ORIGINAL RESEARCH COMMUNICATION

Senescence-Associated MCP-1 Secretion Is Dependent on a Decline in BMI1 in Human Mesenchymal Stromal Cells

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Abstract

Aims: Cellular senescence and its secretory phenotype (senescence-associated secretory phenotype [SASP]) develop after long-term expansion of mesenchymal stromal cells (MSCs). Further investigation of this phenotype is required to improve the therapeutic efficacy of MSC-based cell therapies. In this study, we show that positive feedback between SASP and inherent senescence processes plays a crucial role in the senescence of umbilical cord blood-derived MSCs (UCB-MSCs). **Results:** We found that monocyte chemoattractant protein-1 (MCP-1) was secreted as a dominant component of the SASP during expansion of UCB-MSCs and reinforced senescence *via* its cognate receptor chemokine (c-c motif) receptor 2 (CCR2) by activating the ROS-p38-MAPK-p53/p21 signaling cascade in both an autocrine and paracrine manner. The activated p53 in turn increased MCP-1 secretion, completing a feed-forward loop that triggered the senescence program in UCB-MSCs. Accordingly, knockdown of *CCR2* in UCB-MSCs significantly improved their therapeutic ability to alleviate airway inflammation in an experimental allergic asthma model. Moreover, BMI1, a polycomb protein, repressed the expression of *MCP-1* by binding to its regulatory elements. The reduction in BMI1 levels during UCB-MSC senescence altered the epigenetic status of *MCP-1*, including the loss of H2AK119Ub, and resulted in derepression of *MCP-1*. **Innovation:** Our results provide the first evidence supporting the existence of the SASP as a causative contributor to UCB-MSC senescence and reveal a so far unappreciated link between epigenetic regulation and SASP for maintaining a stable senescent phenotype. **Conclusion:** Senescence of UCB-MSCs is orchestrated by MCP-1, which is secreted as a major component of the SASP and is epigenetically regulated by BMI1. *Antioxid. Redox Signal.* 24, 471–485.

Introduction

HUMAN UMBILICAL CORD blood-derived mesenchymal stromal cells (UCB-MSCs) have been considered the standard choice for conventional clinical use because of their easy isolation, multipotent ability to differentiate into diverse types of cells (*e.g.*, myocytes, adipocytes, bone osteocytes, chondrocytes, neurons, and hepatocytes), expandable *ex vivo*

culture, little or no immunogenicity due to a lack of HLA-DR expression, and low tumorigenicity (13, 23). To obtain sufficient cells for clinical application, reliable and efficient expansion of MSCs *in vitro* is required. However, the expansion of UCB-MSCs according to traditional culture techniques comprising serum use, culture onto plasticware, and continuous exposure to oxygen results in a progressive loss of proliferative and differentiation potential with each

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Innovation

Several reports have demonstrated that umbilical cord blood-derived mesenchymal stromal cells (UCB-MSCs) during long-term expansion become susceptible to senescence paralleled with an altered secretory phenotype, termed the senescence-associated secretory phenotype (SASP). However, to what extent and by what means the SASP contributes to the senescence of adult tissue stem cells remain unknown. We showed that monocyte chemoattractant protein-1 (MCP-1) is a crucial component of the SASP in human UCB-MSC senescence and determines their responses to allergic asthma treatment *in vivo*. MCP-1 induction is epigenetically regulated by a reduction in BMI1 in senescent UCB-MSCs. The interplay between BMI1 and MCP-1 consolidates the senescence program in UCB-MSCs, possibly determining their therapeutic potency.

passage. Similar to primary tissue cells, UCB-MSCs initiate a permanent cessation of cell division, termed senescence, after approximately 50 population doublings (PDs) (19, 49).

Cellular senescence is triggered by the gradual accumulation of DNA damage and epigenetic alterations that can directly affect the expression of senescence-associated genes (48). Due to incomplete and erratic DNA replication, senescent cells create an intrinsic biological clock for cellular aging and converge on the so-called Hayflick limit of cell division at the molecular level (7, 19, 37, 52). The nuclei of senescent cells are characterized by irreversible changes, resulting in an aberrant nuclear shape (30), loss of heterochromatin-associated protein (*e.g.*, heterochromatin protein-1) structures (22), altered patterns of histone modification that are frequently observed in aged cells (46), and transcriptionally repressive heterochromatin structure called senescence-associated heterochromatin foci, which induces the stable repression of E2F-target genes and represses some growth-promoting genes through the recruitment of the retinoblastoma (Rb) tumor suppressor (36). Furthermore, the conventional cell culture protocol that uses 21% of ambient oxygen could generate reactive oxygen species (ROS) (26), which induce aging-associated cellular responses by activating various signaling pathways, such as tumor protein p53 (p53), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF κ B), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT), and extracellular signal-regulated kinase (ERK)/c-Jun kinase (JNK)/p38 mitogen-activated protein kinase (p38-MAPK) (15). Although it is suggested that the culture conditions and intrinsic nuclear alterations could cross talk during the senescence process, the detailed mechanism remains to be determined.

Recently, it has been reported that cellular senescence is paralleled by a striking increase in the secretion of many factors that participate in intercellular signaling, termed the senescence-associated secretory phenotype (SASP) (16, 29, 44). Although several groups have reported that the execution of cellular senescence involves, and often requires, the secretion of a plethora of factors, it is not immediately clear which soluble factors are major contributors to the SASP and what exact role it plays in cellular senescence (4). In the

present study, we show for the first time that senescent UCB-MSCs secrete monocyte chemoattractant protein-1 (MCP-1) as a dominant secreted chemokine that positively relays senescence signaling *via* its cognate receptor chemokine (c-c motif) receptor 2 (CCR2) and reinforces senescence by increasing the protein levels of p53 and p21 *via* ROS or p38-MAPK signaling. Accordingly, the knockdown (KD) of CCR2 significantly improves the therapeutic outcome of UCB-MSCs by decreasing both cellular and inflammatory mediators in a severe asthma animal model. Moreover, BMI1 protein, a member of the polycomb repressor complex-1 (PRC1), binds to regulatory elements of the *MCP-1* locus. Accordingly, a decrease in BMI1 during UCB-MSC senescence derepresses the transcription of *MCP-1*. Our results thus provide the first evidence supporting the existence of the SASP in senescent UCB-MSCs and contribute novel insights into the role of SASP signaling, which is a causative contributor to UCB-MSC senescence and positively connects the intrinsic and extrinsic circuitry of the process.

Results

Long-term expansion of UCB-MSCs induces cellular senescence

During long-term expansion of UCB-MSCs (more than 11 passages), the cells reached a senescent phase and stopped proliferating *in vitro*, although the growth rate depended on the donor and passage number (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). According to the criteria described in previous reports (2, 24), UCB-MSCs were categorized into early, intermediate, and late phases according to the senescence status. The boundary of each phase corresponded with a marked decrease in PD and the breakpoint of the cumulative PD curve (Supplementary Fig. S1A). Compared with early-phase cells, late-phase UCB-MSCs showed reduced telomerase activity and an enlarged morphology with strong staining of senescence-associated beta-galactosidase (SA- β -gal) (Supplementary Fig. S1B, C). They also exhibited severe defects at the level of proliferation and multilineage differentiation, such as osteogenesis (confirmed by von Kossa staining or alkaline phosphatase [ALP] activity), chondrogenesis (Safranin O staining or glycosaminoglycan [GAG] assay), and adipogenesis (Oil red O staining) (Supplementary Fig. S1D, E). In gene expression analysis, expression of p53 phosphorylated at Ser392, indicative of p53 activation, and the p53 downstream gene p21 began to increase from the intermediate phase, showing full activation in the late phase. Phosphorylated pRb protein, which is observed during cell cycle progression, progressively decreased from the intermediate phase and was barely detected in the late phase (Supplementary Fig. S1F). Notably, the expression of *BMI1*, which suppresses senescence-triggering genes in adult stem cells (33, 34, 39), was 0.5 ± 0.2 -fold lower in late-phase UCB-MSCs than in early-phase cells (Supplementary Fig. S1G). The expression of surface antigens characteristic of MSCs was largely unaffected during UCB-MSC senescence (Supplementary Fig. S2). Taken together, these results indicate that *ex vivo* expansion of UCB-MSCs up to the late phase induces senescence and results in the loss of stem cell properties, including stemness and differentiation capability, through similar pathways to those of other tissue cells (11). Of note, UCB-MSCs exhibit the heterogeneous kinetics of senescence progress.

Senescent UCB-MSCs possess a secretion profile

The senescent cells showed their characteristic protein secretion profile (secretome), referred to as the SASP (16). MSCs secrete a variety of cytokines and growth factors with paracrine and autocrine activities that can determine their therapeutic outcomes (20, 42). Thus, we hypothesized that senescent UCB-MSCs would also use a similar SASP phenomenon to actively regulate the senescence process. To address this hypothesis, we performed a cytokine array containing 200 proteins (interleukins, 35; chemokines, 11; other cytokines, 154; Supplementary Fig. S3A) using conditioned medium (CdM) from early- and late-phase UCB-MSCs. The levels of five proteins—MCP-1, interleukin-6 (IL-6), neurotrophin 4 (NT-4), chemokine cxc motif ligand 16 (CXCL16), and Fas-ligand (FASLG)—were markedly increased in the CdM of late-phase cells. Of these, the increase in IL-6 and MCP-1 was the highest (Fig. 1A). When we measured their mRNA expression levels, only the *MCP-1* transcript was significantly increased in the late phase of UCB-MSCs compared with early-phase cells (Fig. 1B).

To further validate the association between MCP-1 secretion and UCB-MSC senescence, we quantified the amount of MCP-1 protein in CdM of UCB-MSCs derived from 10 individual donors. As shown in Figure 1C, the se-

cretion of MCP-1 gradually increased as the passage number increased in all of the UCB-MSCs tested. Notably, according to the amount and rate of the increase in MCP-1 secretion with each passage, UCB-MSCs could be classified into two groups. These groups showed significant differences in basal MCP-1 secretion at the initial passage (group I vs. group II: 76.7 ± 10.8 pg/ml vs. 21.6 ± 11.0 pg/ml; $p < 0.05$) and the increase from the initial passage to the final passage (group I vs. group II: 10.6 ± 4.1 -fold vs. 5.7 ± 2.3 -fold; $p < 0.01$). Particularly, the cells in group I ceased to proliferate sooner and characteristically showed a faster senescence pattern with a lower cumulative PD and shorter time to doubling cessation than the cells in group II (group I vs. group II: 63.9 ± 6.7 PD vs. 119.7 ± 20.2 PD; $p < 0.01$) (Fig. 1D). In addition, the levels of both BMI1 transcript and protein were significantly higher in group II than in group I (Fig. 1E, F). We also observed significantly increased secretion of IL-6 protein in the late-phase UCB-MSCs (Supplementary Fig. S3B). However, this tendency varied considerably depending on the donor, which could be the reason for the minor change in the IL-6 transcript (Fig. 1B). Taken together, these data demonstrate that long-term cultivation of UCB-MSCs stimulates the SASP, which releases MCP-1 protein and determines the heterogeneity regarding the senescence process in UCB-MSCs.

FIG. 1. Senescent umbilical cord blood-derived mesenchymal stromal cells (UCB-MSCs) display senescence-associated secretory phenotype (SASP). (A) Cytokine array analysis using conditioned medium collected from early- and late-phase UCB-MSCs (UCB #3). Two spots in membrane 1 showing significant increases in the late-phase group are marked with circles. (B) qPCR analysis of *MCP-1* and *IL-6* in early- and late-phase UCB-MSCs (UCB #3, 6, 10). The levels of the genes were normalized to those of β -actin, with the expression levels in the early phase defined as 1 (mean $\pm$ SEM; $n = 4$; $**p < 0.01$). (C) Based on the monocyte chemoattractant protein-1 (MCP-1) protein secretion profile, 10 cases of UCB-MSCs were divided into two groups showing significant differences in the basal level of MCP-1 secretion at the initial passage and a fold increase from the initial passage to the final passage: group I (UCB #1 to #4) and group II (UCB #5 to #10). (D, E) The cumulative population doublings (PD) (D) and expression levels of the *BMI1* transcript (E) and protein (F) in group I (UCB #3) and group II (UCB #10) UCB-MSCs at early (passage 4 or 5) and late (passage 10 or 14) phases. The expression level of BMI1 protein was normalized to that of β -actin (BMI1/ β -actin). Data are shown as the median $\pm$ interquartile range (D, $n = 6$; Mann-Whitney U test) or mean $\pm$ SEM (E, $n = 4$; F, $n = 6$; two-way ANOVA). $**p < 0.01$, $***p < 0.001$ compared with group II. $##p < 0.01$, $###p < 0.001$.

FIG. 2. MCP-1 stimulates the senescence phenotypes of UCB-MSCs. (A, B) Percentage of cells with positive SA-β-gal staining (A) and growth kinetics (B) in cells exposed to human recombinant MCP-1 for 48 h at each passage from the intermediate phase up to the late phase. Data are shown as the mean ± SEM ($n=3$; two-way ANOVA). (C) Western blot analysis of cell cycle inhibitors, including phospho-p53 and p21. β-actin was used as a loading control. The expression level of P-p53 protein normalized to that of β-actin (P-p53/β-actin) is shown as median ± interquartile range ($n=5$; Kruskal–Wallis test). (D, E) Quantitative analysis of SA-β-gal activity (D) and cell growth (E) in cells transfected with control (siControl) or MCP-1 siRNA (siMCP-1) at the indicated passage number. Data are shown as mean ± SEM ($n=3$; two-way ANOVA). (F) After 3 weeks of osteogenic induction, the level of osteogenic differentiation was quantified by measuring the percentage of cells positive for von Kossa staining (median ± interquartile range; $n=6$; Kruskal–Wallis test). (G) Western blot analysis of cell cycle inhibitors in intermediate-phase (passage 11) cells treated with MCP-1 siRNA for 24 or 48 h. The expression level of P-p53 protein is shown as mean ± SEM ($n=7$; one-way ANOVA). (H) qPCR and Western blot analysis of BMI1 in naïve cells or cells (UCB-MSC #6) transfected with MCP-1 or control siRNA. The level of BMI1 transcript was normalized to that of β-actin, with the level of expression in the early phase defined as 1 (median ± interquartile range; $n=5$; Kruskal–Wallis test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. P, passage.

MCP-1 increases senescence in UCB-MSCs

To examine the causative role of MCP-1 in UCB-MSC senescence, 100 ng/ml of recombinant MCP-1 protein (54, 56) was added for 48 h to UCB-MSCs at each passage from the intermediate phase (average P9–11), in which cells showed prominent growth arrest and MCP-1 secretion, up to the late phase. Compared with the untreated control, MCP-1 treatments significantly increased SA- β -gal activity with passaging by up to eight-fold (Fig. 2A and Supplementary Fig. S4A), concomitantly with a considerable delay in cell growth (Fig. 2B). MCP-1 treatment activated the p53-p21 signaling cascade, increasing the protein level of p53 phos-

phorylated at Ser392 and total p21 (Fig. 2C). In contrast, the pRb pathway was largely unaffected by MCP-1 treatment.

The use of siRNA to silence *MCP-1* in UCB-MSCs (Supplementary Fig. 4B) significantly blocked both SA- β -gal activity induction and delayed cell growth (Fig. 2D, E, and Supplementary Fig. S4C). Moreover, *MCP-1* KD UCB-MSCs showed restored osteogenic differentiation with passaging (Fig. 2F and Supplementary Fig. S4D). At the molecular level, *MCP-1* KD cells showed attenuated activation of the p53-p21 pathway (Fig. 2G) and, simultaneously, a 2.2-fold increased expression of *BMI1* transcript, which increased the level of BMI1 protein (Fig. 2H). Taken together, these data demonstrate that MCP-1 protein secreted as

FIG. 3. CCR2 is a crucial mediator of UCB-MSC senescence. (A) Flow cytometric analysis of CCR2 expression in early, intermediate, and late passages of UCB-MSCs. The percentage of CCR2⁺ cells is shown on the right side of the FACS histogram (mean $\pm$ SEM; $n=5$; one-way ANOVA). (B–E) Western blot analysis of CCR2 protein (B), SA- β -gal staining (C), cell growth kinetics (D), and colony-forming assay (E, F) in UCB #6 cells at the indicated passage (P) number treated with isotype (control IgG) or CCR2 blocking antibody (CCR2 Ab) for 48 h. (F) CFU-F activity was quantified as colony counts as the percentage of seeded cells and is shown as median $\pm$ interquartile range ($n=6$; Kruskal–Wallis test). (G–I) SA- β -gal staining (G) and expression of the cell cycle inhibitor proteins (p21 and P-p53) (H) and *BMI1* transcript and protein (I) in UCB #6 cells at the indicated passage number transfected with scrambled control (siControl) or *CCR2* (siCCR2) siRNA. β -actin was used as a control for Western blot analysis. The level of *BMI1* transcript relative to the value of naïve cells is shown as the median $\pm$ interquartile range ($n=5$; Kruskal–Wallis test). (H) The relative level of P-p53 to β -actin was quantified and is shown as the median $\pm$ interquartile range ($n=3$; Kruskal–Wallis test). Data for SA- β -gal staining (C, G, $n=5$) and cell growth (D, $n=3$) are shown as mean $\pm$ SEM (two-way ANOVA). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

a component of the SASP plays a causative role in inducing the senescence of UCB-MSCs.

MCP-1-induced UCB-MSC senescence is mediated by its canonical receptor, CCR2

MCP-1 is a proinflammatory cytokine that exerts its biological effects through its cognate receptor, CCR2 (5, 45). The expression of CCR2 in UCB-MSCs was lower in the early phase, but it was considerably upregulated in the intermediate phase and further increased in the terminal senescent phase (Fig. 3A, B). Importantly, treatment with CCR2 blocking antibody significantly reduced SA- β -gal expression (Fig. 3C) and enhanced the growth rate (Fig. 3D) and colony-forming capacity (Fig. 3E, F) of UCB-MSCs in the intermediate phase. The significance of CCR2 was further validated by the fact that CCR2 KD cells had decreased SA- β -gal activity (Fig. 3G) and p53-p21 pathway activation (Fig. 3H). However, they had a 1.9-fold increased expression of *BMI1* transcript, which simultaneously increased the level of BMI protein (Fig. 3I). Furthermore, overexpression of *CCR2* in early-phase UCB-MSCs remarkably increased cellular senescence, as indicated by induction of SA- β -gal activity (Supplementary Fig. S5A), activation of the p53-p21 signaling cascade, and down-regulation of *BMI1* expression (Supplementary Fig. S5B). Notably, silencing of *MCP-1* less affected the senescence triggered by overexpression of CCR2. These results collec-

tively suggest that CCR2 is a key signaling factor for the secreted MCP-1 that triggers the cellular senescence of UCB-MSCs.

Autocrine/paracrine-positive loop of MCP-1 secretion through CCR2

Cellular senescence can be stably sustained by the autocrine/paracrine-positive regulatory loop of secreted SASP factors (28, 43). To test whether the MCP-1 and CCR2 signaling cascade can use a similar mechanism, we first examined whether MCP-1 and CCR2 could regulate each other's expression. Exposure of intermediate-phase UCB-MSCs to MCP-1 protein increased the expression of CCR2 in a time-dependent manner (Fig. 4A, B). Similarly, the repression of CCR2 by either siRNA or a blocking antibody decreased the secretion of MCP-1 by 1.5-fold (Fig. 4C), suggesting positive feedback between MCP-1 and CCR2.

Next, to determine whether the MCP-1 and CCR2 cascade can trigger UCB-MSC senescence as a paracrine action, we cocultured intermediate- and late-phase UCB-MSCs in a Transwell chamber that prevents direct cell-cell contact due to its small pore size (1 μ m). The SA- β -gal activity in intermediate-phase cells was increased by coculture with late-phase senescent cells. However, SA- β -gal activity was significantly reduced when the late-phase cells in the upper chamber were treated with *MCP-1* or *CCR2* siRNA (Fig. 4D).

FIG. 4. Positive feedback between MCP-1 and CCR2 in autocrine and paracrine manner. (A, B) Increased CCR2 expression analyzed by flow cytometry (A) and Western blot (B) following MCP-1 (100 ng/ml) treatment of UCB-MSC #10 cells. (C) Secretion of MCP-1 was examined following inhibition of CCR2 by blocking antibody (CCR2 Ab) or siRNA (siCCR2). (D) Representative images of SA- β -gal staining (upper panel) and quantification of SA- β -gal-positive cells (lower panel) in intermediate-phase cells (UCB #10; passage 9) after coculture with late-phase cells transfected with the indicated siRNA for 48 h. Note that a Transwell chamber with a small pore size (1 μ m) blocks physical contact between cells in the upper and lower chambers. The numbers of SA- β -gal-positive cells of the intermediate phase in the lower chamber were measured. Data are shown as the mean $\pm$ SEM ($n = 5$; one-way ANOVA). *** $p < 0.001$. Scale bar: 50 μ m.

FIG. 5. Reactive oxygen species (ROS) and the p38-MAPK pathway mediate MCP-1-dependent UCB-MSC senescence. (A, B) Quantification of ROS production by long-term expansion of UCB-MSC #10 cells (A) and by treatment with recombinant MCP-1 protein (B) in the presence or absence of CCR2 blocking antibody (CCR2 Ab). Data are shown as the median $\pm$ interquartile range (A, $n=4$; Mann-Whitney U test) or mean $\pm$ SEM (B, $n=5$; one-way ANOVA). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C) Activation of p38-MAPK, a mediator of ROS signaling, was measured by Western blot. The relative level of P-p38 to T-p38 was quantified and is shown as the mean $\pm$ SEM ($n=3$; one-way ANOVA). * $p<0.05$, ** $p<0.01$ compared with 0 h. (D–F) The effects of a chemical inhibitor of p38-MAPK, SB203580 (E), and N-acetyl-L-cysteine (NAC) (F) on phosphorylated p53 and p21 expression were measured by Western blot. (D) The relative level of P-p53 to β -actin was quantified and is shown as the median $\pm$ interquartile range ($n=3$; Kruskal-Wallis test). (G and H) Treatment with SB203580 or NAC prevented the increase in SA- β -gal staining (H) and the downregulation of *BMI1* transcript and protein (G) induced by MCP-1 treatment. Data for *BMI1* expression (G) and SA- β -gal staining activity (H) are shown as the mean $\pm$ SEM of at least five independent experiments in UCB-MSC #10 cells (one-way ANOVA). * $p<0.05$, ** $p<0.01$ compared with MCP-1 treatment. Scale bar: 50 μ m.

Together, these results demonstrate that the autocrine/paracrine amplification feedback between MCP-1 and CCR2 is essential to the progression of UCB-MSC senescence.

Role of oxidative stress and p38-MAPK in MCP-1-mediated UCB-MSC senescence

Oxidative stress is a central contributor to senescence-like cell arrest (12,18) and is induced by the MCP-1 and CCR2 cascade in immune cells (17). Accordingly, the level of ROS was significantly increased by long-term expansion of UCB-MSCs (Fig. 5A) and treatment with MCP-1 recombinant protein. However, this increase was prevented by CCR2 blocking antibody (Fig. 5B). Moreover, p38-MAPK, a crucial mediator of ROS-induced senescence, was activated from 8 h and peaked at 18 h after MCP-1 stimulation (Fig. 5C). Importantly, treatment with SB203580, a chemical inhibitor of p38-MAPK, significantly abrogated the increase in phosphorylated p53 and p21 proteins induced by MCP-1 stimulation (Fig. 5D, E). Addition of N-acetyl-L-cysteine (NAC), an ROS scavenger, also markedly suppressed the

activation of both p38-MAPK and p53-p21 cascades (Fig. 5D, F). Accordingly, SA- β -gal activity (Fig. 5H) as well as the decrease in the levels of *BMI1* transcript and protein (Fig. 5G) in the MCP-1-treated UCB-MSCs was significantly abrogated by the interference of p38-MAPK or ROS. Thus, these results suggest a significant role of ROS in MCP-1-mediated UCB-MSC senescence.

Blocking the MCP-1-CCR2 cascade enhances the therapeutic capacity of UCB-MSCs in an asthma animal model

Next, we investigated the significance of MCP-1-mediated UCB-MSC senescence under *in vivo* conditions. To address this issue, we compared the therapeutic outcome of UCB-MSCs stably expressing shRNA targeted against *CCR2* and scrambled shRNA using a severe murine asthma model. Before injection of the cells, we confirmed the decreased level of CCR2 protein and SA- β -gal activity in *CCR2* KD UCB-MSCs (Fig. 6A, B). Furthermore, silencing of CCR2 cells significantly prevented the MCP-1-induced senescent

FIG. 6. Improved therapeutic outcomes of UCB-MSCs by *CCR2* KD in a murine allergic asthma model. All animals were sensitized and challenged with PBS (Control) or OVA and polyI:C (Poly IC-induced asthma). Fifteen days after the first sensitization, mice were intravenously injected with 3×10^5 UCB-MSCs (UCB #6, passage 8) stably expressing the scrambled (shControl) or *CCR2* (shCCR2) shRNA. Twenty-four hours after the final challenge, blood, lung tissue, and BALF were collected. (**A**, **B**) Expression of *CCR2* protein (**A**) and the activity of SA- β -gal (**B**) according to *CCR2* KD. (**C**) Number of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in BALF. (**D**) Hematoxylin and eosin staining of lung tissues (magnification, $\times 40$). Photomicrographs with higher magnification ($\times 200$) are shown in the lower right-hand corner of each panel. (**E**) Quantification of IL-8 in BALF ($n = 9$). (**F**) Immunohistochemical detection of human β -2 microglobulin (green) in lung tissues (magnification, $\times 100$). Nuclei were stained with DAPI (blue). Scale bar: 50 μ m. (**G**) The number of human β -2 microglobulin-expressing cells was assessed from at least seven randomly selected fields. Data are represented as the median $\pm$ interquartile range (**B**, $n = 9$; Mann-Whitney *U* test) or mean $\pm$ SEM ($n \geq 5$; one-way ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns; nonsignificant.

phenotypes, including activation of SA- β -gal and p53-p21 cascades (Supplementary Fig. S5C, D). Similar to previous reports (3, 31), the OVA- and polyIC-sensitized mice had significantly increased levels of cellularity and inflammatory cells, including macrophages, neutrophils, and lymphocytes, in bronchoalveolar lavage fluid (BALF) (Fig. 6C) and around the bronchial and vascular area in lung tissues (Fig. 6D). These parameters were reduced by administration of both control and *CCR2* KD UCB-MSCs. However, *CCR2* KD cells more effectively attenuated the infiltration of inflammatory cells in BALF and lung tissues than control UCB-MSCs (Fig. 6C, D). When inflammatory cytokines were measured, injection of UCB-MSCs reduced the level of IL-8 proteins in BALF regardless of the expression level of *CCR2*, with a greater reduction in *CCR2* KD UCB-MSC-injected mice (Fig. 6E). The expression levels of IL-5, IFN- γ , and IL-17 were largely unaffected in the BALF of all of the groups tested (data not shown). When we compared the number of engrafted cells by staining the lung tissue with antibody specific to human β 2 microglobulin, *CCR2* KD UCB-MSCs yielded greater engraftment capacity in the lung than control cells (Fig. 6F, G). Thus, these results demonstrate that repression of the MCP-1 and *CCR2* cascade reinforces the ability of UCB-MSCs to alleviate the inflammatory response in the experimental allergic asthma model.

Decline in BMI1 during UCB-MSC senescence derepresses the transcription of MCP-1

Next, we investigated whether the cell-intrinsic senescent program could be linked to the MCP-1-mediated SASP. According to our molecular analysis, p53, p21, and BMI1 were consistently implicated in the MCP-1-mediated SASP observed in senescent UCB-MSCs. The significance of p53 was further confirmed by the finding that ectopic expression of p53 or treatment with nutlin-3, a p53 activator, remarkably increased the secretion of MCP-1 in early-phase UCB-MSCs (Supplementary Fig. S6).

BMI1, a transcriptional repressor, prevents the premature activation of senescence-associated genes, including *INK4a* and *ARF*, in a variety of adult stem cells by binding to its target loci (8, 34, 39). Thus, we tested whether *MCP-1* expression could be epigenetically regulated by BMI1 protein during UCB-MSC senescence. According to DNase I hypersensitivity and the ChIP-seq database (histone modifications and two transcription repressors, EZH2 and HDAC1) of the entire *MCP-1* locus, which is available via the ENCODE project with the UCSC genome browser (<http://genome.cse.ucsc.edu/cgi-bin/hgTracks>) (Supplementary Fig. S7A), we selected four putative sites and performed chromatin immunoprecipitation (ChIP) analysis to examine the binding of BMI1 to them. We found that BMI1 strongly bound to the *MCP-1* locus in early-phase UCB-MSCs, but its enrichment was significantly decreased in senescent late-phase cells (Fig. 7A). Indeed, the distal (–7 kb) and proximal (within–1.5 kb) promoter regions in late-phase cells lost the monoubiquitination at lysine 119 of histone2A (H2AK119Ub) that is catalyzed by BMI1 (Fig. 7A), concomitant with the increase in trimethylation at lysine-4 of histone3 (H3K4me3), a transcriptionally active histone modification (Supplementary Fig. S7B).

Finally, to better support a role for BMI1 protein in maintaining the repressive epigenetic status of the *MCP-1*

locus in early-phase UCB-MSCs, we established stable cells expressing *BMI1* shRNA (Supplementary Fig. S8A). As previously reported, *BMI1* KD UCB-MSCs exhibited the typical senescent phenotype, such as an enlarged and flattened morphology and increased SA- β -gal activity (Supplementary Fig. S8B, C). As expected, the promoters of *MCP-1* in *BMI1* KD cells lost the recruitment of BMI1 and the concomitant H2AK119Ub histone modification (Fig. 7B). The epigenetic change in the *MCP-1* locus in *BMI1* KD cells coincided with derepression of *MCP-1* transcription (Fig. 7C), which resulted in increased secretion of MCP-1 protein (Fig. 7D). The enhanced secretion of MCP-1 protein in BMI1 KD cells triggered cellular senescence, as judged by the increase in SA- β -gal activity (Fig. 7E) and the activation of the p53-p21 signaling pathway (Fig. 7F). Most importantly, the senescent phenotypes shown in *BMI1* KD cells were significantly rescued after silencing of *MCP-1* (Fig. 7E, F). Taken together, these results demonstrate that BMI1, using a similar mechanism to that seen in the *INK4* locus, is responsible for sustaining the repressive epigenetic stability in the promoter of *MCP-1*, which plays a key role in SASP-mediated stimulation of senescence of UCB-MSCs.

Discussion

Extensive *ex vivo* expansion by long-term cultivation is required to acquire sufficient numbers of cells for MSC-based therapies. Our present study demonstrated that senescent features of UCB-MSCs that develop through expansion in culture were stimulated by MCP-1, a main mediator of the SASP in UCB-MSCs whose expression was epigenetically regulated by the BMI1 polycomb group protein. In addition, depending on basal MCP-1 secretion, UCB-MSCs exhibited heterogeneous susceptibility to senescence during sustained culture (Fig. 1C), and the expression level of *CCR2* was strongly associated with the senescent phenotype of UCB-MSCs (Fig. 3). These results suggest that the status of MCP-1 and *CCR2* signaling activity could be adopted as an index for assessing the commitment of the cell to senescence. The MCP-1 and *CCR2* signaling activity could also be a good predictor of susceptibility to senescence in response to environmental stresses, which is critical for their therapeutic outcomes. Indeed, in an allergic asthma animal model, *CCR2* KD in UCB-MSCs led to greater beneficial effects by not only improving the engraftment capacity of infused stem cells but also reducing the airway inflammation response (Fig. 6). Moreover, since senescence-associated MCP-1 secretion was also seen in bone marrow-derived MSCs (data not shown), the roles of the MCP-1-centered SASP in other adult stem cells, such as hematopoietic stem cells (HSCs) and neural stem cells (NSCs), should be investigated to verify whether the SASP could be exploited to regulate the senescence progress of adult stem cells. Interestingly, extracellular vesicles released from bone marrow-derived MSCs play a beneficial paracrine role in reversing injury to renal (9, 10) and lung (41) tissues. The secreted extracellular vesicles, enclosed in a lipid bilayer vesicle with a diameter of ~50–100 nm, can alter the phenotype of neighboring cells by delivering proteins, mRNA, microRNA, and long noncoding RNA contained in the extracellular vesicle to target cells (41). Thus, elucidation of a potential role of extracellular vesicles in the SASP in UCB-MSCs could be required for further studies.

FIG. 7. BMI1 epigenetically represses MCP-1 in UCB-MSCs. (A) The *MCP-1* locus marked with the regions of DNase I hypersensitivity and enriched with H3K4me3, H3K27me3, EZH2, and HDAC1 is described in Supplementary Figure S7A. The location of primer sets used in the ChIP assay is marked with a line. ChIP analysis for early- (passage 4) and late- (passage 13) passage UCB-MSCs (UCB #10). The enrichment of BMI1 (left panel) or H2AK119Ub (right panel) is represented as the ratio of the bound to the unbound fraction (B/UB) of ChIP products. (B) ChIP for BMI1-bound (left panel) or H2AK119Ub-modified (right panel) chromatin in UCB-MSCs (UCB #10) stably expressing empty (shControl) or *BMI1* (shBMI1) shRNA. Data are shown as the mean $\pm$ SEM ($n=3$; two-way ANOVA). $*p<0.05$, $**p<0.01$. (C, D) Quantification of *MCP-1* transcript (C) or secreted MCP-1 protein (D) following *BMI1* knockdown in UCB-MSCs (UCB #10). Data are represented as the median $\pm$ interquartile range (C, $n=5$; Kruskal–Wallis test) or mean $\pm$ SEM ($n=3$; two-way ANOVA). $**p<0.01$, $***p<0.001$ compared with shControl. (E, F) SA- β -gal staining (E) and expression of the cellular senescence marker proteins (P-p53, p21, and BMI1) (F) in UCB #6 cells transfected with *BMI1* shRNA (shBMI1) in the presence or absence of *MCP-1* shRNA (shMCP-1). β -actin was used as a control for Western blot analysis. Quantitative data for SA- β -gal staining (E) are shown as the mean $\pm$ SEM ($n=5$; one-way ANOVA). $***p<0.001$.

As a general cause of senescence, oxidative stress and activation of its associated signaling cascades (*e.g.*, p38-MAPK, NF- κ B, C/EBP β , p53, p21) could be generated by chronic exposure to extrinsic stresses. The secreted MCP-1 protein, acting through the CCR2 receptor, increased ROS and in turn activated p38-MAPK and p53/p21 signaling (Fig. 5A–F). Notably, p53, activated by either ectopic expression or its chemical activator, significantly increased the secretion of MCP-1 (Supplementary Fig. S6). Thus, oxidative stress acts as a pivotal second messenger in consolidating MCP-1-mediated stem cell senescence through positive feedback amplification. Indeed, treatment with NAC effectively prevented the senescence of UCB-MSCs after exposure to recombinant MCP-1 protein (Fig. 5G, H). It is well recognized that stem cells in the mesenchymal niches in the body have a relatively low concentration of oxygen at 1%–8% (6, 32). Thus, 21% ambient oxygen would produce oxidized biological macromolecules and result in the generation of ROS (26). Indeed, UCB-MSCs maintained under hypoxic (5% oxygen) conditions showed improved cell growth capacity (Supplementary Fig. S9A) and expression of BMI1 protein (Supplementary Fig. S9B), but reduced induction of the p53-p21 signaling cascade (Supplementary Fig. S9B) and MCP-1 protein secretion (Supplementary Fig. S9C) compared with cells under normoxic (21% oxygen) conditions. These results suggest that the culture conditions relevant to the cellular level of ROS could be a practical strategy to retard the progression of cellular senescence during *ex vivo* expansion of UCB-MSCs.

In addition, we suggest BMI1 as another possible candidate that mediates this positive feedback because its expression in UCB-MSCs was decreased by MCP-1 and because BMI1 stably maintained the repressive epigenetic status of the *MCP-1* locus in early-phase UCB-MSCs (Fig. 7). Since *BMI1* is downregulated in replicative senescent fibroblasts, both cell-intrinsic and -extrinsic stimuli converged on reduced BMI1 activity, which could stably reprogram the stressed cells to adopt a senescent phenotype by an epigenetic mechanism.

BMI1 is highly expressed in numerous adult stem cells to protect them against premature aging by epigenetically repressing aging-associated gene expression (38). Indeed, a deficiency of *Bmi-1* in HSCs and NSCs results in premature aging and a decreased regenerative capacity (34, 39). The best-characterized target of *Bmi-1* is the *Ink4* locus, which, by alternative splicing, encodes two important tumor suppressors, *p16Ink4a* and *p19Arf* (8). As *Bmi-1* tends to be repressed during aging, the *Ink4* locus genes become progressively derepressed in several tissues (27) and stem cells (47) from older individuals. Notably, deletion of *Ink4a* or *Arf* from *Bmi-1*^{−/−} mice partially rescued the self-renewal capability of HSCs and NSCs (33,51), suggesting that an additional pathway must also function downstream of *Bmi-1* in regulating stem cell aging. The present study provides experimental evidence that *MCP-1* is a novel target gene for BMI1 protein to orchestrate stem cell senescence (Fig. 7).

Chronic inflammation has long been regarded as a causative contributor to age-associated degenerative ailments, including diabetes mellitus, atherosclerosis, osteoarthritis, neurodegenerative diseases, and cancers, which are major potential targets of MSC-based cell therapeutics (40). Chronic inflammatory responses redirect the protective inflammatory response to

danger signals in the acute phase of inflammation into a self-destructive, damaging, and deregulated proliferation-prone environment. In this regard, chronic inflammation might be the origin site of cancer, and inflammatory microenvironments could be more common in aged tissues. Given that cell therapies are being clinically administered to treat chronic degenerative diseases with persistent inflammation, such an inflammation-prone microenvironment might affect the final outcome of cell therapies by affecting the senescence program of MSCs in a cell-autonomous or noncell-autonomous manner (1, 28, 43). Several studies have reported that a long-lasting deviant profile of inflammatory molecules, including MCP-1, was causatively involved in the initiation and maintenance of aging-related diseases and aging processes (21, 35, 50).

With this in mind, several new angles on the preparation of next-generation MSC-based cellular therapies should be considered. First, ready-to-use MSC-based cell therapies should be cautiously monitored to remove contamination from senescent cells, which could be conducted *via* a surrogate marker such as MCP-1 and CCR2 that can reveal how far the cells have committed to senescence. Even a small population of senescent cells in the therapy may trigger a stress response inside the other young healthy cells that reinforces itself through a vicious loop. Accordingly, the fewer the cells undergoing senescence in the therapy, the greater the benefit to patients. Once the senescence program in MSCs begins, functional compromise of their multipotency and consecutive loss of therapeutic competence inevitably result. Therefore, screening and depletion of the CCR2-expressing population could be of tremendous value when monitoring the quality of the expanded UCB-MSCs. Notably, these important concepts for MSC therapy were experimentally proven *in vivo* using the severe murine asthma model (Fig. 6). Thus, cell therapies should be furnished as interfered MCP-1/CCR2 signaling acting on the senescence program, although the preparation of such customized cell therapies would be costly and challenging.

In the present study, we provide the first evidence supporting the existence of the SASP as a causative contributor to UCB-MSC senescence and positive feedback between the intrinsic and extrinsic circuitry of the process. Therefore, our current results provide important insights into an enhanced and integrative understanding of both the functional aspects and regulation of the SASP, which may open up new therapeutic modalities for the preparation of next-generation MSC-based cell therapies.

Materials and Methods

Cell culture

This study was approved by the Institutional Review Board of MEDIPOST Co., Ltd. UCB was collected from umbilical veins after neonatal delivery with maternal informed consent (Supplementary Table S1). UCB harvests were processed within 24 h of collection. UCB was separated by isolating mononuclear cells with Ficoll-Hypaque solution (density, 1.077 g/cm³; Sigma, St. Louis, MO). The separated mononuclear cells were washed, suspended in Minimum Essential Medium α modification (MEM α ; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco), and seeded at a concentration of 5×10^5 cells/cm². Cultures were maintained at 37°C in a humidified atmosphere (21%

O₂) containing 5% CO₂ with a change of culture medium twice a week (55). To maintain cells under hypoxic conditions, the oxygen concentration in the chamber was maintained at 5% with a residual gas mixture consisting of 5% carbon dioxide and balanced nitrogen. Primary bone marrow MSCs (five lots) were purchased from Lonza (PT-2501; Basel, Switzerland).

Reagents

Human recombinant MCP-1 protein (279-MC) was purchased from R&D Systems (Minneapolis, MN). The p38-MAPK inhibitor, SB203580, was obtained from Sigma. Anti-CCR2 antibody (ab32144; Abcam, Cambridge, MA) was used to block CCR2 stimulation. NAC, used to assess antioxidant effects, was purchased from Sigma. Detailed information on the reagents is provided in Supplementary Table S2.

Growth kinetics

Expansion during time in culture was measured using the trypan blue exclusion method. Each passage was cultured for 5 days and cells were obtained with trypsin-EDTA (Gibco), counted, and reseeded at a density of 2000 cells/cm². Culture medium was replaced twice weekly. The PD was calculated for each passage by dividing the logarithm of the fold increase value obtained at the end of the passage by the logarithm of 2 (24). The PD and cumulative PD were continuously monitored until cells ceased to proliferate between 11 and 19 passages (Supplementary Fig. S1A). Cell growth in the experimental conditions was depicted as the fold increase in the cell number for each passage. A colony-forming unit-fibroblast (CFU-F) assay was performed by seeding 100 cells in a 35-mm culture dish (BD Biosciences, San Jose, CA) and incubating them in humidified 5% CO₂ at 37°C; culture medium was exchanged every 3 days. After 2 weeks, the dishes were washed twice with phosphate-buffered saline (PBS; Gibco), fixed with 100% methanol, and stained with 3% crystal violet (Sigma). The number of colonies was counted.

Detection of the intracellular ROS level

ROS stress was assessed using a fluorescent probe (carboxy-H2DCFDA; C400; Invitrogen, La Jolla, CA) as previously described (53). Briefly, cells seeded in a T75 tissue culture flask at a density of 2000 cells/cm² were maintained for 5 days before incubating with 10 μ M C400 for 1 h. The ROS fluorescence intensity was analyzed using an FACSCalibur flow cytometer, and the ROS level was represented as the percentage of C400-stained cells.

Evaluation of the multilineage differentiation potential

Multilineage potential was assessed by incubating cells under specific conditions to induce differentiation into osteocytes, chondrocytes, and adipocytes (25). The procedures used are detailed in the Supplementary Materials and Methods.

Cytokine antibody array and enzyme-linked immunosorbent assay

To obtain CdM, cells were serum starved for 24 h, washed, and then cultivated for a further 24 h with fresh serum-free medium. CdM was collected and clarified by centrifugation

at 200 g for 5 min. The presence of cytokines within the harvested CdM was detected using a commercially available proteome profiler array membrane, the Human Inflammatory Cytokine Array C2000 and Analysis Tool (AAH-CYT-2000) (RayBiotech, Norcross, GA). The detail of array maps in each membrane is described in Supplementary Figure S3A. Human MCP-1 (DCP00) and IL-6 (D6050) protein were quantified by an enzyme-linked immunosorbent assay (ELISA; R&D Systems) according to the manufacturer's protocol. Results were obtained by measuring absorbance at 450 nm and were normalized per 1×10^4 cell number.

Real-time polymerase chain reaction

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to cDNA using oligo-dT primers (Invitrogen) and superscript II polymerase (Invitrogen). Amplification was performed with the LightCycler real-time polymerase chain reaction (PCR) (Roche, Mannheim, Germany) system using LightCycler TaqMan Master Mixtures (Roche), sequence-specific probes (Roche), and specific primers (Supplementary Table S3). Relative quantities of the mRNA of interest were calculated by the comparative threshold cycle ($2^{-\Delta\Delta C_t}$) method and normalized to those of β -actin. Values are expressed as fold increases relative to the levels of uninduced cells, defined as 1.

RNA interference

siRNAs for MCP-1, CCR2, and control were purchased from Dharmacon (Chicago, IL). The siRNA pool consisted of four duplexes (Supplementary Table S3). Cells were treated with control siRNA (50 nM), MCP-1 siRNA (50 nM), or CCR2 siRNA (50 nM) for 24 h using DharmaFECT reagent (Dharmacon). When cells were examined at multiple passages, *de novo* transfection of siRNAs was performed at each passage. To establish UCB-MSCs stably expressing MCP-1, CCR2, or *BMII* shRNA, the shRNA was cloned into pSicoR lentiviral vector (Addgene plasmid 12084). Lentivirus was produced by a four-plasmid transfection system according to the manufacturer's instructions (Invitrogen) and concentrated by precipitation using polyethylene glycol 6000. The concentrated virus was infected into UCB-MSCs with 10 μ g/ml polybrene (Invitrogen). The cells were further maintained for at least 3 weeks in the presence of 1 μ g/ml puromycin (Invitrogen) for selection, and the effect of ectopic expression was examined by quantitative PCR (qPCR) and Western blotting. The target sequences for each shRNA are indicated in Supplementary Table S3.

Overexpression and activation of p53 and CCR2

To overexpress the p53 protein, the coding sequence of full-length human p53 was cloned into the pCDNA3 expression vector. Cells were then transfected using transfection reagent (Invitrogen). For p53 activation, cells were treated with 10 μ M nutlin-3 for 24 h (Sigma). Human CCR2 cDNA amplified by PCR using a human CCR2 open-reading frame clone (BC074751; Dharmacon) as a template was cloned into the pLenti7.5/V5 TOPO lentiviral vector (Invitrogen). Five days after infection of lentivirus containing

the *CCR2*-expressing cassette, the effects of ectopic expression of *CCR2* were examined using Western blot analysis.

SA- β -gal staining

SA- β -gal staining was used as a biomarker of senescence in UCB-MSCs. SA- β -gal activity was qualitatively assessed with a histochemical staining kit (CS0030; Sigma) according to the manufacturer's instructions, followed by inverted microscopy. The percentage of senescent cells was represented by the number of stained cells per the total number of cells.

Telomerase activity assay

Telomerase activity was measured by a telomeric repeat amplification protocol (TRAP) assay with a telomerase PCR ELISA kit (TeloTAGGG Telomerase PCR ELISA, 11854666910; Roche) according to a published protocol (14). Approximately 2×10^5 cells were harvested for each reaction and centrifuged at 3000 g for 10 min at 4°C. The absorbance for the final product was acquired by measuring absorbance at 450 nm.

Western blotting

Cell extracts were prepared in buffer containing 9.8 M urea, 4% CHAPS, 130 mM dithiothreitol, 40 mM Tris-HCl, and 0.1% sodium dodecyl sulfate. Protein concentrations were measured by a bicinchoninic acid assay (Sigma). Protein extracts (15 μ g) were separated by SDS-PAGE and the resolved proteins were transferred to nitrocellulose membranes. Each membrane was incubated with anti-phospho-p53 (Ser392) (#9281), anti-p53 (#9282), anti-CCR2 (ab32144; Abcam), anti-p21 (#3688), anti-phospho-Rb (Ser780) (#9307), anti-Rb (#9309), anti-phospho-p38 (Thr180/Tyr182) (#9211), anti-p27 (#3688) (Cell Signaling, Danvers, MA), anti-BMI1 (39993; Active Motif, Carlsbad, CA), or anti- β -actin (A5441; Sigma). The density of signals for the indicated proteins was measured and quantified using NIH ImageJ software.

Asthma animal model

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Ulsan College of Medicine. To generate a murine model of severe asthma, 6-week-old BALB/c mice (OrientBio, Gapyong, Gyeonggi-do, Korea) were sensitized and challenged by intranasal administration with 75 μ g OVA (Sigma) and 10 μ g polyI:C (Calbiochem) at days 0, 1, 2, 3, 7, 14, 21, 22, and 23. Mice were injected with 3×10^5 UCB-MSCs by intravascular injection at day 15. BALF, lymph nodes, and lung tissues were obtained from the mice 24 h after the last immunization. The number of monocytes, eosinophils, neutrophils, and lymphocytes and the concentrations of IL-10 and IL-8 in BALF were measured as previously described (3, 31). For histopathological evaluation, the lungs were perfused with 5 ml PBS through the right ventricle and inflated with 1 ml PBS through the trachea. The inflated lungs were fixed by immersing them in 10% neutral buffered formalin solution for 24 h. Fixed lung tissues were embedded in paraffin and sectioned to 4 μ m thickness and the magnitude of inflammation around the bronchial and vascular area was examined by hematoxylin and eosin staining. The engraftment of the infused UCB-MSCs was determined by immunofluorescence

analysis of human B2-microglobulin (ab15976; Abcam) visualized using an FITC-labeled secondary antibody. Nuclei were counterstained using 4',6-diamino-2-phenylindole (DAPI; Sigma).

ChIP assay

ChIP analysis was performed using a Magna ChIP™ G kit (Upstate-Millipore, Billerica, MA) according to the manufacturer's instructions. The procedures used are detailed in the Supplementary Materials and Methods section.

Statistical analyses

Data are reported as mean $\pm$ standard error of the mean (SEM) if the total sample size was more than 20 or as median $\pm$ interquartile range if the total sample size was smaller than 20 and were analyzed by GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). Differences and significance were verified by one-way or two-way ANOVA, followed by Bonferroni *post hoc* tests when the total sample size was more than 20. Otherwise, we performed a nonparametric Kruskal-Wallis and Mann-Whitney *U* test for comparing three and two groups, respectively. All *p*-values less than 0.05 were considered statistically significant.

Supplementary Data

Supplementary data include Supplementary Materials and Methods, nine figures, and three tables and can be found with this article online.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ALP	= alkaline phosphatase
BALF	= bronchoalveolar lavage fluids
CCR2	= chemokine (c-c motif) receptor 2
CdM	= conditioned medium
ChIP	= chromatin immunoprecipitation
H2AK119Ub	= monoubiquitination at lysine-119 of histone2A
H3K4me3	= trimethylation at lysine-4 of histone3
HSCs	= hematopoietic stem cells
IL-6	= interleukin-6
KD	= knockdown
MAPK	= mitogen-activated protein kinase
MCP-1	= monocyte chemoattractant protein-1
MSCs	= mesenchymal stromal cells
NAC	= N-acetyl-L-cysteine
NSCs	= neural stem cells
PCR	= polymerase chain reaction
PDs	= population doublings
Rb	= retinoblastoma
ROS	= reactive oxygen species
SASP	= senescence-associated secretory phenotype
SA-β-gal	= senescence-associated beta-galactosidase
UCB-MSCs	= umbilical cord blood-derived MSCs