (6 mice were sacrificed at each time point in each group). Collagen content was measured by Masson's tri-

chrome staining (**b–e**) and hydroxyproline determination (**f**). **b** Mice given saline alone. **c** Bleomycin injury with CSF3 treatment. **d** Bleomycin injury with saline control. **e** The percentage of collagen staining (indicating fibrosis) was determined by microscopy. **f** Hydroxyproline content in lung tissue from animals treated with bleomycin and CSF3. ** $p < 0.01$. BLM = Bleomycin. $\times 200$.

CSF3 has been widely used as a mobilizing agent of bone marrow stem cells for cancer patients with leukopenia after chemotherapy. In this study, CSF3 was administered to bleomycin-injured mice whose bone marrow was reconstituted with bone marrow from GFP transgenic mice. With the aid of GFP visualization, we found that CSF3 mobilized bone marrow-derived cells to the injured lung tissue, although the increase in GFP-positive bone marrow-derived cells in the lung was transient. These data were consistent with previous reports that CSF3 can significantly increase the numbers of mesenchymal stem cells both in bone marrow and peripheral blood [41, 42] and in injured tissues [43, 44].

Furthermore, we did not find any adverse effects such as tissue injury or tumor formation with the use of CSF3 for 3 months in mice under the experimental conditions used (data not shown).

Next, we determined whether the mobilization of bone marrow-derived cells plays a role in lung repair. We found that the mobilization of bone marrow-derived cells by CSF3 significantly increased mouse survival and attenuated lung inflammation and collagen deposition. Additionally, we showed that earlier intervention with CSF3 was more effective during tissue injury and repair. These data were consistent with previous reports. For example, recombinant human CSF3 mobilized bone mar-

Fig. 5. Delayed CSF3 treatment was less effective. **a** C57Bl/6J mice were given either bleomycin or saline intratracheally. Fourteen days after bleomycin injury, CSF3 or saline was administrated intraperitoneally daily for 10 consecutive days. Lung tissues were harvested 42 days (**b–d**) or 21, 28 and 42 days (**e, f**) after bleomycin injury (6 mice were sacrificed at each time point in each group). Collagen content was measured by either Masson's tri-

chrome staining (**b–e**) or hydroxyproline determination (**f**). **b** Mice given saline alone. **c** Bleomycin injury with CSF3 treatment. **d** Bleomycin injury with saline control. **e** The percentage of collagen staining (indicating fibrosis) was determined by microscopy. **f** Hydroxyproline content in lung tissue from animals treated with bleomycin and CSF3. ** $p < 0.01$. BLM = Bleomycin.

row stem cells to damaged brain tissue, induced the release of a neurotrophic factor and reduced brain tissue damage [43, 44]. In addition, CSF3 mobilized bone marrow stem cells to myocardial infarction sites and promoted cardiac myocyte repair [35, 45].

Our data suggest that the improvement in lung injury and secondary fibrosis are related to the bone marrow-derived cells mobilized by CSF3. These bone marrow-derived cells, including mesenchymal stem cells, entered injured lung tissue and played some role in repairing local lung tissue. Our data (fig. 1e) show that CSF3 administration significantly increased the number of GFP-positive cells in injured lung tissue compared to the non-CSF3-

treated groups. CSF3 can enhance the migration and proliferation of mesenchymal stem cells [46]. The next question is how do the bone marrow-derived cells mobilized by CSF3 affect lung injury and fibrosis? TGF- β 1 is thought to play a central role in the pathogenesis of lung fibrosis [11, 12]. TGF- β 1 increases the synthesis of ECM and decreases its degradation [13, 14]. On the other hand, the Th1 cytokine IFN- γ limits lung fibrosis [17, 18, 38]. Our data showed that the expression of TGF- β 1 but not IFN- γ in injured lung tissue decreased after CSF3 therapy. We speculated that the bone marrow-derived cells mobilized by CSF3 may change the microenvironment of injured lung with regard to TGF- β 1 and other immune and in-

Fig. 6. CSF3 affected cytokines after bleomycin-induced lung injury. C57Bl/6J mice were given bleomycin intratracheally. One day after bleomycin injury, CSF3 or saline was administrated intraperitoneally daily for 10 consecutive days. Lung tissues were

harvested 7, 14 and 28 days after bleomycin injury (6 mice were sacrificed at each time point in each group). TGF-β (a) and IFN-γ (b) levels in lung tissues were measured with respective ELISAs. * $p < 0.05$. BLM = Bleomycin.

Fig. 7. CSF3 modulated MMP9 and TIMP1 after bleomycin-induced lung injury. C57Bl/6J mice were given bleomycin intratracheally. One day after bleomycin injury, CSF3 or saline was administrated intraperitoneally daily for 10 consecutive days. Lung

tissues were harvested 7, 14 and 28 days after bleomycin injury (6 mice were sacrificed at each time point in each group). MMP9 (a) and TIMP1 (b) levels in lung tissues were measured with respective ELISAs. * $p < 0.05$. BLM = Bleomycin.

inflammatory mediators [11–13]. The reduction of TGF-β1 in the microenvironment of the injured lung may impact on the synthesis and degradation of ECM and thus affect the lung injury and fibrosis. Furthermore, we found that MMP9 and TIMP1 expression changed after CSF3 treatment. MMPs are a main enzymatic system that degrades ECM, while TIMPs negatively regulate MMP activity.

Our data showed that MMP9 was increased and TIMP1 was decreased in lung tissue after CSF3 treatment, indicating that the MMP/TIMP balance was shifted to favor degradation of ECM, leading to the alleviation of lung fibrosis.

Overall, these data suggest that CSF3 plays a role in lung injury by mobilizing bone marrow-derived cells, in-

cluding mesenchymal stem cells, to injured lung tissue and that these stem cells can alter the microenvironment of the injured lung. Within this microenvironment, TGF- β 1, and possibly other mediators, can affect the repair of lung injury and fibrosis induced by bleomycin. However, it remains to be determined whether the expression of TGF- β 1 and other components of the injured lung microenvironment are directly affected.

In summary, we have presented evidence to confirm that mobilization of bone marrow stem cells by CSF3 increased the survival rate, reduced lung inflammation and attenuated pulmonary fibrosis in a bleomycin-induced lung injury and fibrosis model in mice. We found that earlier intervention with CSF3 was more beneficial. Moreover, we found that CSF3 treatment reduced TGF- β 1 and TIMP1 and increased MMP9 expression. The mechanism by which CSF3 attenuates lung injury and fibrosis may be related to a modulation of the local microenvironment, including alterations in the expression of cytokines and growth factors, ECM changes by influ-

encing MMP/TIMP expression and altered immunological functions. Further studies are warranted to investigate the mechanism(s) by which bone marrow-derived cell mobilization by CSF3 attenuates tissue injury. Nevertheless, this represents a new way to promote tissue repair which may be much more clinically useful than mesenchymal stem cell transplantation in combating lung injury and fibrosis.

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