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# The Maintenance of Lymphatic Vessels in the Cornea Is Dependent on the Presence of Macrophages

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**PURPOSE.** It has been shown previously that the presence in the cornea of antigen-presenting cells (APC), such as macrophages (MPS) and lymphangiogenesis, is a risk for corneal transplantation. We sought to determine whether the existence of lymphatic vessels in the non-inflamed cornea is associated with the presence of MPS.

**METHODS.** Flat mounts were prepared from corneas of untreated C57BL/6, CD11b<sup>-/-</sup>, F4/80<sup>-/-</sup>, and BALB/c mice, and after suture placement or corneal transplantation, observed by immunofluorescence for the presence of lymphatic vessels using LYVE-1 as a marker of lymphatic endothelium. Innate immune cells were detected in normal mouse corneas using CD11b, F4/80, CD40, as well as MHC-class II. Digital images of the flat mounts were taken using a spot image analysis system, and the area covered by lymphatic vessels was measured using NIH Image software.

**RESULTS.** The number of spontaneous lymphatic vessels in C57BL/6 corneas was significantly greater than in BALB/c corneas ( $P = 0.03$ ). There were more CD11b<sup>+</sup> ( $P < 0.01$ ) and CD40<sup>+</sup>, MHC-class II (+) cells in the C57BL/6 corneas than in BALB/c mouse corneas. MPS depletion via clodronate liposome in C57BL/6 mice led to fewer spontaneous lymphatic vessels and reduced inflammation-induced lymphangiogenesis relative to control mice. Mice deficient in CD11b or F4/80 had fewer spontaneous lymphatic vessels and less lymphangiogenesis than control C57BL/6 mice.

**CONCLUSIONS.** C57BL/6 mouse corneas have more endogenous CD11b<sup>+</sup> cells and lymphatic vessels. The endogenous lymphatic vessels, along with pro-inflammatory MPS, account for the high risk of corneal graft rejection in C57BL/6 mice. CD11b<sup>+</sup> and F4/80<sup>+</sup> MPS appear to have an important role in of the formation of new lymphatic vessels. (*Invest Ophthalmol Vis Sci.* 2012;53:3145-3153) DOI:10.1167/iovs.11-8010

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**B**lood and lymphatic vessels are critical to organ and tissue maintenance. In particular, the lymphatic system is central to the tissue fluid homeostasis, and to the return of macromolecules and immune cells to the blood stream. However, some tissues, including the cornea, have neither vascular nor lymphatic vessels. The lack of a vascular supply is essential to maintaining the transparency of the cornea. The cornea obtains its nutrients by diffusion from the aqueous fluid and the tears. The mechanisms that underlie the avascularity of the cornea may be related to the immune-privilege of the eye.<sup>1,2</sup> In addition, the presence of anti-angiogenic factors, such as sFlt, VEGFR3, and thrombospondin-1, have been associated with avascularity of the cornea.<sup>1-3</sup>

The wound healing that follows corneal transplantation involves the growth of blood and lymphatic vessels as part of the acute inflammatory response. The lymphatics provide a route for the transport of antigens by antigen-presenting cells (APC) via the efferent pathway to the regional lymph node. After antigen presentation, activated T cells return to the cornea via the blood vessels, a process referred to as the afferent pathway.<sup>4-6</sup> Corneal transplantation in the absence of vascularization has the lowest rejection rates and graft survival approaches 90%.<sup>7,8</sup> In contrast, vascularized corneas have much higher rejection rates with survival below 50%.<sup>8,9</sup> It is well known that host C57BL/6 allo-corneal transplantations have a higher rejection rate than host BALB/c allo corneal transplantations.<sup>10</sup> This difference in rejection rate can be explained, at least in part, by differential immunological responses between BALB/c and host C57BL/6 mouse strains.<sup>11</sup>

MPS and other innate immune cells (some expressing MHC-class II antigens) are present in the corneal epithelial layer and stroma,<sup>12-16</sup> especially in the peripheral cornea (limbus).<sup>17</sup> Following the induction of inflammation, MPS are activated and the expression of MHC-class II, as well as co-stimulatory molecules, such as CD80 (B7.1), CD86 (B7.2), and CD40, is increased.<sup>17,18</sup> We and others have found that activated MPS can contribute to lymphatic vessels in inflammation-associated lymphangiogenesis.<sup>19-21</sup> Based on these observations, we postulated that the differences in endogenous MPS might account for the observed difference in graft rejection rate.

## METHODS

### Mouse Corneal Transplantation and Corneal Suture Placement Models

Male BALB/c and C57BL/6 mice (Taconic Farms, Germantown, NY and Crea Japan, Shizuoka, Japan), CD11b<sup>-/-</sup> (B6.129S7-*Irf1*<sup>tm1Ts/J</sup> mice; The Jackson Laboratory, Bar Harbor, ME),<sup>22</sup> and F4/80<sup>-/-23</sup> mice were used at 8-12 weeks of age. All animal protocols were approved by Schepens Animal Care and Use Committee, and the Committee for Animal Research of Kyoto Prefectural University of Medicine in accordance with the Association for Research in Vision and Ophthal-

mology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

For the corneal transplantation, each animal was anesthetized by an intraperitoneal (IP) injection of 3 mg ketamine and 0.007 mg xylazine before the surgical procedure. The central 2-mm portion of the donor cornea was excised with Vannas scissors and secured in recipient graft beds with 8 interrupted 11-0 nylon sutures (MANI Inc, Tochigi, Japan).<sup>24</sup> For the suture placement, three 11-0 nylon sutures were placed intrastromally using stromal incursions that encompassed more than 120 degrees of the corneal circumference. To obtain a standardized angiogenic response, the outer edge of the suture was placed halfway between the limbus and the line outlined by the 2-mm trephine; the inner edge was equidistant from the 2-mm trephine. Sutures were left in place for seven days.

### Whole-Mount Determination of Blood and Lymphatic Vessels

Mice were euthanized by CO<sub>2</sub> inhalation, and the corneas were excised, rinsed three times in PBS, and fixed in acetone for 1 hour. The corneas were rinsed once in PBS, blocked with 2% BSA containing PBS, and incubated with rabbit anti-mouse LYVE-1 antibody (1:200; a lymphatic endothelium-specific hyaluronic acid receptor; RELIATECH, Wolfenbüttel, Germany).<sup>25,26</sup> The corneas were washed, blocked, and stained with Cy3-conjugated secondary antibody (1:100; Jackson ImmunoResearch Laboratories, Westgrove, PA). For visualization, stained whole-mount corneas were viewed under a Zeiss Axiophot microscope, and a Leica TSC-SP2 inverted and an upright confocal laser-scanning microscope. Digital images of the flat mounts were taken using a spot image analysis system, and the area covered by lymphatic vessels positive for LYVE-1<sup>25,26</sup> was measured using NIH Image software. The total corneal area was outlined using the innermost vessel of the limbal arcade as the border; the area of lymphatic vessels within the cornea was calculated and normalized to the total corneal area. Results are expressed as a percentage of the cornea covered by vessels.

### Whole-Mount Determination of CD11b Positive Cells

For the whole mount corneal staining, corneas were removed and fixed with acetone for 1 hour, then rinsed in PBS, blocked with 2% BSA containing PBS, incubated with FITC, PE-conjugated CD11b (BD-Pharmingen, San Diego, CA) or anti-mouse F4/80 (1:100). Rat (DA) IgG<sub>2b</sub>, κ (1:100, BD Pharmingen) was used as an isotype control. Corneas then were washed, blocked, and stained with FITC or PE conjugated CD40 and MHC-class II (Ia<sup>d</sup>; BALB/c, Ia<sup>b</sup>; C57BL/6; BD-Pharmingen). Double-stained whole mount corneas were visualized and photographed as described above. The same laser status was used when taking pictures in BALB/c and C57BL/6 mouse cornea. CD11b<sup>+</sup> cells were counted 300 μ from the corneal margin for the entire circumference of the cornea (limbal area).

### Systemic and Local Depletion of MPS Using Clodronate Liposomes

Systemic and local depletion of monocytes/MPS was accomplished as described previously.<sup>27</sup> Cl2MDP (clodronate; a gift of Roche Diagnostics GmbH, Mannheim, Germany) was injected via tail vein (200 μL) and into subconjunctival space (40 μL) of C57BL/6 mice three times in a week (days 0, 3, and 5). Control mice received liposomes containing PBS at the same time points.

### Immunofluorescence Staining for F4/80, LYVE-1, and 4',6-Diamidino-2-Phenylindole (DAPI)

After fixation with 4% paraformaldehyde (PFA) at 4°C for 24 hours, the corneas were washed three times with PBS, placed into Tissue-Tek, and

frozen -80°C for 24 hours. Sections (4 μm) were fixed with 99% cold acetone for two minutes, washed three times with PBS, then blocked with 0.3% Triton-x100 and 2% BSA containing PBS for 30 minutes. The corneas were incubated with primary antisera rabbit anti-F4/80 (1:1000; Serotec, Oxford, UK), MHC class II (1:1000; eBioscience) or LYVE-1 overnight at 4°C, then washed, blocked and the secondary Cy3-conjugated donkey anti-rabbit (1:2500; Jackson ImmunoResearch, Westgrove, PA) or FITC-conjugated goat anti-rat (1:2500, Jackson ImmunoResearch) was added for 30 minutes, followed by washing. Then, the corneas were washed with PBS, and mounting with VECTASHIELD with DAPI (Vector, Burlingame, CA).

## RESULTS

### Presence of Innate Immune Cells in the Inflamed Cornea

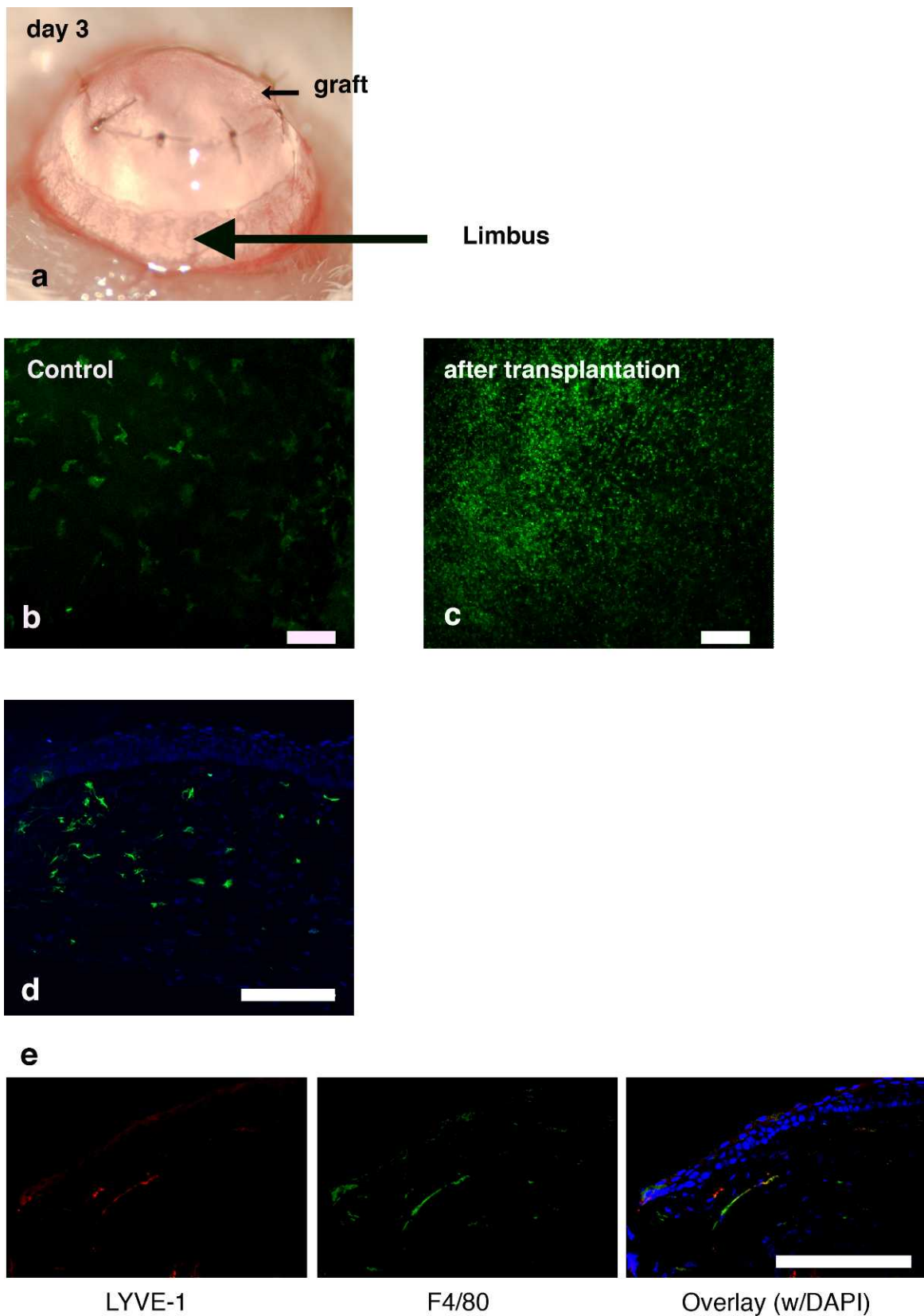
We demonstrated previously the development of lymphatic vessels in the cornea after transplantation.<sup>19</sup> At day three after transplantation, lymphatic vessels began to grow into corneal stroma. Corneal lymphangiogenesis has been shown to be associated with CD11b<sup>+</sup> MPS.<sup>19</sup> Therefore, we investigated the presence of MPS in cornea at day three after transplantation. We used the corneal transplantation model of inflammation to assess macrophage infiltration into corneal stroma. Three days after BALB/c syngeneic corneal transplantation (Fig. 1a), the mice were euthanized, and the corneas were dissected from the limbus, fixed, and stained with anti-CD11b-FITC conjugated antibody. In the naïve, non-transplanted cornea there was a small number of CD11b<sup>+</sup> cells, primarily in the limbal area (Fig. 1b). In contrast, inflamed corneas, such as after transplantation, contained large numbers of CD11b<sup>+</sup> cells (Fig. 1c). Similarly, sections of inflamed corneas revealed many MHC class II<sup>+</sup> cells (Fig. 1d). Moreover, some of the F4/80<sup>+</sup> cells expressed LYVE-1 and had aligned with lymphatic vessels (Fig. 1e).

### Comparison of CD11b<sup>+</sup> in BALB/c and C57BL/6 Mouse Corneas

The presence in the corneal stroma of bone marrow-derived CD11b<sup>+</sup> MPS has been reported previously.<sup>28</sup> Quantification revealed significantly more CD11b<sup>+</sup> cells in C57BL/6 mouse corneas than in BALB/c mouse corneas ( $P < 0.01$ : C57BL/6  $1439 \pm 203SE$ , BALB/c  $465 \pm 74 SE$ ). MHC class II<sup>+</sup> cells were located at limbal area under non-inflamed condition. As for CD11b<sup>+</sup> cells, the number of MHC class II<sup>+</sup> cells in C57BL/6 mice was higher than in BALB/c mice (Figs. 2a, b). Most of the CD11b<sup>+</sup> cells were in the stromal layer of the limbus. These cells also expressed CD40 and MHC-class II marker (Fig. 2c). There was a relatively small number of CD40 expressing cells in BALB/c mice corneas (data not shown). These results indicate that C57BL/6 mouse corneas have higher numbers of endogenous activated (MHC class II and CD40 +) CD11b<sup>+</sup> cells than BALB/c mouse corneas, suggesting a low level of chronic MPS activation in the limbal areas of C57BL/6 mice.

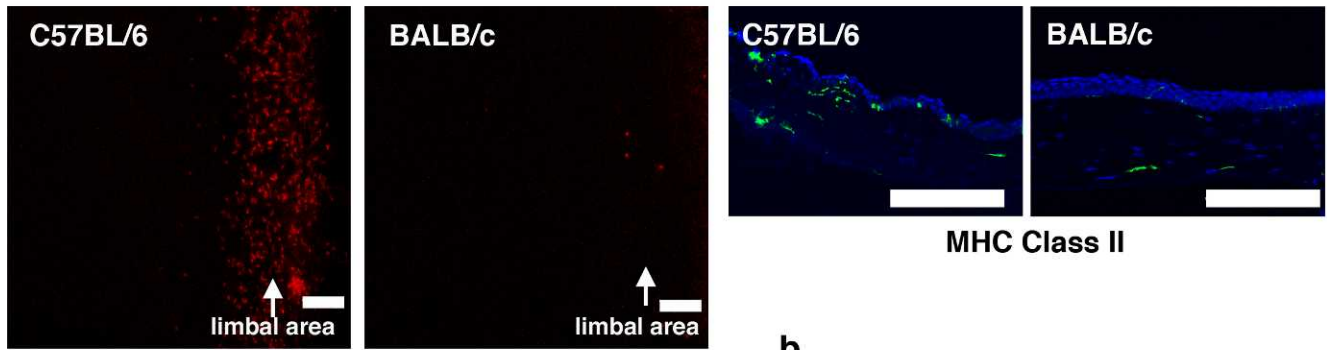
### Comparison of Blood and Lymphatic Vessels in Naïve BALB/C and C57BL/6 Mouse Corneas

It has been reported previously that the lymphangiogenic response in the cornea is strain-dependent.<sup>29</sup> In addition, the presence of spontaneous lymphatic vessels has been demonstrated in normal mouse cornea.<sup>30</sup> Although the cornea generally is considered to be devoid of blood and lymphatic vessels (Figs. 3a, b), the use of appropriate markers (LYVE-1) revealed lymphatic vessels (Figs. 3c, e) in the corneal stroma of

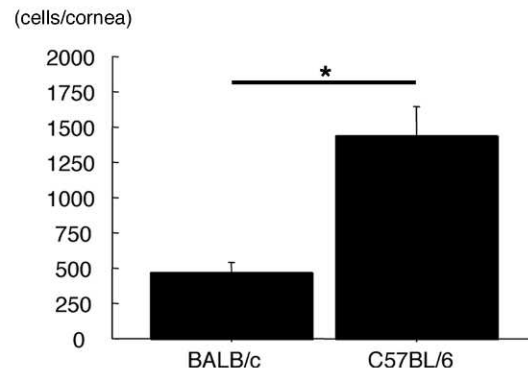


**FIGURE 1.** Macrophage and MHC class II+ cell density in the cornea. (a) Microscopic image, and (b, c) flat mounts of corneas stained for the presence of CD11b (+) cells. (b) Normal BALB/c corneal limbus and (c) host BALB/c corneal limbus on day 3 after corneal transplantation. Scale bar is 80  $\mu$ m. (d) Section of limbus at day 3 after corneal transplantation. Green label (FITC) indicated MHC class II staining, blue label indicates DAPI staining. Scale bar is 150  $\mu$ m. (e) Corneal lymphatic vessels at the limbal cornea stained with LYVE-1 (red), F4/80 (green), and DAPI (blue). Scale bar is 150  $\mu$ m.

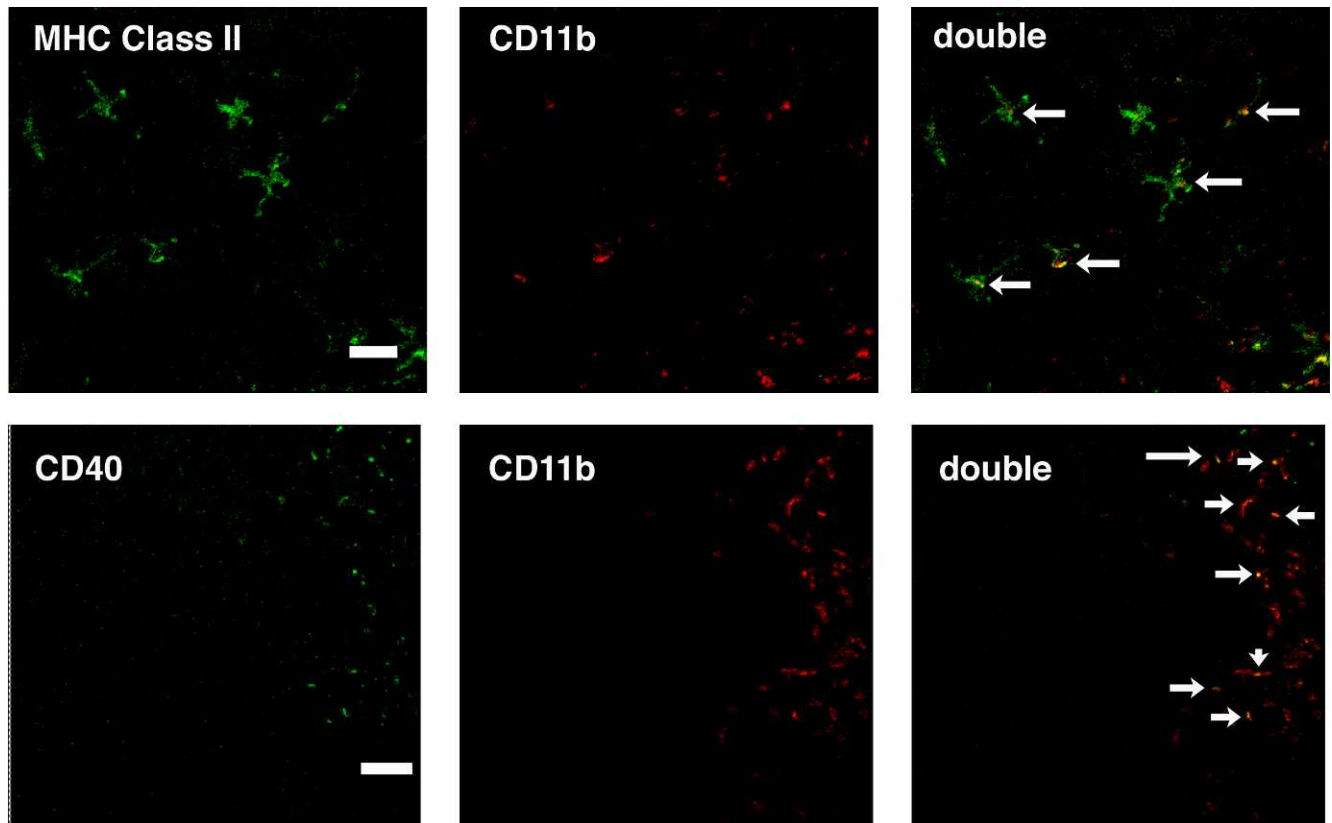
**a**



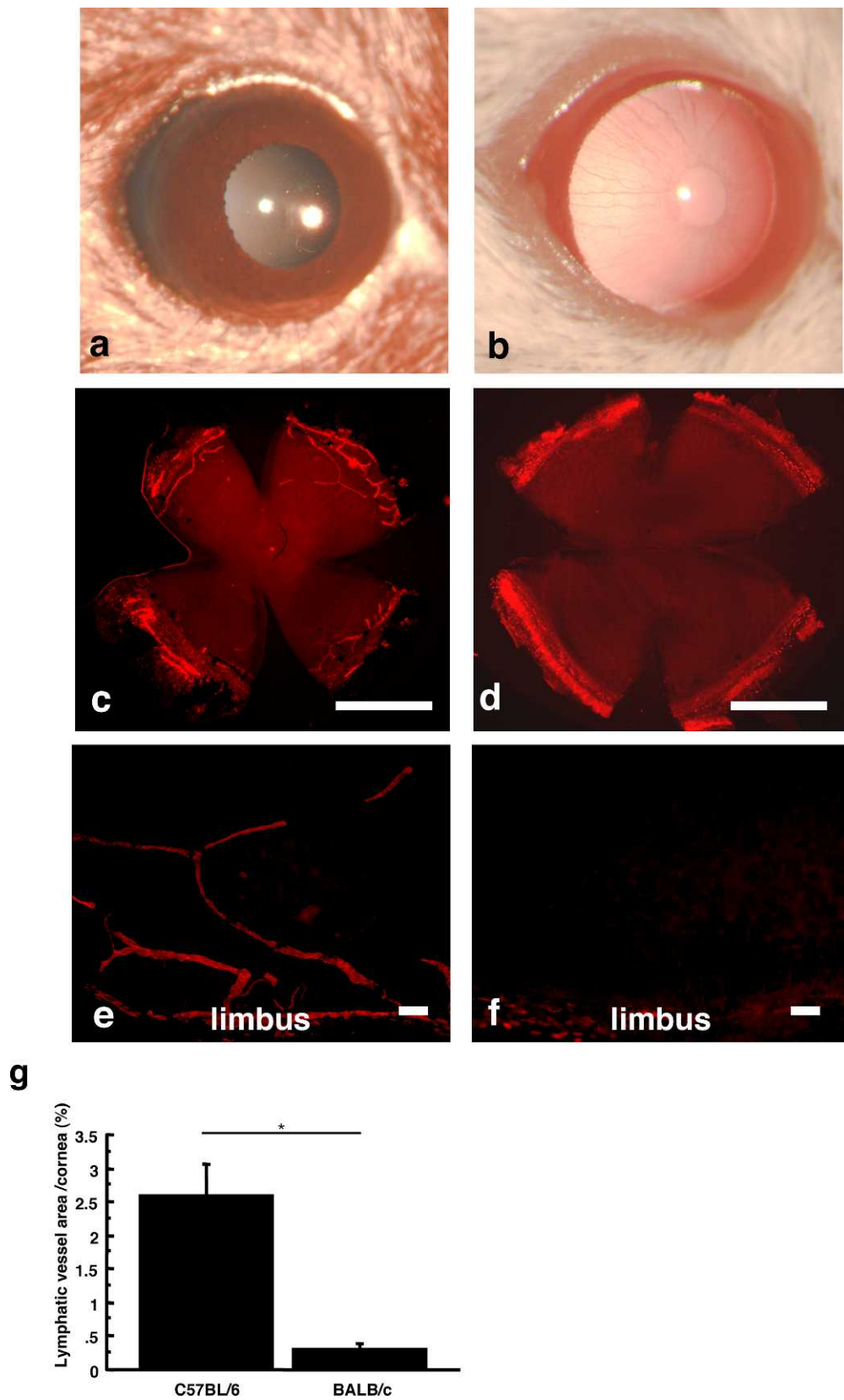
**b**



**c**



**FIGURE 2.** Comparison of CD11b (+) cell density in C57BL/6 and BALB/c mouse corneas. (a) Whole mount staining of the corneal limbus of normal C57BL/6 and BALB/c mouse, higher magnification of corneal limbus in normal C57BL/6 and BALB/c mouse. Sections of limbus of normal C57BL/6 and BALB/c mouse. *Green label* (FITC) indicates MHC class II staining, *blue label* indicates DAPI staining. (b) Comparison of CD11b + cell density between normal C57BL/6 and BALB/c mice (\* $P < 0.05$ ). (c) Corneal limbus of C57BL/6; MHC-class II (*green*), CD11b, overlay of (MHC-class II), and (CD11b), CD40 (*green*), CD11b (*red*), overlay of (CD40), and (CD11b). Scale bars are: (a) top 2 figures, 80  $\mu$ m; (a) lower 2 figures, 20  $\mu$ m; sections, 150  $\mu$ m; (c) 40  $\mu$ m.



**FIGURE 3.** Comparison of lymphatic vessel area in corneal limbus between C57BL/6 and BALB/c mice. **(a)** Normal C57BL/6 mouse. **(b)** Normal BALB/c cornea. **(c, e)** LYVE-1 labeling of lymphatic vessels (*red*) in C57BL/6 cornea. **(d, f)** LYVE-1 labeling of lymphatic vessels in BALB/c cornea. **(g)** Comparison of lymphatic vessel area in corneal limbus between C57BL/6 and BALB/c mice ( $P = 0.03$ ,  $n = 5$ ). Scale bars are **(c, d)** 1 mm and **(e, f)** 100  $\mu$ m.

6–8-week-old naïve C57BL/6 mice. In contrast, BALB/c mice (Figs. 3d, f), which are “low responders” for lymphatic vessel formation, had significantly fewer lymphatic vessels in cornea (Fig. 3g, \* $P = 0.03$ ).

### The Effect of MPS Depletion on Lymphangiogenesis in C57BL/6 Mouse

We showed previously that clodronate depletion of MPS leads to reduced lymphangiogenesis in the inflamed cornea.<sup>19</sup> However, to our knowledge the effect of MPS depletion on the non-inflamed cornea has not been investigated. Above, we described the presence of activated MPS expressing CD40 or MHC-class II in C57BL/6 mouse cornea. We, therefore, sought to determine whether clodronate deletion of MPS in C57BL/6 corneal stroma would influence the presence of the endogenous lymphatic vessels. Systemic and local clodronate treatment reduced the number of endogenous lymphatic vessel structures in C57BL/6 mice. Some of the lymphatic vessels were seen to be separate from limbal lymphatic vessels (Figs. 4a, b;  $P < 0.05$ ). Moreover, clodronate treatment also led to fewer CD11b<sup>+</sup> cells in lymphatic vessels (some of the CD11b<sup>+</sup> cells expressed both CD11b and LYVE-1) in C57BL/6 mouse cornea, compared to the untreated corneas (Fig. 4c). These results suggested that activated endogenous MPS contributed to the lymphatic vessels seen in C57BL/6 mouse corneas.

### Spontaneous Lymphatic Vessel and Lymphangiogenesis in CD11b<sup>-/-</sup> and F4/80<sup>-/-</sup> Mice

CD11b<sup>-/-</sup> mice have been reported to exhibit impaired corneal wound healing under inflammatory conditions.<sup>31</sup> However, the possible role of endogenous corneal lymphatic vessels and lymphangiogenesis has not been investigated to our knowledge. Examination of corneas from CD11b<sup>-/-</sup> and F4/80<sup>-/-</sup> mice revealed that the area of endogenous lymphatic vessels was smaller in CD11b<sup>-/-</sup> and F4/80<sup>-/-</sup> mice than in control C57BL/6 mice (Fig. 5a). Using the corneal suture model of inflammation to assess lymphangiogenesis in CD11b<sup>-/-</sup>, F4/80<sup>-/-</sup>, and wild C57BL/6 mice, we found that the extent of lymphangiogenesis in CD11b<sup>-/-</sup> and F4/80<sup>-/-</sup> mice corneas was less than in wild type mice (Fig. 5b). These results indicated that F4/80<sup>+</sup> or CD11b<sup>+</sup> cells are important for maintenance of the endogenous lymphatic vessels and for the process of lymphangiogenesis in the cornea.

## DISCUSSION

The cornea is one of the few tissues devoid of blood and lymphatic vessels. However, our results indicated the presence of lymphatic vessels in the corneal stroma, particularly in the limbal area. The endogenous lymphatic vessels were especially prominent in C57BL/6 mice, but were not detected in corneas of BALB/c, nor were they seen in corneas of F4/80<sup>-/-</sup> or CD11b<sup>-/-</sup> mice, which have low functional MPS. We demonstrated previously that the formation of lymphatic vessels in the cornea correlates well with the number of activated CD11b<sup>+</sup> MPS.<sup>19</sup> Furthermore, we have shown that under inflammatory conditions CD11b<sup>+</sup> MPS secrete the VEGF-A and VEGF-C.<sup>32</sup> Elimination of these cells by treatment with clodronate liposomes led to the suppression of inflammation-induced corneal hem- and lymphangiogenesis to levels less than control mice.<sup>32</sup>

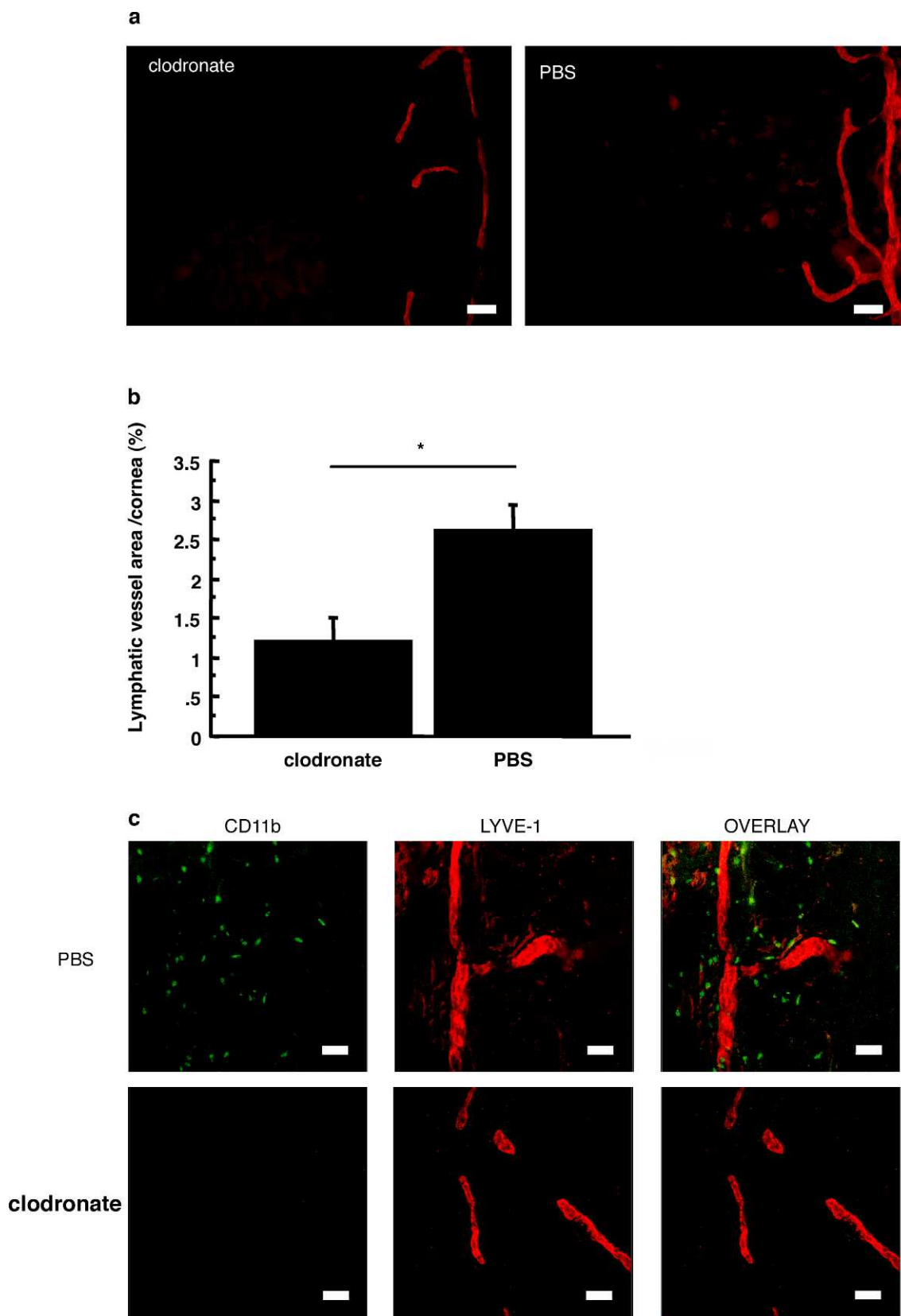
Host C57BL/6 mice are known to have a higher rejection rate after allogeneic corneal transplantation than host BALB/c mice (50% for C57BL/6 to BALB/c, and 80% for BALB/c to C57BL/6).<sup>11</sup> However, the reason for this difference is not well

understood. One possible explanation is the presence of some, as yet unidentified, class II<sup>+</sup> cells in the cornea whose number or immunogenicity differ between these two strains of mice. Alternatively, the two species might have the same number of these cells, but the frequency of their migration from the eye to the lymph node could be different.<sup>11</sup> Our data support the former explanation and demonstrated that C57BL/6 mice have a higher risk of corneal transplantation rejection because they contain relatively large numbers of activated CD11b<sup>+</sup> cell and lymphatic vessels compared to BALB/c mice. The larger number of CD11b<sup>+</sup> or MHC class II<sup>+</sup> cells in C57BL/6 mouse cornea would result in a more rapid and effective detection of alloantigens than in BALB/c mice.

The involvement of these cells in the rejection reaction is supported by the fact that MPS depletion via clodronate liposomes led to a decrease in rejection<sup>33</sup> and a suppression of inflammation-induced lymphangiogenesis.<sup>32</sup> Mice depleted of MPS by clodronate treatment exhibit no alloantigen tolerance and delayed type hypersensitivity is not suppressed.<sup>33</sup> In naïve (non-inflamed) corneas, intravenous/subconjunctival clodronate liposome injection led to a reduced number of lymphatic vessels and CD11b<sup>+</sup> cells in the C57BL/6 corneal stroma, suggesting that CD11b<sup>+</sup> cell may contribute to the maintenance of lymphatic vessels in these mice.

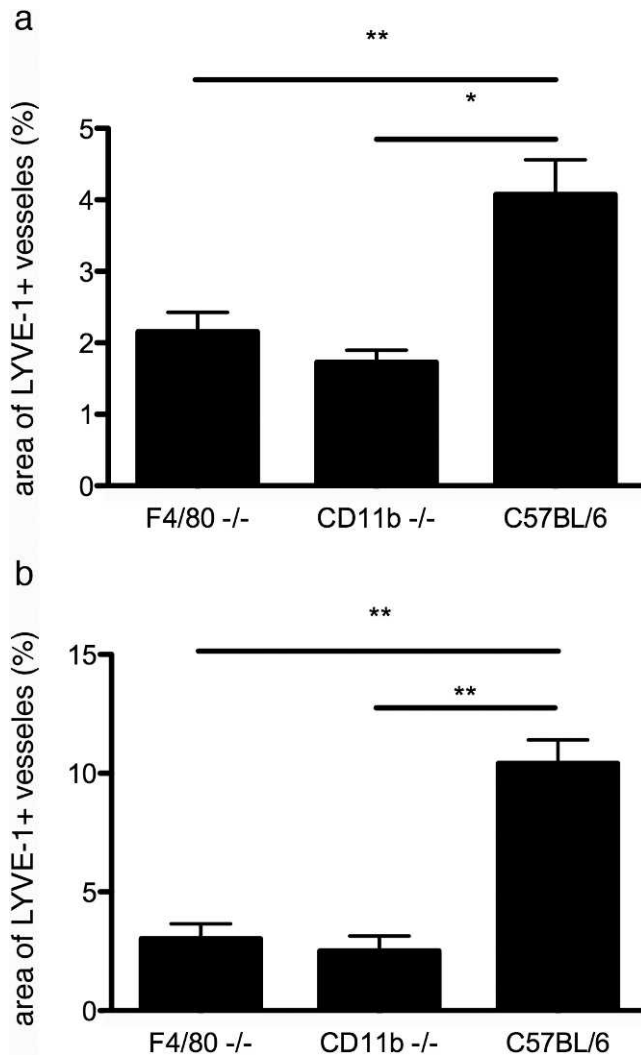
The role of MPS as a source of cytokines and growth factors, and as a phagocyte during wound healing, has been well documented.<sup>34</sup> We showed previously that MPS have a key role in the induction of lymphatic vessels under pathological conditions in cornea and skin. Some of the MPS that express lymphatic-specific markers, such as LYVE-1, podoplanin, and prox-1 contributed to the formation of lymphatic vessels.<sup>19,35</sup> In addition, we observed that MPS formed lymphatic vessel-like tubes in vitro in a density-dependent manner.<sup>19</sup> Accordingly, the lower number of MPS appeared to contribute to the reduced formation of lymphatic structures in the granulation tissue of wounds under diabetic conditions.<sup>35</sup>

The precise role of CD11b<sup>+</sup> cells during inflammation is not well understood. MPS express F4/80 and CD11b. F4/80 is a prototypic MPS membrane glycoprotein that is highly restricted to mature resident MPS subpopulations.<sup>36</sup> F4/80<sup>-/-</sup> mice do not display any apparent abnormality, indicating that F4/80 is dispensable for the development of mouse tissue MPS. However, a functional requirement for F4/80 in the production of TNF-alpha, IL-12 and IFN-gamma after exposure of the mouse spleen cells to *Listeria* has been demonstrated.<sup>37</sup> Thus, we speculate that F4/80 may function under pathological conditions. Also, CD11b is the alpha subunit of the predominant beta2 integrin expressed on monocyte/MPS. CD11b mediates many functions of myeloid cell, including adhesion, migration, chemotaxis, and phagocytosis.<sup>38</sup> Neutralization of CD11b using antibodies reduced the leukocyte recruitment under inflammatory conditions.<sup>39</sup> Consistent with this concept, the delay of corneal wound healing in CD11b<sup>-/-</sup> mouse might be mediated by dysfunction or reduced recruitment of monocyte/MPS.<sup>31</sup> We have shown that MPS infiltration (accumulation) into an inflamed site is important for the induction of lymphangiogenesis.<sup>35</sup> The reduced accumulation of MPS in CD11b<sup>-/-</sup> and F4/80<sup>-/-</sup> mice, resulting in fewer lymphatic vessels than wild type, is consistent with this observation. Our data indicate that endogenous lymphatic vessels in the corneas are maintained by activated MPS and, thus, we speculate that the number of MPS and lymphatic vessels in C57BL/6 mouse corneal limbus might contribute to the observed differences in graft rejection between C45BL/6 mice and BALB/c mice. In addition, it is likely that polymorphisms, epigenetic changes, and/or other systemic processes also may influence graft rejection in humans, and any of these may provide targets for future therapeutic modulation.



**FIGURE 4.** The effect of macrophage depletion on lymphangiogenesis in C57BL/6 mouse cornea. **(a)** Immunofluorescence of C57BL/6 mouse corneal flat mounts following clodronate liposome treatment and PBS liposome treatment. **(b)** Comparison of lymphatic vessel area in corneal limbus following clodronate and PBS liposome treatment ( $*P < 0.05$ ). **(c)** LYVE-1 (red) and CD11b (green) immunofluorescent double labeling of corneas following clodronate or PBS liposome treatment. Scale bars are **(a)** 100  $\mu$ m and **(c)** 40  $\mu$ m.





**FIGURE 5.** Endogenous lymphatic vessels and lymphangiogenesis in F4/80<sup>-/-</sup> and CD11b<sup>-/-</sup> mouse corneas. Quantification of (a) spontaneous lymphatic vessels \* $P=0.0061$ , \*\* $P=0.0051$ , no significant difference between F4/80<sup>-/-</sup> and CD11b<sup>-/-</sup>,  $n=5$ . (b) Lymphangiogenesis at day seven after suture placement, \*\* $P=0.0095$ , no significant difference between F4/80<sup>-/-</sup> and CD11b<sup>-/-</sup>,  $n=5$ .

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