Macrophage Depletion Impairs Corneal Wound Healing after Autologous Transplantation in Mice

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Abstract

Purpose: Macrophages have been shown to play a critical role in the wound healing process. In the present study, the role of macrophages in wound healing after autologous corneal transplantation was investigated by depleting local infiltrated macrophages.

Methods: Autologous corneal transplantation model was used to induce wound repair in Balb/c mice. Macrophages were depleted by sub-conjunctival injections of clodronate-containing liposomes (Cl_2MDP-LIP). The presence of $CD11b^+$ $F4/80^+$ macrophages, α-smooth muscle actin$^+$ (α-SMA^+) myofibroblasts, $CD31^+$ vascular endothelial cells and NG_2^+ pericytes was examined by immunohistochemical and corneal whole-mount staining 14 days after penetrating keratoplasty. Peritoneal macrophages were isolated from Balb/c mice and transfused into conjunctiva to examine the recovery role of macrophages depletion on wound healing after autologous corneal transplantation.

Results: Sub-conjunctival Cl_2MDP-LIP injection significantly depleted the corneal resident phagocytes and infiltrated macrophages into corneal stroma. Compared with the mice injected with PBS-liposome, the Cl_2MDP-LIP-injected mice showed few inflammatory cells, irregularly distributed extracellular matrix, ingrowth of corneal epithelium into stroma, and even the detachment of donor cornea from recipient. Moreover, the number of macrophages, myofibroblasts, endothelial cells and pericytes was also decreased in the junction area between the donor and recipient cornea in macrophage-depleted mice. Peritoneal macrophages transfusion recovered the defect of corneal wound healing caused by macrophages depletion.

Conclusions: Macrophage depletion significantly impairs wound healing after autologous corneal transplantation through at least partially impacting on angiogenesis and wound closure.

Introduction

Wounds normally heal in an orderly manner through the inflammatory phase, proliferation phase and remodeling phase, which are overlapping and involve many cell types, such as neutrophils, macrophages, fibroblasts and endothelial cells [1]. Infiltrated monocytes and macrophages are key cells in the inflammatory phase and clean the wound bed through the phagocytosis of wound debris and necrotic cells. Moreover, they generate free radicals and produce proinflammatory cytokines to recruit more inflammatory cells to the wound site. In addition, the soluble mediators released are involved in the recruitment and activation of fibroblasts and endothelial cells in preparation for the subsequent proliferative and remodeling phase. Fibroblasts are one of the major cells in the proliferative and remodeling phases, and they migrate and accumulate in the provisional matrix of the wound site and secrete and deposit collagen, proteoglycans and other components of the extracellular matrix (ECM) for tissue

matrix remodeling. Moreover, activated fibroblasts can differentiate into myofibroblasts, which are characterized by the expression of the putative contractile marker α-SMA and are central for wound closure. The interactions between myofibroblasts and the surrounding extracellular matrix may be critical for the final result of wound healing. Inappropriate ECM synthesis and deposition can cause fibrosis and scar formation, which results in the deformation of surrounding parenchymal cells [1–4].

The cornea, a highly organized avascular tissue located in the anterior part of the eye, has been widely used as a model for the investigation of wound healing [5–7]. During corneal wound healing, inflammatory cells infiltrate into the corneal stroma, and quiescent corneal keratocytes are activated and differentiated into fibroblasts and myofibroblasts to repair damaged tissue architecture [8]. In addition, new vessels gradually grow from the limbal region to the central cornea, known as corneal neovascularization. Delayed wound healing often results in chronic inflammation,

recurrent epithelial defects, neovascularization and corneal ulceration [9].

The role of macrophages in corneal wound healing has been described in several corneal injury models, such as alkali burn and allogeneic corneal transplantation [10–12]. However, the specific role of macrophages in wound healing after autologous corneal transplantation is poorly understood. According to the known function of macrophages in wound healing, we hypothesize that macrophages may play key roles in corneal wound healing after autologous corneal transplantation, and the depletion of infiltrated macrophages into the cornea may impair the repair process. In the present study, we used the liposome-mediated macrophage depletion method to deplete infiltrated phagocytes into the cornea, evaluated the effect of macrophage depletion on the impairment of corneal wound healing after penetrating keratoplasty, including wound closure and angiogenesis.

Materials and Methods

Animals

Sixty 6- to 8-week-old Balb/c mice (Beijing Pharmacology Institute, Chinese Academy of Medical Sciences) were used for the experiments. All animal experiments were carried out in accordance with The Chinese Ministry of Science and Technology Guidelines on the Humane Treatment of Laboratory Animals (vGKFCZ-2006-398) and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Animal Care and Use Committee on the Ethics of the Shandong Eye Institute (Permit Number: SEIRB-2011-81170815). All surgery was performed on only one eye of each mouse under sodium pentobarbital anesthesia, and the mice were sacrificed with an overdose of 10% chloral hydrate.

Macrophage depletion

Liposomes encapsulated with dichloromethylene diphosphonate (Cl$_2$MDP; Sigma, St. Louis, MO) or phosphate-buffered saline (PBS) were prepared and injected as previously described [13,14]. The final concentration of Cl$_2$MDP was 2.5 mg/ml suspension. Mice were injected sub-conjunctivally with 10 μl Cl$_2$MDP-LIP (n = 30) under anesthesia on days 7 and 2 before penetrating keratoplasty. Control mice were pretreated with PBS-LIP (n = 30). After phagocytosis and disruption of the phospholipid bilayers, Cl$_2$MDP is released into the macrophage cytoplasm, which leads to apoptosis. The depletion of infiltrated macrophages in corneal stroma was evaluated by immunohistochemistry as described below.

Macrophage transfusion

Peritoneal macrophages were collected by lavage of the peritoneal cavity of naïve Balb/c mice as previously described [15]. As briefly, the fluid from peritoneal cavity after the injection of ice-cold phosphate-buffered saline was centrifuged, and the cell pellet was suspended in RPMI-1640 supplemented with 10% fetal bovine serum. Macrophages were collected by culturing the peritoneal exudate cells for 2 hours at 37°C followed by the removal of non-adherent cells. For the transfusion of macrophages, 10^5 cells were injected sub-conjunctivally after 48 hours of penetrating keratoplasty.

Autologous corneal transplantation

Mouse autologous corneal transplantation was performed as described previously, with some modifications [16,17]. Briefly, 1% atropine eye oil was applied 1 day and 0.5% tropicamide mixed with 0.5% phenylephrine hydrochloride eye drops was used 2–3 times/hour before surgery to dilate the pupil. A 2-mm trephine was used to score the donor cornea before the removal of the corneal button using Vannas scissors. The autologous corneal button was sewn in place using 8 interrupted sutures with 11-0 nylon sutures (Mani, Japan). The anterior chamber was reformed with sterile air. To prevent self-injury to eyes, temporary tarsorrhaphy was performed for 3 days. A topical antibiotic (ofloxacin, Santen, Japan) was applied immediately after surgery and once a day thereafter. The eyes that underwent surgery were monitored at 3, 7, 14, 21 and 28 days, the sutures were removed 10 days after penetrating keratoplasty.

Histology

Eyes were collected for histology after 14, 21 and 28 days of corneal surgery. The samples were fixed in 4% formalin and embedded in paraffin. Continuous 4 μm sections were stained with hematoxylin & eosin (H.E. staining). The presence of vascular vessels and inflammatory cells and the corneal fiber distribution in the junction between the donor and recipient cornea were observed by light microscopy.

Immunofluorescence and whole-mount staining

Cornea whole-mount and immunofluorescence staining was performed as previously described [18,19]. Briefly, full-thickness corneal flat mounts and frozen sections were fixed in acetone for immunofluorescence staining. To block nonspecific staining, the samples were incubated with normal goat serum for 20 minutes at room temperature and incubated with primary antibodies overnight at 4°C. Subsequently, they were incubated with secondary antibodies for 1 hour at room temperature. Each step was followed by 3 wash steps with 0.02 M PBS for 5 minutes. The following primary antibodies were used in the present study: rat anti-F4/80 (MCA497GA, AbD Serotec, Oxford, UK), anti-CD11b (101201, BioLegend, San Diego, CA), anti-α-smooth muscle actin (ab5694, Abcam, Cambridge, MA), anti-CD31 (550274, BD Pharmingen, San Jose, CA), and rabbit anti-NG2 chondroitin sulfate proteoglycan (AB5320, Millipore, Billerica, MA). The secondary antibodies included fluorescein-conjugated goat anti-rat IgG (ZF-0315, ZSGB-Bio, Beijing, China) and rhodamine-conjugated goat anti-rabbit IgG (ZF-0316, ZSGB-Bio, Beijing, China). Finally, the flat mount or sections were examined under a confocal microscope (TE2000-U; Nikon, Tokyo, Japan) or epifluorescence microscope (E800; Nikon, Tokyo, Japan).

Results

Effect of Cl$_2$MDP-LIP sub-conjunctival injection on macrophage depletion

CD11b and F4/80 are two commonly used markers for the detection of mouse macrophage [20,21]. Immunofluorescence showed a few scattered F4/80$^+$CD11b$^+$ macrophages in the central corneas of mice injected with PBS-LIP (n = 5). However, there was no specific staining (2 days after the second clodronate liposome injection) in the corneas that were previously injected with clodronate liposomes, which suggests that the sub-conjunctival injection of Cl$_2$MDP-LIP (n = 5) effectively depleted corneal resident phagocytes (Fig. 1). In addition, corneal flat mount staining after 14 days of penetrating keratoplasty showed a number of F4/80+ macrophages recruited near the suture sites and around the junction between the graft and recipient cornea (Fig. 2A, B). Section staining showed that the macrophages clustered in the superficial corneal stromal layer (Fig. 2C), while few macrophages were found in the junction and suture area in

Cl_2MDP-LIP-injected mice (Fig. 2D–F). The expression of CD11b also showed a similar distribution as F4/80 in PBS-LIP- and Cl_2MDP-LIP-injected mice (data not shown). The results showed that Cl_2MDP-LIP sub-conjunctival injection depleted not only corneal resident phagocytes, but also the infiltrated macrophages into cornea after autologous corneal transplantation for at least 14 days.

Effect of macrophage depletion on myofibroblast differentiation

As a critical factor involved in wound contraction, myofibroblasts are characterized by α-SMA expression and are regulated by macrophages through the cytokines and chemokines released [22,23]. Immunofluorescence staining of the corneal flat mounts after 14 days of penetrating keratoplasty revealed the strong expression of α-SMA in the junction area between the graft and the recipient cornea in the PBS-LIP-injected mice (Fig. 3A), while the mice injected with liposomes containing Cl_2MDP showed no staining (Fig. 3D). Moreover, α-SMA staining and myofibroblast accumulation were mainly observed in the junction area and parallel to the corneal surface in the control mice (Fig. 3B,C), while in the Cl_2MDP-LIP-treated mice, α-SMA expression was significantly lower, and the fibers in the junction area were more disorganized than in the control mice (Fig. 3E,F).

Effect of macrophage depletion on corneal neovascularization

We evaluated the effect of macrophage depletion on corneal neovascularization caused by autologous corneal transplantation. As showed by slit microscopy observation, mice injected with PBS-LIP assumed significant vessel growth to the suture site (Fig. 4A), while the mice with depleted macrophages showed weaker neovascularization than control mice at 7 days after surgery (Fig. 4E). Immunofluorescence staining of corneal flat mounts showed CD31-positive vascular endothelial cells and NG_2-positive pericytes in the cornea junction in PBS-treated controls (Fig. 4B–D), while there was no obvious expression of CD31 or NG_2 in the junction of Cl_2MDP-LIP-treated mice (Fig. 4F–H). The results suggest that the local depletion of macrophages inhibited neovascularization after autologous corneal transplantation.

Effect of macrophage depletion and transfusion on corneal wound healing

The mouse autologous corneal transplantation model was used in the present study to exclude the interference of immune rejection caused by allogeneic transplantation. Slit-lamp photographs (100%, 25/25) and histological staining showed that the graft healed well after 14 days of penetrating keratoplasty in the PBS-LIP-treated mice (Fig. 5A). The graft was closely linked with the recipient bed, with apparent inflammatory cells and fibroblasts in the junction region between the graft and recipient cornea. The collagen fibers were dense and distributed parallel to stromal layers (Fig. 5B, C). However, the graft cornea showed apparent edema and a space between the recipient bed in the Cl_2MDP-LIP-treated mice (64%, 16/25) (Fig. 5D). Few inflammatory cells and fibroblasts were recruited into the junction between the graft and recipient cornea. The collagen fibers were small, thin, and irregularly distributed (Fig. 5E, F). Moreover, several mice showed the onset of wound dehiscence, with the graft detached from the recipient cornea (36%, 9/25), and the ingrowth of corneal epithelial cells into the junction (Fig. 5G–I). In addition, the mice with macrophage transfusion into conjunctiva showed significant enhanced wound healing (Fig. 6C, F), almost similar to the control mice injected with PBS-LIP (Fig. 6A, D), while the macrophage-depleted mice still showed significant defective wound healing and even the disclosure of eye content (Fig. 6B, E) after 21 and 28 days of corneal transplantation.
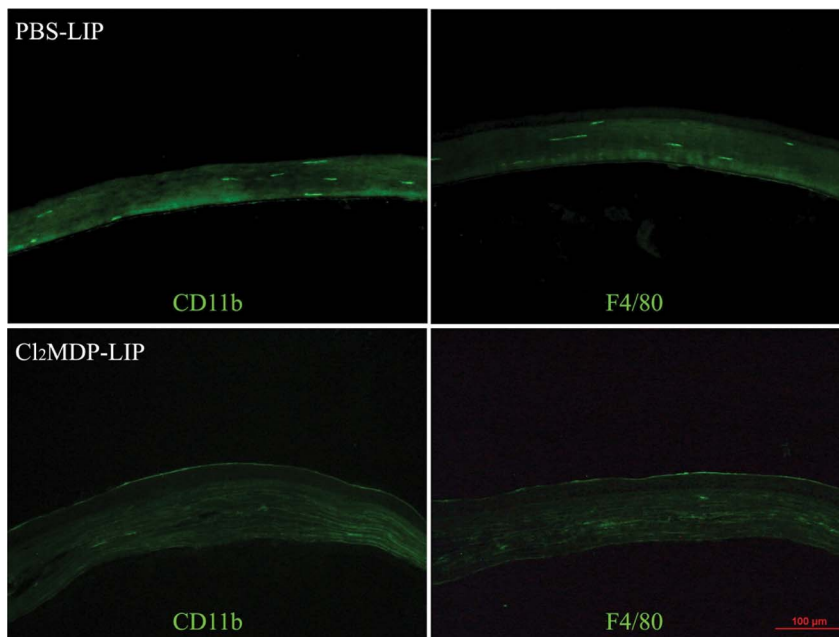

Figure 1. Sub-conjunctival Cl_2MDP-LIP injection depleted corneal resident macrophages. Immunofluorescence staining of cornea after 2 days of liposome injection showed a few F4/80+CD11b+ macrophages in the central cornea of mice injected with PBS-LIP, while there was no specific staining in mice injected with clodronate liposomes.
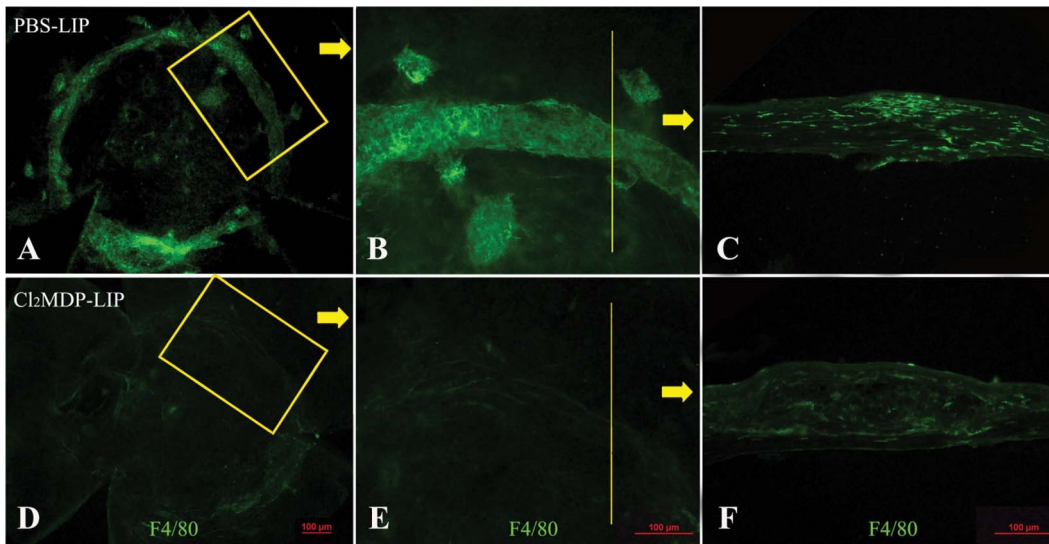doi:10.1371/journal.pone.0061799.g001

Figure 2. Sub-conjunctival Cl$_2$MDP-LIP injection prevented the infiltration of macrophages. Corneal flat mount staining after 14 days of penetrating keratoplasty showed a number of F4/80$^+$ macrophages near the suture sites and around the junction between the graft and recipient cornea (A, B). Section staining primarily revealed staining clustered in the superficial corneal stromal layer (C), while few macrophages could be found in the junction and suture area in Cl$_2$MDP-LIP-injected mice (D–F).
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Discussion

Macrophages have been confirmed to play a central role during all phases of wound healing [24,25]. During the inflammatory phase, macrophages play a role in antigen presentation, phagocytosis and the production of inflammatory cytokines and growth factors that facilitate wound healing [26,27]. During the proliferative phase, they stimulate the proliferation of fibroblasts, endothelial cells and epithelial cells to promote ECM formation, epithelialization and neovascularization [28]. Subsequently, macrophages contribute to the remodeling of newly formed ECM by releasing degrading enzymes during the remodeling phase [29]. In

the present study, for the first time, we depleted the corneal infiltrated macrophages through the sub-conjunctival injection of clodronate liposomes and evaluated the impairment of corneal wound healing after autologous corneal transplantation. The results showed that the corneal macrophage-depleted mice exhibited low inflammatory cell infiltration, irregular extracellular matrix alignment, intrastromal invasion of the corneal epithelium, and the detachment of donor corneas. Moreover, the number of macrophages, myofibroblasts, endothelial cells and pericytes was decreased in the junction area between the donor and recipient cornea. In addition, macrophages transfusion recovered the defect
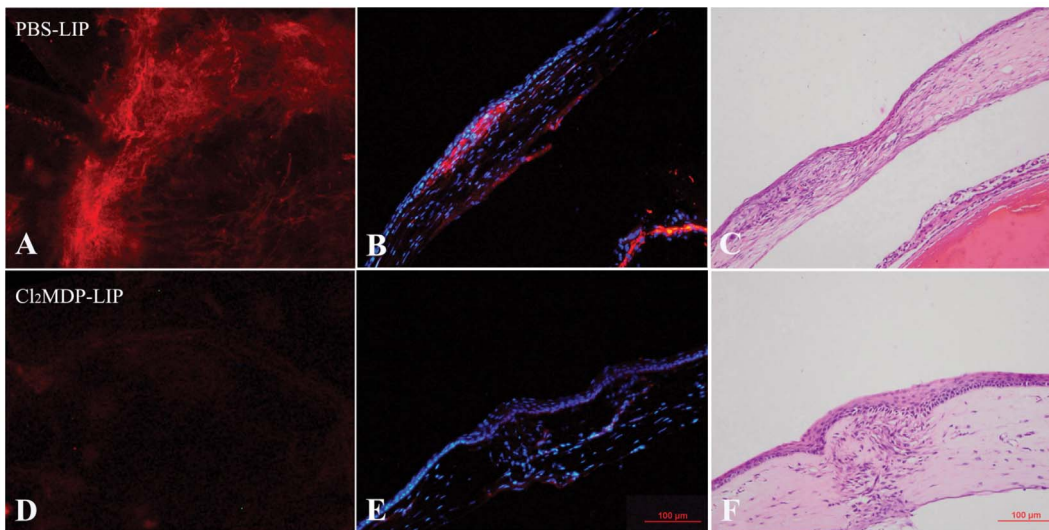

Figure 3. Effect of macrophage depletion on myofibroblast differentiation. Corneal flat mount staining after 14 days of penetrating keratoplasty showed that α-SMA was strongly expressed in the junction area between the graft and recipient cornea (A) and parallel to the corneal surface (B, C) in the PBS-LIP-injected mice, while in the Cl$_2$MDP-LIP-treated mice, α-SMA expression was significantly lower (D), and the fibers in the junction area were more disorganized than in the control mice (E,F).
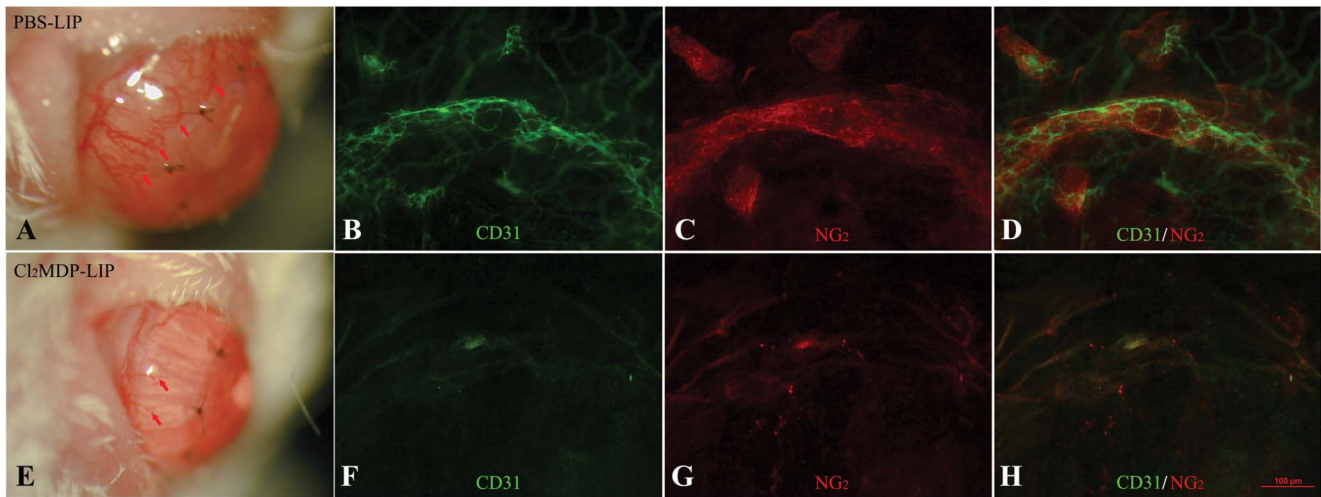doi:10.1371/journal.pone.0061799.g003

Figure 4. Effect of macrophage depletion on corneal neovascularization. Corneal flat mount staining after 7 days of corneal transplantation showed that both CD31 (endothelial cell marker) and NG_2 (pericyte marker) were strongly expressed in the junction area between the graft and recipient cornea in PBS-LIP-injected mice, while the expression was significantly lower in the Cl_2MDP-LIP-treated mice.
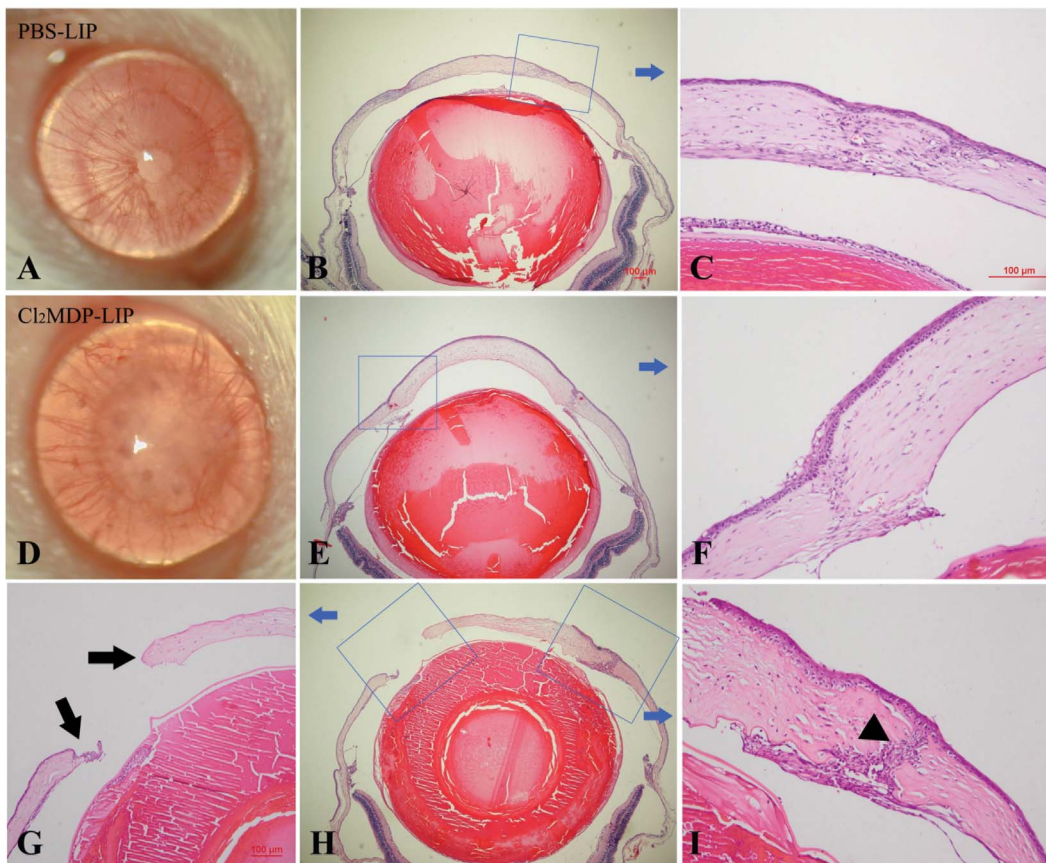doi:10.1371/journal.pone.0061799.g004

Figure 5. Effect of macrophage depletion on corneal wound healing. In mice injected with PBS-LIP, the graft healed well at 14 days after surgery (A), with apparent inflammatory cells and fibroblasts in the junction region between the graft and recipient cornea (B, C), and the collagen fibers were dense and distributed parallel to stromal layers (C). However, the graft showed apparent edema (D) and space between the recipient bed (E) in Cl_2MDP-LIP-treated mice. Few inflammatory cells and fibroblasts were recruited into the junction, the collagen fibers were irregularly distributed (F), the onset of wound dehiscence was observed, with the graft detached from the recipient cornea (G, H), and there was ingrowth of corneal epithelial cells into the junction (I).
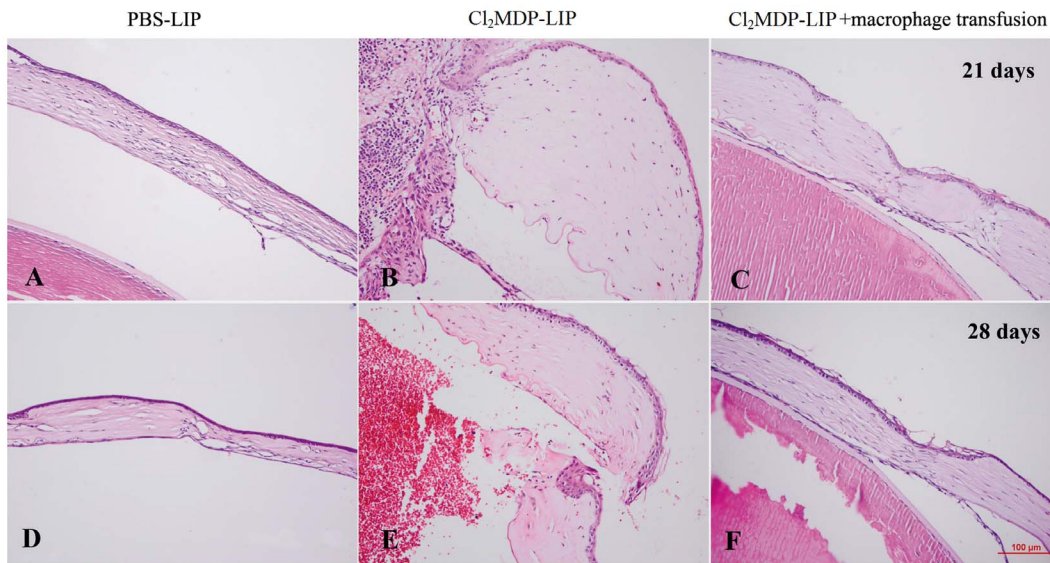doi:10.1371/journal.pone.0061799.g005

Figure 6. Effect of macrophage transfusion on corneal wound healing. Forty-eight hours after penetrating keratoplasty in mice injected with Cl$_2$MDP-LIP, 10^5 peritoneal macrophages from naïve Balb/c mice were transfused into conjunctiva. After 21 and 28 days later, the mice with macrophage transfusion into conjunctiva showed significant enhanced wound healing (C, F), almost similar to the control mice injected with PBS-LIP (A, D), while the macrophage-depleted mice still showed significant defective wound healing and even the disclosure of eye content (B, E). doi:10.1371/journal.pone.0061799.g006

of corneal wound healing caused by the depletion of corneal macrophages.

The delivery of liposome containing dichloromethylene-bis-phosphonate has been used popularly to deplete macrophages from liver and spleen [30], kidney [31], lung [32] and cornea [14,33,34] in vivo. The method is specific for the depletion of phagocytic cells, since the liposomes can be phagocytosis only by phagocytic cells. Moreover, the Cl$_2$MDP cannot be easily escaped from phagocytes or enter non-phagocytic cells if leakage from liposome, and free Cl$_2$MDP is not toxic by itself [35]. For cornea, several articles had described the repeated sub-conjunctival injection of Cl$_2$MDP-LIP could prevent corneal allograft rejection via the depletion of infiltrated macrophages from conjunctiva in rats [33]. In the previous study, we and Van Klink also found that local depletion of macrophages may impair the immune response and aggravate fungal and acanthamoeba keratitis [14,34]. From the depletion effect of local macrophage, Cl$_2$MDP-LIP sub-conjunctival injection can deplete corneal local phagocytic cells and infiltrated macrophages for about 14 days, since we found there are only a few F4/80+ macrophages in corneal stroma after 14 days of Cl$_2$MDP-LIP injection. Moreover, we also checked the cell apoptosis by TUNEL staining on the cornea of 3–21 days after penetrating keratoplasty, and found the control PBS-LIP injected mice showed more TUNEL stained cells than the Cl$_2$MDP-LIP injected mice, as well as the infiltration of inflammatory cells in corneal stroma (data not shown). In the homeostatic corneas, there are residential macrophages but no residential polymorphonuclear leukocytes [36–40]. The results suggests the decreasing of macrophages in cornea of Cl$_2$MDP-LIP injected mice after penetrating keratoplasty was caused by the depletion of resident corneal phagocytes and infiltrated macrophages from conjunctiva. It should be mentioned that neutrophils were not depleted by sub-conjunctival injection of Cl$_2$MDP-LIP, since we didn't find the difference of neutrophil population in the corneal wound healing after penetrating keratoplasty (data not shown). However, although commonly used to deplete monocytes in the circulation and macrophages in tissues, Cl$_2$MDP-LIP administration also depletes dendritic cells that also engulf clodronate [41]. Further study needs to confirm the specific role of macrophage and dendritic cells in corneal wound healing by using transgenic mice expressing diphtheria toxin receptors (DTR) under the promoters for either CD11b or CD11c that selectively deplete macrophage or dendritic cells [42,43].

During stromal wound healing, fibroblasts differentiate into myofibroblasts that share similar features with smooth muscle cells, including prominent intracellular microfilament bundles and in vitro contractile responses to smooth muscle agonists [44]. Previous reports have described that the expression of α-SMA is directly correlated with corneal wound contraction, and the absence of myofibroblasts is associated with continued widening of the wound space after incisional procedures [45]. These results are similar to our results in corneal wound healing after penetrating keratoplasty. Macrophage-depleted mice showed reduced α-SMA expression, wound dehiscence and the intrastromal invasion of corneal epithelial cells, which suggested that macrophage-depleted mice exhibited impaired myofibroblast differentiation during corneal wound healing. Among the several cytokines that regulate myofibroblast differentiation and wound contraction by corneal fibroblasts, TGF-β, which is produced by infiltrated macrophages, may play a critical role during corneal wound healing. Moreover, macrophages also produce platelet-derived growth factor (PDGF), acidic and rich in cysteine (SPARC) and insulin-like growth factor-1 (IGF-1), which are involved in the process [46–49]. In addition, we confirmed that the elimination of macrophages resulted in the reduction of corneal neovascularization, including the number of pericytes that enclose endothelial cells to strength newly formed vessels. The reduction of the ingrowth of new vessels from the limbal region may be associated with diminished VEGF and bFGF in the wound sites of macrophage-depleted corneas [19,50,51]. Moreover, the suture removal at 10 days after penetrating keratoplasty was also critical for the regression of corneal neovascularization.

In conclusion, the present results showed that macrophage-depleted mice impaired corneal wound healing after autologous

corneal transplantation, which was recovered by peritoneal macrophage transfusion. However, several issues need to be explored in the further study: (1) sub-conjunctival injection of Cl$_2$MDP-LIP can only deplete the infiltrated macrophages in a short period, further study should be done to confirm the effect of infiltrated macrophages on corneal wound healing by using systemic injection of Cl$_2$MDP-LIP. (2) Considering the limited merit of exploring the role of macrophages in autologous corneal transplantation-mediated wound healing, dendritic cells or macrophage selectively-depleted mice and corneal scratch model should be used to confirm the role of various phagocytes (neutrophils, dendritic cells or macrophages) in corneal wound healing. (3) Although we found the peritoneal macrophage

transfusion could recover defect of corneal wound healing after macrophage depletion, more study should be performed by using the purified and fluorescence-labeled macrophages (such as macrophages from GFP transgenic mice) to demonstrate the detailed mechanism.

Author Contributions

Conceived and designed the experiments: SWY LSX ZQJ. Performed the experiments: LSX LB JHR WY QML DHY. Analyzed the data: LSX ZQJ. Contributed reagents/materials/analysis tools: SWY. Wrote the paper: ZQJ LSX.

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