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## Mesenchymal stem cells modulate differentiation of myeloid progenitor cells during inflammation

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### Abstract

Mesenchymal stem cells (MSCs) possess distinct immunomodulatory properties and have tremendous potential for use in therapeutic applications in various inflammatory diseases. MSCs have been shown to regulate pathogenic functions of mature myeloid inflammatory cells, such as macrophages and neutrophils. Intriguingly, the capacity of MSCs to modulate differentiation of myeloid progenitors to mature inflammatory cells remains unknown to date. Here, we report the novel finding that MSCs inhibit the expression of differentiation markers on myeloid progenitors under inflammatory conditions. We demonstrate that the inhibitory effect of MSCs is dependent on direct cell-cell contact and that this intercellular contact is mediated through interaction of CD200 expressed by MSCs and CD200R1 expressed by myeloid progenitors. Further, using an injury model of sterile inflammation, we show that MSCs promote myeloid progenitor frequencies and suppress infiltration of inflammatory cells in the inflamed tissue. We also find that downregulation of CD200 in MSCs correlates with abrogation of their immunoregulatory function. Collectively, our study provides unequivocal evidence that MSCs inhibit differentiation of myeloid progenitors in the inflammatory environment via CD200-CD200R1 interaction.

### Keywords

mesenchymal stem cells; myeloid progenitor cells; immunoregulation; differentiation; inflammation

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## Introduction

During hematopoiesis, myeloid lineage-committed progenitors derived from hematopoietic stem cells (HSCs) in the bone marrow give rise to mature myeloid cells such as macrophages and neutrophils [1]. The bone marrow is also home to non-hematopoietic stromal cells such as mesenchymal stem cells (MSCs), which, in addition to providing a niche and trophic support for HSCs, maintain hematopoiesis by sustaining a part of the HSC population in an undifferentiated quiescent state through release of soluble factors and intercellular interactions [2, 3].

Acute inflammatory stresses lead to deviation of hematopoiesis toward preferential induction of committed myeloid progenitors and their subsequent differentiation into mature macrophages and neutrophils [4]. The highly proliferative capacity of myeloid progenitors plays a central role in inflammation-induced myelopoiesis, restoring consumed macrophages and neutrophils at the site of inflammation [5]. Despite the critical role of mature myeloid cells in host defense and resolution of inflammation, excessive innate immune response can have deleterious effects on tissue homeostasis and lead to undesired tissue damage.

In addition to supporting hematopoiesis, MSCs are characterized by their self-renewal and multilineage differentiation potential and unique immunoregulatory properties [6]. Studies on the interaction between MSCs and immune cells have shown that MSCs can regulate functions of mature innate immune cells, including polarization of inflammatory macrophages into an anti-inflammatory phenotype and enhancement of the phagocytic capacity of neutrophils [7, 8].

Although much is known about the regulatory role of MSCs on function of mature myeloid cells, information regarding potential regulatory interactions between MSCs and myeloid progenitor cells is lacking. Given the central role of myeloid progenitors in inflammation, regulating differentiation of these precursors into pathogenic myeloid cells could effectively inhibit inflammatory response at an earlier stage. In this study, we sought to determine whether MSCs can inhibit the differentiation of myeloid progenitors into mature inflammatory cells during inflammation. Specifically, we demonstrate that MSCs inhibit differentiation of myeloid progenitors and maintain these cells in an immature state. Using both *in vitro* co-culture assays and an *in vivo* model of injury-induced sterile inflammation, we show that MSCs exert immunoregulatory effects on myeloid progenitors in a cell-cell contact dependent manner – a process mediated through CD200-CD200R1 interaction.

## Materials and Methods

### Animals

Six- to eight-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were used in the experiments. Mice were kept in a pathogen-free environment at the Schepens Eye Research Institute Animal Facility. The protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Isolation, expansion, and characterization of mesenchymal stem cells

Bone marrow was harvested from femur bones of euthanized C57BL/6 mice. Using the previously described plastic adherence method of MSC cultivation [9, 10], bone marrow cells were cultured at 37°C in murine MesenCult basal medium and supplement (Stem Cell Technologies, Vancouver, BC, Canada). Cells were passaged at every three to five days intervals. Before using in experiments, MSCs from third passage were characterized phenotypically for the expression of MSC markers (CD45<sup>-</sup>CD34<sup>-</sup>SCA1<sup>+</sup>CD29<sup>+</sup>) using flow cytometry, and functionally by in vitro differentiation using adipocytes using MesenCult adipogenic stimulatory supplements (Stem Cell Technologies). Oil-red-O (Sigma-Aldrich, St. Louis, MO) staining was used to confirm the differentiation of MSCs into the adipocytes. MSCs from third passage were used in both in vitro and in vivo experiments.

### Myeloid progenitor cell characterization and isolation

Single cell suspensions from spleen, bone marrow, and draining submandibular lymph nodes harvested from C57BL/6 mice were stained with fluorochrome-conjugated monoclonal antibodies to CD14 (#123308), CD11b (#101210), CD34 (#119307), c-kit (#105817), and FcγR2/3 (#101327) (Biolegend, San Diego, CA, USA) for characterization of myeloid progenitors. Due to higher frequencies of myeloid progenitors in the spleen, CD14<sup>+</sup>CD11b<sup>-</sup> progenitors were then isolated from the spleen by flow sorting (MoFlo XDP, Beckman Coulter). Purity of isolated myeloid progenitors (>95%) was determined by flow cytometry. Isolated spleen-derived progenitors were characterized for the expression of progenitor and mature myeloid cell markers before being used in in vitro experiments, as described later in Flow cytometry method.

### Co-culture and transwell assays

Isolated spleen-derived myeloid progenitors ( $2 \times 10^5$  cells) were cultured with or without in vitro expanded MSCs ( $4 \times 10^4$  cells) for 72 hours in the presence of 100ng/mL IFN $\gamma$ , 100 ng/mL IL-1 $\beta$ , or 10 ng/mL GM-CSF (Biolegend, San Diego, CA, USA) as inflammatory or hematopoietic growth factor stimuli. For the indirect co-culture, MSCs were first cultured in a monolayer on 6.5 mm transwell inserts with 0.4  $\mu$ m pore size (Corning, NY, USA) and then co-cultured with isolated myeloid progenitors at the ratio of 1:5 MSCs to myeloid progenitors in the presence of 100ng/mL IFN $\gamma$  for 72 hours.

### shRNA transfection

MSCs ( $1.5 \times 10^6$  cells) were plated in a 75 cm<sup>2</sup> flask and incubated for 18–24 hours to reach to 60–70% confluency. The cells were then washed and transfected with CD200-specific or non-specific control shRNA using transfection reagent in shRNA transfection media according to the protocol suggested by the manufacturer (Santa Cruz Biotechnology, Dallas, TX). After overnight incubation, transfection media was replaced with normal MSC growth culture media and cells were cultured for additional 2 days. Knockdown efficiency of shRNA was validated by real-time PCR using CD200-specific primers 48 hours after transfection (Supplement Fig. S4).

## Corneal injury model

Corneal injury was induced in mice as described previously [11, 12]. Briefly, Mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine. Central cornea of deeply anesthetized mice was marked by a 2mm trephine. Using the tip of a hand-held motor brush (AlgerBrush II, Alger Company Inc., Lago Vista, TX), total corneal epithelium and anterior stroma were removed mechanically to create corneal injury. Upon completion of the procedure, triple antibiotic ointment was applied to the injured eyes, and a subcutaneous injection of Buprenorphine was given to mice to minimize injury-induced pain. To study the therapeutic effect of MSCs on corneal inflammation, mice were randomly divided into injury only or MSC (wild-type or CD200 shRNA)-recipient groups, with  $n=5$  in each group. In vitro expanded and characterized MSCs or CD200 shRNA-treated MSCs ( $0.5 \times 10^6$  cells suspended in 100 $\mu$ L sterile saline) were injected into the tail veins of mice 1-hour post injury. Mice were euthanized 48 hours post injury to collect corneas for flow cytometry, real-time PCR, and fluorescence microscopy analyses as described later.

## Flow cytometry

Flow cytometry was performed to characterize the phenotype of in vitro expanded MSCs and myeloid progenitors, to evaluate in vitro differentiation of myeloid progenitors, and to quantify the frequencies of CD45<sup>+</sup> and myeloid progenitors in the cornea. Cultured MSCs in single cell suspension were stained with conjugated monoclonal antibodies to CD45 (#103115), CD34 (#119307), Sca-1 (#108107), CD29 (#102207), CD11b (#101210), c-Kit (#105817), CD105 (#120407), CD31 (#102407), and CD200 (#123807). Single cell suspensions were prepared from bone marrow, spleen, and draining submandibular lymph nodes and were stained with conjugated monoclonal antibodies to CD14 (#123308), CD11b (#101235), CD34 (#119307), c-kit (#105817), Fc $\gamma$ RII/III (#101327), Ly6G (#127627), Ly6C (#128007), and CD200R1 (#123907). Single cell suspensions of cultured myeloid progenitors were stained with conjugated monoclonal antibodies to CD11b (#101210), Ly6G (#127627) and CD11c (#117329). Corneas were harvested 48 hours post injury and were digested in RPMI media (Lonza, Walkersville, MD) containing 2 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO) and 2 mg/mL DNase I (Roche, Basel, Switzerland) for 45 minutes at 37 °C and then filtered through a 70- $\mu$ m cell strainer. Corneal single cell suspensions were then stained with conjugated monoclonal antibodies to CD45 (#103133), CD34 (#119307), CD14 (#123308) and CD11b (#101210). All the antibodies with their matched isotype controls were purchased from Biolegend (San Diego, CA, USA). Stained cells were analyzed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA).

## RNA isolation, RT-PCR, and quantitative real-time PCR

Corneas were harvested at 48 hours post injury from each group, and mRNA was isolated using the RNeasy Micro Kit (Qiagen, Germantown, MD, USA). Isolated RNA was reverse transcribed into cDNA using oligo (dT) primer and Superscript TM III (Invitrogen, Grand Island, NY, USA). Real-time PCR was performed using Taqman Universal PCR Mastermix and preformulated primers for PDL-1 (Mm00452054\_m1), VTCN-1 (Mm00628552\_m1), Ceacam-1 (Mm04204476\_m1), CD200 (Mm00487740\_m1), IL-1 $\beta$  (Mm00434228\_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915\_g1)

(ThermoFisher Scientific, Waltham, MA, USA). The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.

### Immunofluorescence and histopathology

Freshly excised corneas were washed in PBS and fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.5% Triton X-100 for 10 minutes. Whole corneas were then immunostained with FITC-conjugated anti-CD14 (#123308) and PE-conjugated anti-CD11b (#101207) (Biolegend, San Diego, CA, USA) overnight at 4°C to detect myeloid progenitors and mounted onto slides with mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized using a confocal microscope (Leica TCS-SP5; Buffalo Grove, IL, USA) at  $\times 20$  magnification. Corneal sections fixed in 4% paraformaldehyde were stained with hematoxylin and eosin. Images were obtained using a bright field microscope (Nikon Eclipse E800; Melville, NY, USA) at  $\times 20$  magnification.

### Statistical analysis

A two-tailed Student's *t*-test was performed and P values  $< 0.05$  were regarded as statistically significant. Results are presented as the mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments. Sample sizes were estimated on the basis of previous experimental studies on corneal injury and inflammation [10, 13].

## Results

### Characterization of myeloid progenitor cells

As immune cells are primarily developed in lymphoid organs, single cell suspensions from bone marrow, spleen, and submandibular lymph nodes were immunostained for flow cytometry analysis as per the gating strategy shown in Supplement Figure S1. First, a population of CD14<sup>+</sup>CD11b<sup>-</sup> cells was identified (Fig. 1A) and gated for further characterization and for examining the expression of progenitor cell markers, including CD34, c-Kit, and Fc $\gamma$ RII/III, as well as the mature myeloid cell markers, Ly6G granulocytic marker and Ly6C monocytic marker (Fig. 1B). Majority of CD14<sup>+</sup>CD11b<sup>-</sup> cells (~80%) were positive for the expression of CD34, c-Kit and Fc $\gamma$ RII/III progenitor markers, and all (~99%) were negative for Ly6G and Ly6C mature myeloid cell markers (Fig. 1B). Based on our results, we estimate that myeloid progenitors (CD34<sup>+</sup>c-Kit<sup>+</sup>Fc $\gamma$ RII/III<sup>+</sup>CD14<sup>+</sup>CD11b<sup>-</sup>Ly6G<sup>-</sup>Ly6C<sup>-</sup>) constitute  $4.8 \pm 1.09\%$  of bone marrow cells,  $8.7 \pm 0.29\%$  of splenocytes, and  $4.1 \pm 0.31\%$  of lymph node cells (Fig. 1C).

### MSCs inhibit differentiation of myeloid progenitor cells in vitro

Bone marrow-derived MSCs were cultured and characterized as per criteria defined by The International Society for Cellular Therapy [9, 10]. MSCs were expanded using the plastic adherence method, and were characterized phenotypically for positive expression of SCA1 and CD29 and negative expression of CD45 and CD34 surface markers, and functionally by their ability to differentiate into adipocytes (Fig. 2A). Next, sorted myeloid progenitor cells were cultured with or without MSCs in the presence or absence of inflammatory or hematopoietic growth stimuli such as IFN $\gamma$ , IL-1 $\beta$  or GM-CSF, which have been implicated in myeloid cell differentiation [14–16]. After stimulation with IFN $\gamma$ , IL-1 $\beta$  or GM-CSF,

expression of mature myeloid cell markers, including CD11b (marker for macrophages; also known as macrophage-1 antigen [Mac-1]) and Ly6G (marker for granulocytes) was investigated using flow cytometry to assess progenitor cell differentiation. Our data showed that upon stimulation with IFN $\gamma$ , myeloid progenitors acquire high expression of both CD11b and Ly6G. Further analysis demonstrated a significant reduction (55%) in expression of CD11b by myeloid progenitor cells cultured with MSCs in contrast to those cultured without MSCs (MFI  $4.15 \pm 1.04$  vs.  $9.2 \pm 1.6$ ;  $p = 0.000065$ ) (Fig. 2B), and a significant 58% suppression in expression of Ly6G in progenitors cultured with MSCs compared to progenitors cultured without MSCs (MFI  $9.9 \pm 0.75$  vs.  $23.02 \pm 1.14$ ,  $p = 0.0000013$ ) (Fig. 2C). Strikingly, our data that myeloid progenitors fail to express CD11c in the steady state or upon stimulation, suggest that these myeloid progenitors do not differentiate into dendritic cells (Supplement Fig. S2). Similar to effects of IFN $\gamma$  on myeloid progenitors, stimulation with IL-1 $\beta$  and GM-CSF also resulted in selective expression of CD11b by myeloid progenitors, which was significantly suppressed in myeloid progenitors co-cultured with MSCs (Supplement Fig. S3). Taken together, these data suggest that MSCs suppress acquisition of differentiation markers by myeloid progenitors and maintain these cells in an immature state in an inflammatory environment.

### **MSCs interact with myeloid progenitors in a cell-cell contact-dependent manner**

To delineate whether the inhibitory effect of MSCs on myeloid progenitor differentiation was through direct cell-cell contact or by MSC-secreted soluble factors, MSCs were either cultured in direct contact with isolated myeloid progenitors, or were first plated into transwell inserts and then cultured with myeloid progenitor cells with IFN $\gamma$  stimulation. Expression of CD11b surface marker was assessed using flow cytometry. As shown in Figure 3A, MSCs that were cultured directly with myeloid progenitors significantly suppressed acquisition of CD11b differentiation marker by these cells. However, MSCs in the transwell chamber system failed to suppress CD11b expression by myeloid progenitors, suggesting that the suppressive function of MSCs is dependent on direct cell-cell contact rather than secretion of soluble factors by MSCs. To further explore the molecular mechanism underlying such contact-dependency, we investigated the expression of following cell membrane-bound inhibitory molecules by MSCs using real time PCR: programmed death-ligand 1 (PD-L1), a transmembrane protein, which delivers inhibitory signals to immune cells upon binding with PD-1 expressed by T cells and activated monocyte; v-set domain containing T cell activation inhibitor 1 (VTCN-1) or B7-H4, a transmembrane protein that negatively regulates the function of T cells and neutrophils [17]; CD200 (OX2), a transmembrane glycoprotein that inhibits function of myeloid immune cells [18]; and carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1), a cell adhesion molecule involved in contact-dependent regulation of the innate and adaptive immune responses [19] (Fig. 3B). Significantly higher mRNA expression of CD200 compared to other molecules prompted us to speculate that CD200 may be the critical ligand mediating the immunoregulatory function of MSCs. Our data further demonstrated that MSCs significantly upregulate their expression of CD200 in the inflammatory environment (Fig. 3C). Using flow cytometry, we also confirmed protein expressions of CD200 on MSCs and its receptor, CD200R1 on myeloid progenitor cells (Fig. 3D & E).

### MSCs inhibit differentiation of myeloid progenitors via CD200-CD200R1 interaction

Next, to investigate the role of CD200 in mediating the immunoregulatory function of MSCs *in vitro*, functional expression of CD200 on MSCs was silenced using CD200-shRNA (Supplement Fig. S4). Control-shRNA or CD200-shRNA treated MSCs were then cultured with myeloid progenitors in the presence of IFN $\gamma$ . Our data regarding expression of CD11b demonstrated that CD200-shRNA-treated MSCs had 22% less ability in suppressing myeloid progenitor differentiation compared to control-shRNA-treated MSCs ( $p=0.008$ ) (Fig. 3F). Compromised ability of CD200-shRNA MSCs to suppress myeloid progenitor acquisition of CD11b suggests that expression of CD200 by MSCs is critical for their inhibitory function on myeloid progenitor differentiation.

### CD200 expression in MSCs is indispensable for suppression of inflammation and accumulation of undifferentiated myeloid progenitors in the inflamed tissue

Lastly, we chose a sterile inflammation *in vivo* model of mouse eye injury – a well-established system to study inflammation [13, 20] – to confirm the immunoregulatory effect of MSCs on myeloid progenitors. This well-characterized model provides an excellent system to study inflammation. Simple anatomy of the eye and its paucity of resident immune cells facilitate study of recruited immune cells and their contribution to the inflammatory response [21]. As demonstrated previously [10, 22], we show that MSCs administered systemically home specifically to the injured cornea (Supplement Fig. S5). Interestingly, similar to the bone marrow, spleen and lymph node, we identified a population of CD34<sup>+</sup>CD14<sup>+</sup>CD11b<sup>-</sup> myeloid progenitors in the cornea (Fig. 4A). Our immunofluorescence microscopy results also confirmed the presence of CD14<sup>+</sup>CD11b<sup>-</sup> progenitors primarily in the stromal layer of cornea (Fig. 4B). Similar to lymphoid tissue-derived progenitors, upon stimulation with IFN $\gamma$ , sorted corneal myeloid progenitors expressed CD11b, and MSCs suppressed their acquisition of CD11b *in vitro* (Supplement Fig. S6). To determine the effect of systemic administration of MSCs on myeloid progenitor cell frequencies and tissue inflammation, mice were intravenously injected with control-shRNA or CD200-shRNA-treated MSCs 1 hour after corneal injury induction, followed by harvesting of corneas 48 hours post-injury (Fig. 4C). Our flow cytometry data demonstrated that normal (control-shRNA-treated) MSCs led to a 5-fold increase in the frequencies of corneal myeloid progenitors, while CD200-shRNA-treated MSCs failed to do so, suggesting that MSC expression of CD200 is important for expansion of myeloid progenitor cell frequencies in the inflamed tissue (Fig. 4E). Similar to our previous findings on the anti-inflammatory effect of MSCs in the inflamed tissue, our data demonstrated that normal (control-shRNA-treated) MSCs, but not CD200-silenced MSCs, have a significant suppressive effect on tissue inflammation as evidenced by reduced frequencies of CD45<sup>+</sup> cells (Fig. 4D), decreased expression of inflammatory cytokine IL-1 $\beta$  (Fig. 4G) and less inflammatory cell infiltration in the corneal stroma (Fig. 4F) compared to untreated mice with corneal injury. These findings strongly suggest that MSCs suppress tissue inflammation by reducing inflammatory cell infiltration and by expanding frequencies of myeloid progenitor cells through a CD200-dependent mechanism.

## Discussion

The current study ascribes a novel immunoregulatory function for MSCs on myeloid progenitor cell differentiation. Our data indicate that MSCs inhibit differentiation of myeloid progenitor cells in an inflammatory environment through direct cell-cell contact. Furthermore, we demonstrate that this intercellular contact is mediated by CD200-CD200R1 interaction, and that CD200 expression by MSCs is indispensable for inhibition of myeloid progenitor differentiation and suppression of tissue inflammation.

Myeloid progenitors are precursors of mature myeloid cells, critical effector cells in innate immune response. Upregulation of pro-inflammatory cytokines such as IFN $\gamma$ , IL1 $\beta$  and TNF $\alpha$  during inflammation activates steady state progenitors in the bone marrow to differentiate into myeloid effector cells [4, 23]. Myeloid progenitors are primarily found in the bone marrow and cord blood [24]. Some studies have demonstrated the presence of undifferentiated monocytes and DC precursors in non-bone marrow tissues such as spleen [25, 26]. Our findings demonstrate a population of myeloid progenitors, which in addition to the bone marrow are also present in peripheral lymphoid tissues, including spleen and lymph nodes. These progenitors express high levels of CD34, CD14, c-Kit and Fc $\gamma$ RII/III progenitor markers, which makes them phenotypically similar to early myeloid progenitors such as common myeloid progenitors (CMPs) and granulocyte/macrophage progenitors (GMPs) [27, 28]. CMPs are thought to be precursors of common DC progenitors, which eventually give rise to DCs [27]. Our data, however, demonstrate that the myeloid progenitors identified do not express the DC marker CD11c in the steady or activated states, suggesting that these cells are not DC precursors. Rather, these progenitors acquire high levels of CD11b and Ly6G myeloid markers in the inflammatory milieu, suggesting these myeloid progenitors are phenotypically closer to GMPs that give rise to macrophages and granulocytes [1, 27, 29].

Interestingly, we find that MSCs inhibit acquisition of CD11b and Ly6G differentiation markers on myeloid progenitors. MSCs have been shown to interact with cells of both innate and adaptive immunity [30]. Recent reports on the interaction of MSCs with DC precursors have demonstrated that MSCs inhibit differentiation of peripheral blood-derived CD14<sup>+</sup> monocytes to mature DCs [31, 32]. Here, our data show that MSCs negatively regulate both bone marrow- and peripheral lymphoid tissue-resident myeloid progenitors. MSCs maintain these cells in an undifferentiated quiescent state and further prevent their differentiation into inflammatory cells. MSCs primarily exert their immunoregulatory effects through secretion of paracrine factors such as IDO, IL-10, TGF- $\beta$  and TSG6 [33, 34]. In contrast, we find that MSCs inhibit differentiation of myeloid progenitors mainly through direct cell-cell contact. The results of our study demonstrate that silencing of CD200 expression in MSCs abrogates their ability to suppress myeloid progenitor cell differentiation, suggesting that CD200-CD200R1 interaction is critical for MSCs to exert their immunoregulatory effect. CD200 or Ox-2 is a transmembrane glycoprotein, which binds to its receptor CD200R1 [35]. The CD200R family of receptors consists of 4 isoforms [36], among which CD200R1 is mainly expressed by myeloid cells and T cells [35, 37]. CD200-CD200R1 pathway plays a central role in regulation of innate immune system by inhibiting myeloid cell activation [38, 39]. We show that bone marrow-derived MSCs constitutively express CD200, and significantly



upregulate CD200 expression in response to inflammatory stimuli. These results are consistent with previous studies which demonstrated that IFN $\gamma$  in particular induces CD200 expression in bone marrow-derived stromal cells [40].

Finally, the functional relevance of MSC regulation of myeloid progenitor cell differentiation during inflammation was tested using a standardized mouse cornea model of sterile injury [10, 41]. Similar to the bone marrow, spleen and lymph nodes, we have identified myeloid progenitors residing in the cornea. MSCs have been shown to migrate to the sites of inflammation and promote wound repair [42, 43]. Previously, we showed that systemically administered MSCs home to the inflamed eye, and accelerate wound healing [10, 22]. Here, we show that MSCs suppress infiltration of inflammatory cells and increase the frequencies of corneal myeloid progenitors. Consistent with our *in vitro* findings, systemically administered CD200-shRNA-treated MSCs lose their ability to suppress differentiation of myeloid progenitors and tissue inflammation. The increase in myeloid progenitor frequencies at the inflamed site could be the result of MSC-mediated expansion of corneal resident myeloid progenitors, or due to MSCs inhibiting differentiation of recruited myeloid progenitors from the bone marrow. Early myeloid progenitors have recently been identified as immunosuppressive cells that are capable of inhibiting T cell proliferation [44]. If MSCs promote recruitment of myeloid progenitors to the inflamed tissue, suppression of inflammation could be the cumulative result of MSC- and myeloid progenitor-mediated regulation of the immune response. However, we acknowledge that further experiments will be needed to elucidate the exact mechanism by which MSCs promote myeloid progenitor frequencies at the site of inflammation.

## Conclusion

In conclusion, our findings provide new insight into the immunoregulatory effect of MSCs on myeloid progenitor cell differentiation. Herein, we show that MSCs suppress inflammation not only by regulating inflammatory cell infiltration, but also by preventing differentiation of early myeloid precursors into inflammatory cells. Our data further supports a critical role for CD200 expressed by MSCs in regulating function of myeloid progenitors and thus inhibiting inflammatory response. These observations could provide a framework for the development of potential CD200-based therapeutics that could effectively modulate the generation of innate immune cells and inhibit inflammation at early stage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

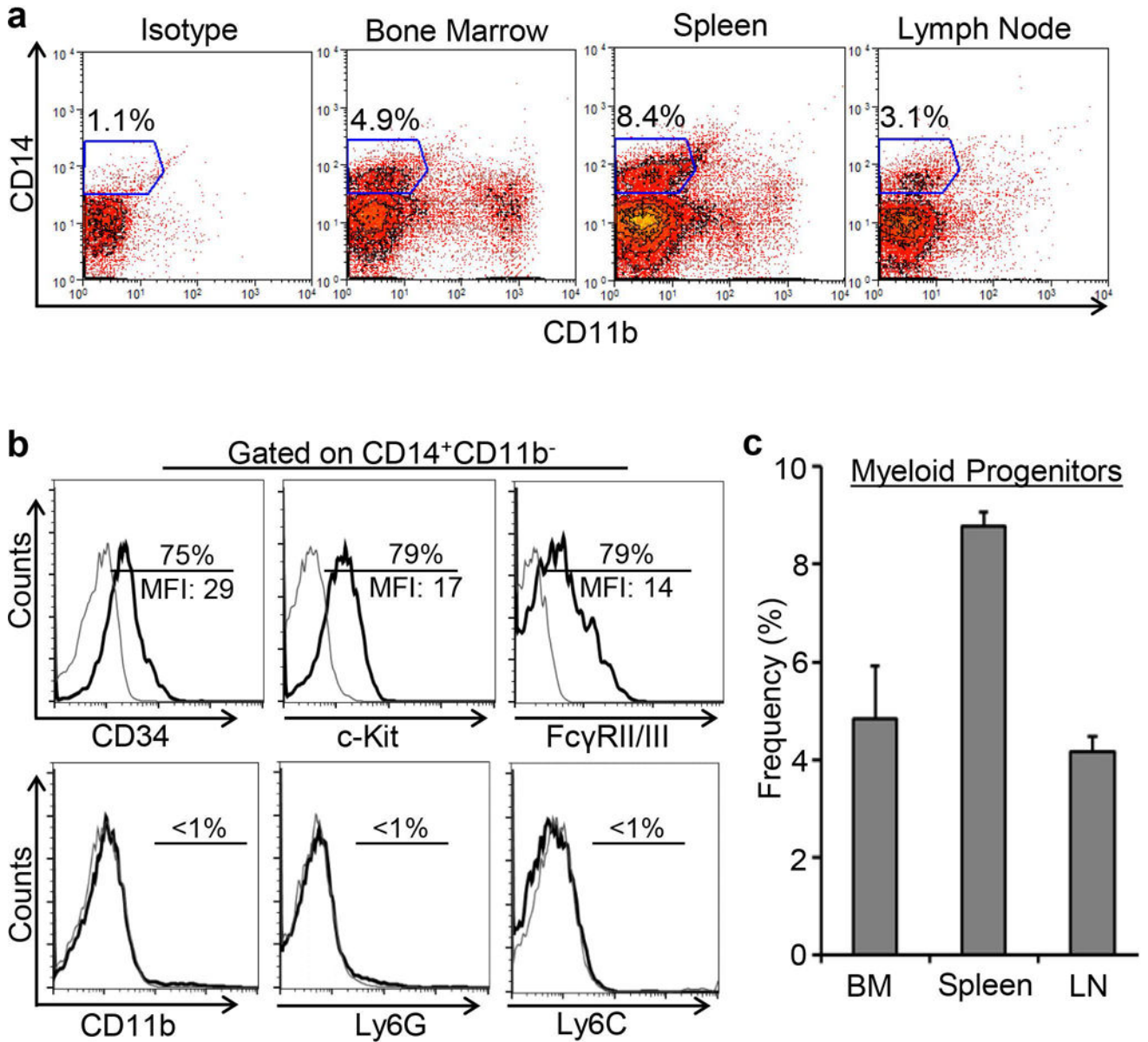
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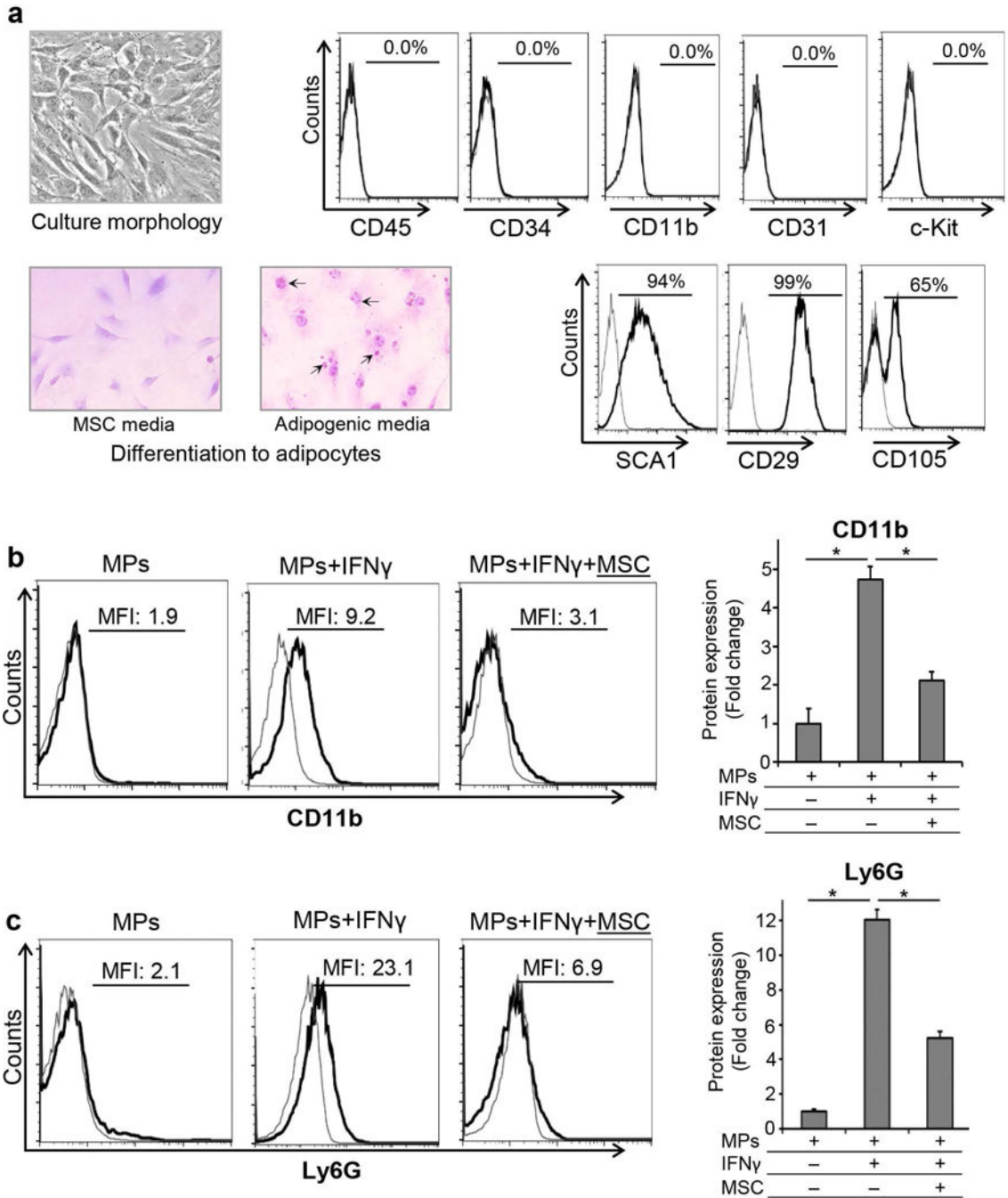
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**Figure 1. Frequencies and phenotypic characterization of myeloid progenitor cells**

**A.** Representative flow cytometric dot plots showing gating strategy for selecting CD14<sup>+</sup>CD11b<sup>-</sup> cells in the bone marrow, spleen, and submandibular lymph nodes. **B.** Representative flow cytometric histograms demonstrating the expression of progenitor markers CD34, c-Kit and FcγRII/III, myeloid marker CD11b, monocytic marker Ly6C, and granulocytic marker Ly6G by CD14<sup>+</sup>CD11b<sup>-</sup> cells. **C.** Bar chart comparing the frequencies of myeloid progenitors in the bone marrow, spleen and lymph node as analyzed by flow cytometry. Representative data from 3 independent experiments are shown and each experiment consisted of 5 animals. Data is represented as mean ± SEM.



**Figure 2. MSCs inhibit differentiation of myeloid progenitor cells in vitro**

**A. Left Panel:** Expansion and characterization of MSCs. Microscopic images of MSCs cultured in MSC or adipogenic media ( $\times 40$  magnification). Oil-Red-O staining after 2 weeks showed red colored fat vacuoles (black arrows) in the cytoplasm of MSCs cultured in adipogenic media, confirming their differentiation into adipocytes. **Right Panel:** Representative flow cytometry plots demonstrating the phenotype of bone marrow derived MSCs as CD45<sup>-</sup>CD34<sup>-</sup>CD11b<sup>-</sup>c-Kit<sup>-</sup>CD31<sup>-</sup>Sca-1<sup>+</sup>CD29<sup>+</sup>CD105<sup>+</sup> cells. **B.** Representative flow cytometric histograms and bar chart demonstrating CD11b expression by myeloid

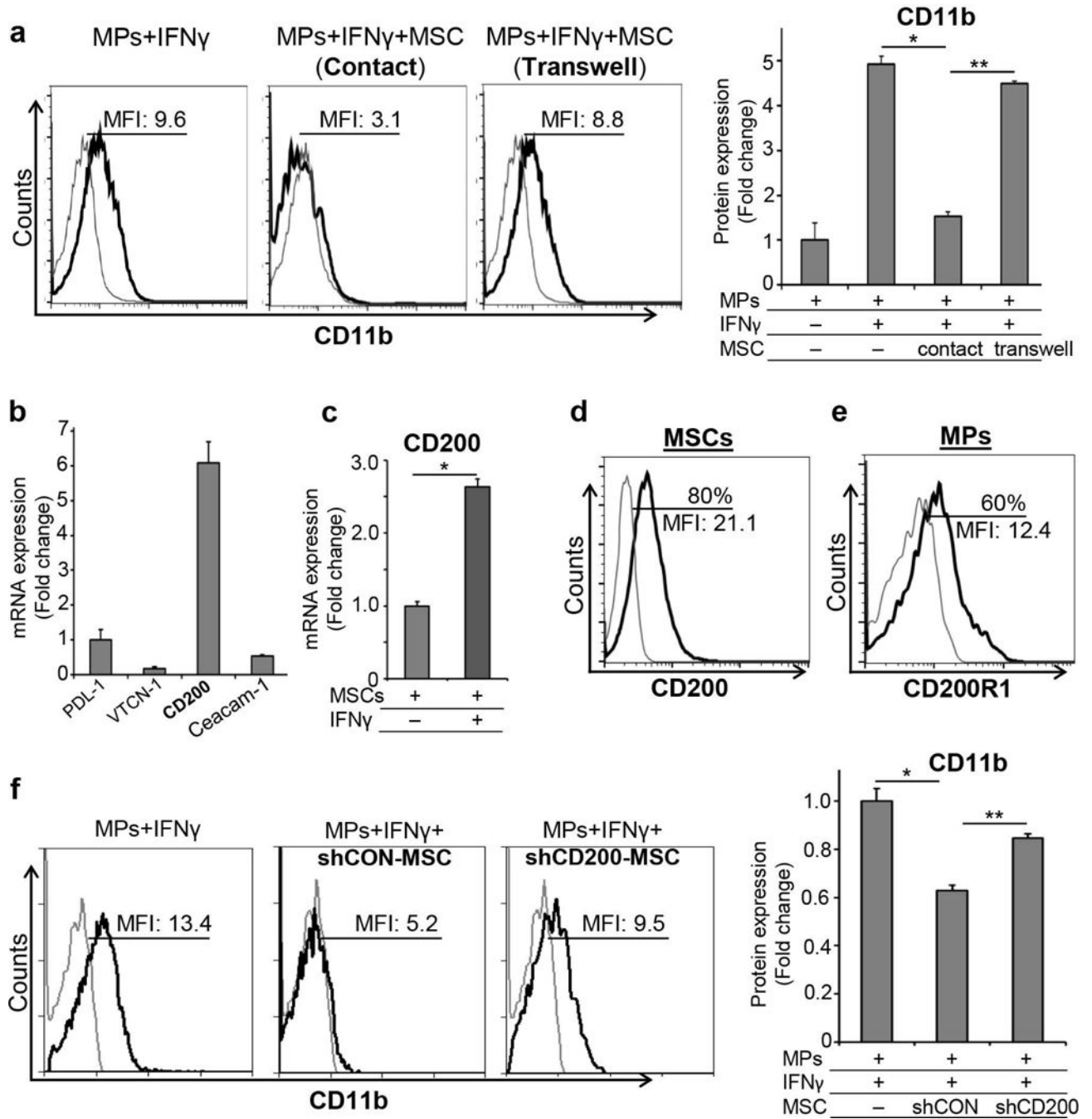
progenitors (MPs) cultured with or without MSCs with IFN $\gamma$  stimulation for 72 hours. C. Representative histograms of flow cytometric data and bar chart showing Ly6G expression by myeloid progenitors (MPs) cultured with or without MSCs with IFN $\gamma$  stimulation for 72 hours. Results are representative of 3 independent experiments. Myeloid progenitors were isolated from a pool of 5–6 animals in each experiment. P values are calculated using student's *t-test* and data is represented as mean  $\pm$  SEM. \* $p < 0.0001$ .

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**Figure 3. MSCs inhibit differentiation of myeloid progenitors in a contact-dependent manner**

**A.** Representative flow cytometric histograms and bar chart demonstrating CD11b expression by myeloid progenitors (MPs) cultured with MSCs either in direct contact or using a transwell chamber system, which separated MSCs from MPs, in the presence of IFN $\gamma$  for 72 hours. Results are representative of 3 independent experiments. Myeloid progenitors were isolated from a pool of 5–6 animals in each experiment. **B.** Real-time PCR analysis of PD-L1, VTCN-1, CD200 and Ceacam-1 mRNA expression levels by resting MSCs. **C.** Real-time PCR analysis of CD200 expression on resting and IFN $\gamma$ -stimulated

MSCs. Representative flow cytometric histograms demonstrating expression of **D.** CD200 on MSCs, and **E.** CD200R1 on myeloid progenitor cells. **F.** Representative flow cytometric histograms and bar chart showing CD11b expression levels in myeloid progenitors cultured with control-shRNA (shCON) or CD200-shRNA (shCD200) MSCs with IFN $\gamma$  stimulation for 72 hours. Results are representative of 3 independent experiments. Myeloid progenitors were isolated from a pool of 5–6 animals in each experiment. P values are calculated using student's *t-test* and data is represented as mean  $\pm$  SEM. \* $p < 0.01$ , \*\*  $p < 0.001$

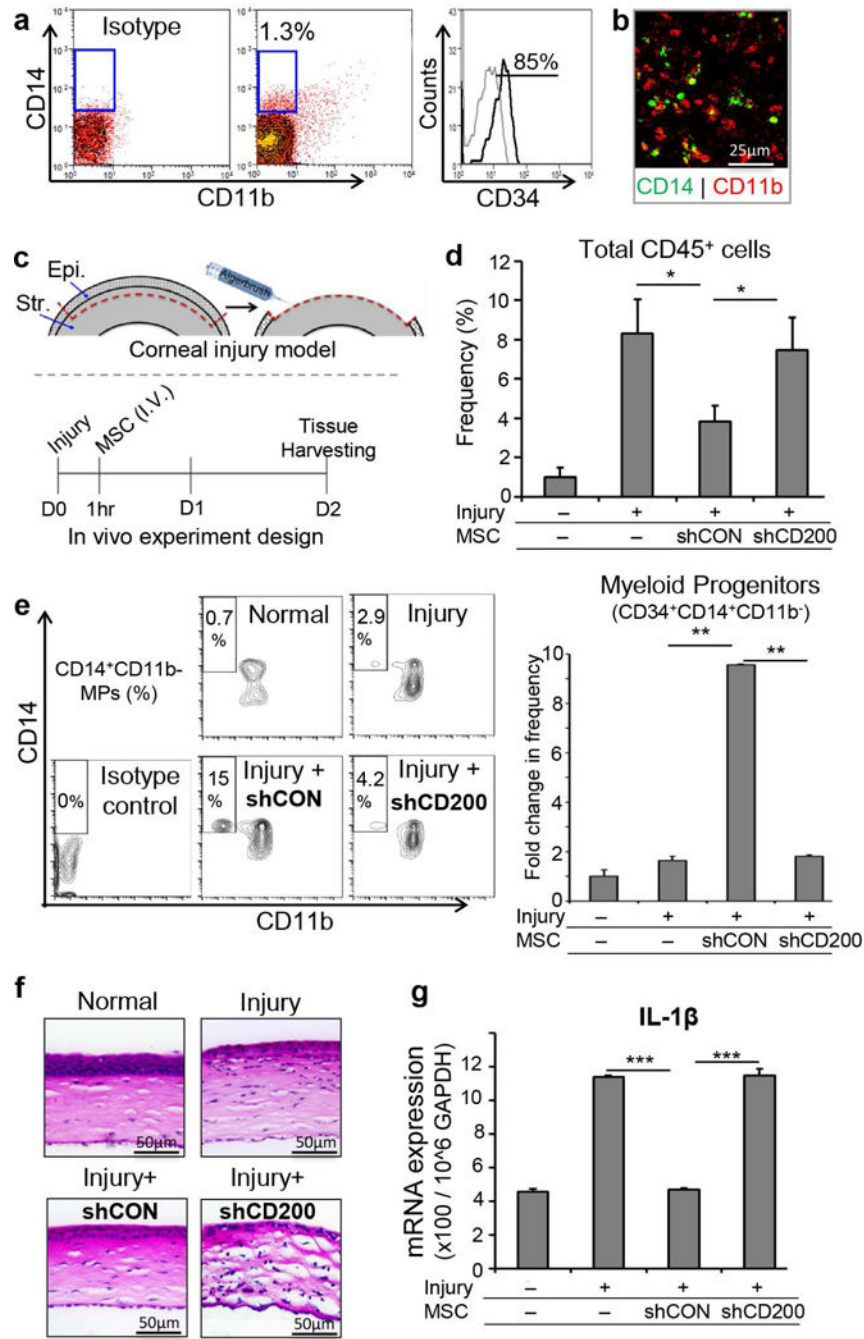
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**Figure 4. MSCs suppress ocular inflammation through expansion of myeloid progenitor cells in a CD200-dependent manner**

**A.** Representative flow cytometric plots demonstrating CD34<sup>+</sup>CD14<sup>+</sup>CD11b<sup>-</sup> myeloid progenitor in the cornea. **B.** Confocal microscopy image (×20 magnification) of corneal whole mount confirming the presence of CD14<sup>+</sup> CD11b<sup>-</sup> cells in the peripheral corneal stroma (Green: CD14, Red: CD11b). **C.** Schematic representation of sterile injury induction in mouse and experiment timeline. Corneal epithelium and anterior stroma are mechanically removed using Algerbrush II. **D.** Bar chart demonstrating the frequencies of infiltrating

corneal CD45<sup>+</sup> cells in naïve mouse, injured mice without systemic MSC treatment, control-shRNA (shCON) MSC-treated and CD200-shRNA (shCD200) MSC-treated mice. **E.** Representative flow cytometric plots and bar chart demonstrating the frequencies of myeloid progenitors in naïve cornea, injured cornea, injured cornea with IV administration of control-shRNA-treated MSCs, and injured cornea with IV administration of CD200-shRNA-treated MSCs 48 hours after injury induction. **F.** H &E staining of corneal cross-sections ( $\times 20$ ) from naïve, untreated, control-shRNA MSC-treated and CD200-shRNA MSC-treated mice demonstrating epithelial and stromal layers and inflammatory cell infiltration. **G.** Real-time PCR analysis of relative expression of IL-1 $\beta$  mRNA in naïve mice, injured mice without systemic MSC treatment, control-shRNA MSC-treated and CD200-shRNA MSC-treated mice. Results are representative of 2 independent experiments. Each group consisted of 4–5 animals in each experiment. P values are calculated using student's *t-test* and data is represented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$