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).[24] 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_2 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).[25,26] 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[25,26] 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_{2b}, 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^d; BALB/c, Ia^b; 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. $CD11b^+$ 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.[27] 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.[19] At day three after transplantation, lymphatic vessels began to grow into corneal stroma. Corneal lymphangiogenesis has been shown to be associated with $CD11b^+$ MPS.[19] 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 $CD11b^+$ cells, primarily in the limbal area (Fig. 1b). In contrast, inflamed corneas, such as after transplantation, contained large numbers of $CD11b^+$ cells (Fig. 1c). Similarly, sections of inflamed corneas revealed many MHC class II^+ cells (Fig. 1d). Moreover, some of the $F4/80^+$ cells expressed LYVE-1 and had aligned with lymphatic vessels (Fig. 1e).

Comparison of $CD11b^+$ in BALB/c and C57BL/6 Mouse Corneas

The presence in the corneal stroma of bone marrow-derived $CD11b^+$ MPS has been reported previously.[28] Quantification revealed significantly more $CD11b^+$ cells in C57BL/6 mouse corneas than in BALB/c mouse corneas ($P < 0.01$: C57BL/6 1439 ± 203SE, BALB/c 465 ± 74 SE). MHC class II^+ cells were located at limbal area under non-inflamed condition. As for $CD11b^+$ cells, the number of MHC class II^+ cells in C57BL/6 mice was higher than in BALB/c mice (Figs. 2a, b). Most of the $CD11b^+$ 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 +) $CD11b^+$ 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 Naive BALB/C and C57BL/6 Mouse Corneas

It has been reported previously that the lymphangiogenic response in the cornea is strain-dependent.[29] In addition, the presence of spontaneous lymphatic vessels has been demonstrated in normal mouse cornea.[30] 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

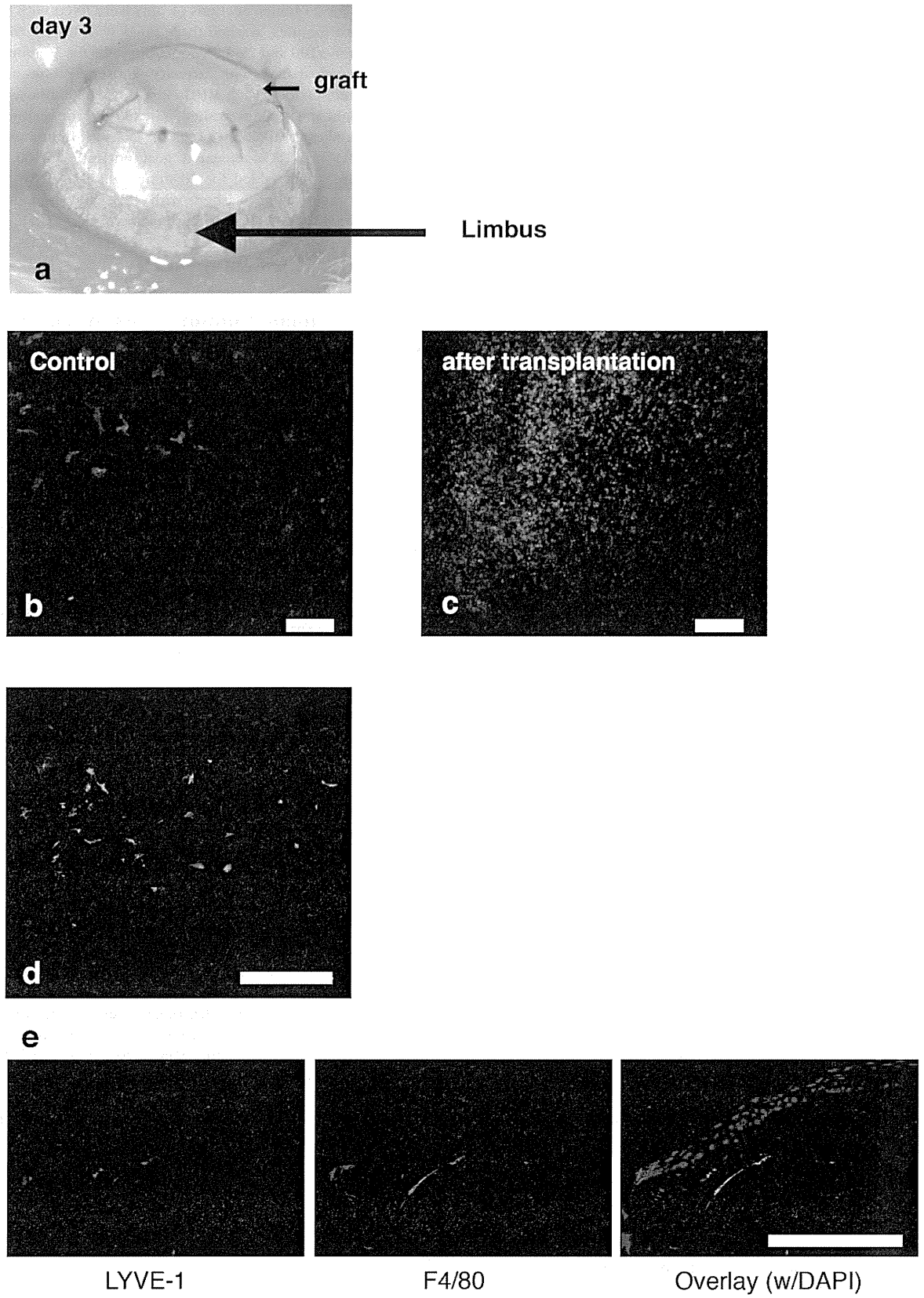

Figure 1. Macrophage and MHC class II+ cell density in the cornea. (**a**) Microscopic image, and (**b**, **c**) flat mounts of corneas stained for the presence of CD11b (+) cells. (**b**) Normal BALB/c corneal limbus and (**c**) host BALB/c corneal limbus on day 3 after corneal transplantation. Scale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

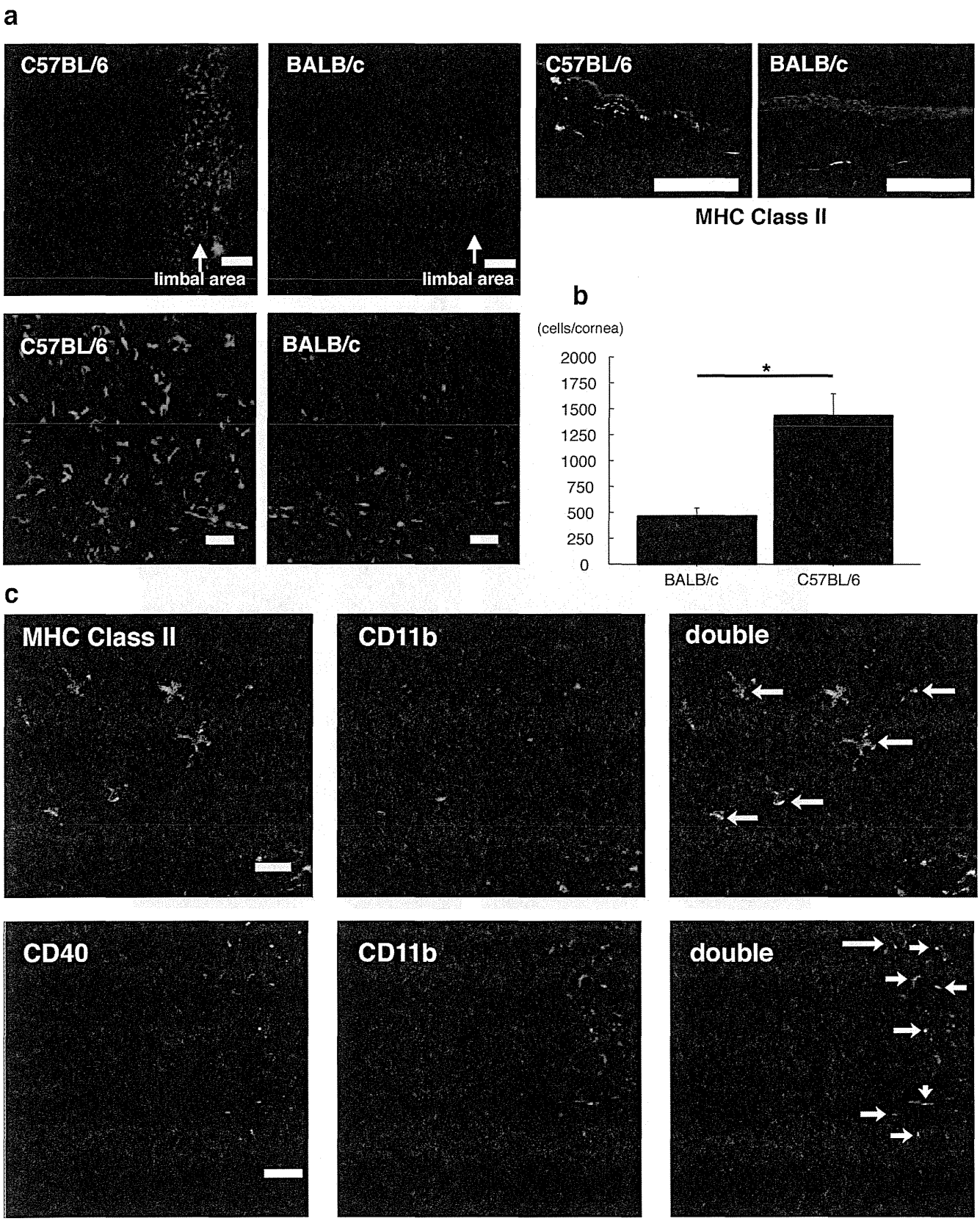

FIGURE 2. Comparison of CD11b (+) cell density in C57BL/6 and BALB/c mouse corneas. (**a**) Whole mount staining of the corneal limbus of normal C57BL/6 and BALB/c mouse, higher magnification of corneal limbus in normal C57BL/6 and BALB/c mouse. Sections of limbus of normal C57BL/6 and BALB/c mouse. *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 μm; (**a**) lower 2 figures, 20 μm; sections, 150 μm; (**c**) 40 μm.

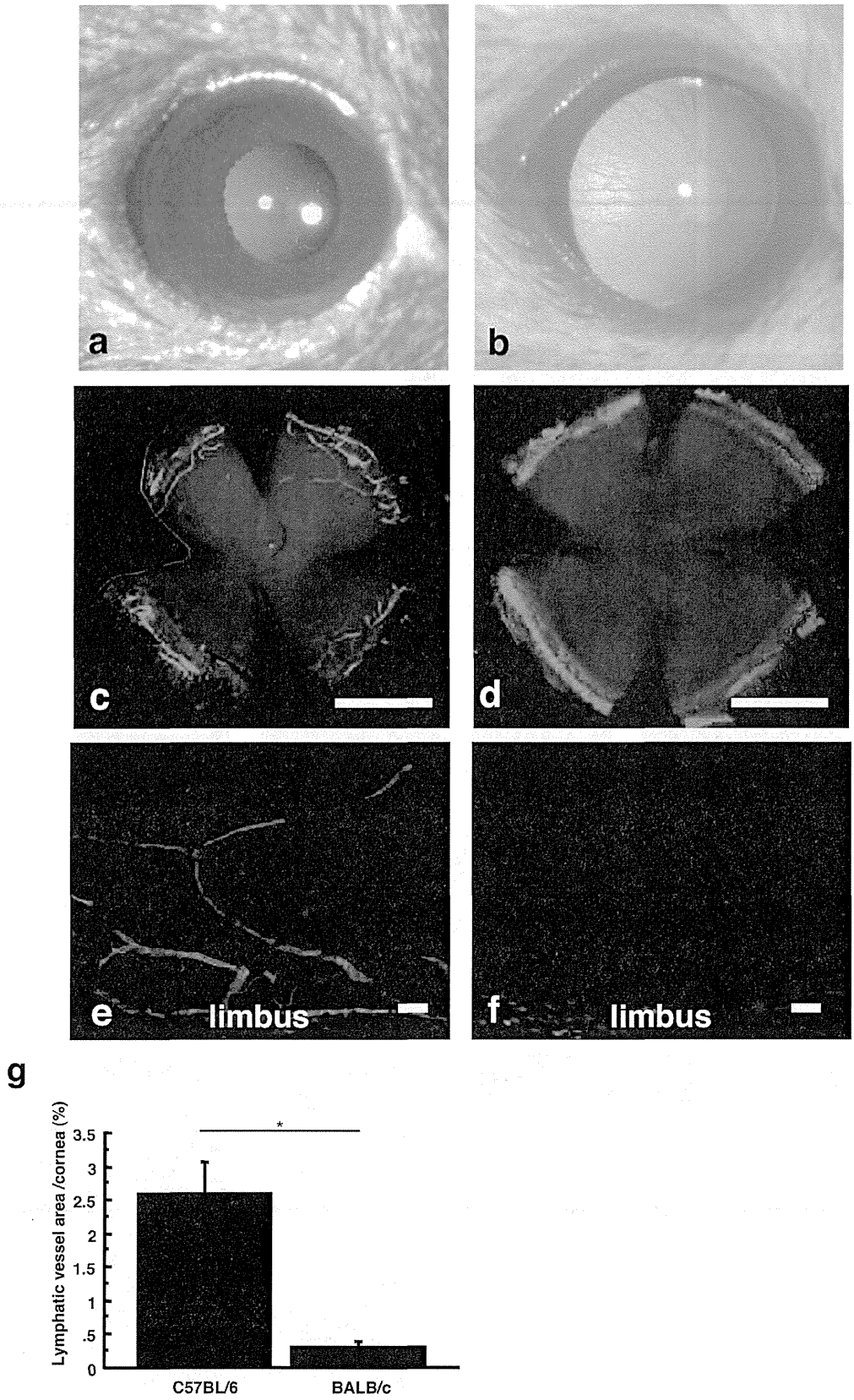

FIGURE 3. Comparison of lymphatic vessel area in corneal limbus between C57BL/6 and BALB/c mice. (**a**) Normal C57BL/6 mouse. (**b**) Normal BALB/c cornea. (**c**, **e**) LYVE-1 labeling of lymphatic vessels (*red*) in C57BL/6 cornea. (**d**, **f**) LYVE-1 labeling of lymphatic vessels in BALB/c cornea. (**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 μm.

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.[19] 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 $CD11b^{+}$ cells in lymphatic vessels (some of the $CD11b^{+}$ 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 $CD11b^{-/-}$ and $F4/80^{-/-}$ Mice

$CD11b^{-/-}$ mice have been reported to exhibit impaired corneal wound healing under inflammatory conditions.[31] However, the possible role of endogenous corneal lymphatic vessels and lymphangiogenesis has not been investigated to our knowledge. Examination of corneas from $CD11b^{-/-}$ and $F4/80^{-/-}$ mice revealed that the area of endogenous lymphatic vessels was smaller in $CD11b^{-/-}$ and $F4/80^{-/-}$ mice than in control C57BL/6 mice (Fig. 5a). Using the corneal suture model of inflammation to assess lymphangiogenesis in $CD11b^{-/-}$, $F4/80^{-/-}$, and wild C57BL/6 mice, we found that the extent of lymphangiogenesis in $CD11b^{-/-}$ and $F4/80^{-/-}$ mice corneas was less than in wild type mice (Fig. 5b). These results indicated that $F4/80^{+}$ or $CD11b^{+}$ 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 $F4/80^{-/-}$ or $CD11b^{-/-}$ 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 $CD11b^{+}$ MPS.[19] Furthermore, we have shown that under inflammatory conditions $CD11b^{+}$ MPS secrete the VEGF-A and VEGF-C.[32] 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.[32]

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).[11] However, the reason for this difference is not well understood. One possible explanation is the presence of some, as yet unidentified, class II^{+} 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.[11] 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 $CD11b^{+}$ cell and lymphatic vessels compared to BALB/c mice. The larger number of $CD11b^{+}$ or MHC class II^{+} 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[33] and a suppression of inflammation-induced lymphangiogenesis.[32] Mice depleted of MPS by clodronate treatment exhibit no alloantigen tolerance and delayed type hypersensitivity is not suppressed.[33] In naïve (non-inflamed) corneas, intravenous/subconjunctival clodronate liposome injection led to a reduced number of lymphatic vessels and $CD11b^{+}$ cells in the C57BL/6 corneal stroma, suggesting that $CD11b^{+}$ 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.[34] 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.[19,35] In addition, we observed that MPS formed lymphatic vessel-like tubes in vitro in a density-dependent manner.[19] 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.[35]

The precise role of $CD11b^{+}$ 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.[36] $F4/80^{-/-}$ 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.[37] 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.[38] Neutralization of CD11b using antibodies reduced the leukocyte recruitment under inflammatory conditions.[39] Consistent with this concept, the delay of corneal wound healing in $CD11b^{-/-}$ mouse might be mediated by dysfunction or reduced recruitment of monocyte/MPS.[31] We have shown that MPS infiltration (accumulation) into an inflamed site is important for the induction of lymphangiogenesis.[35] The reduced accumulation of MPS in $CD11b^{-/-}$ and $F4/80^{-/-}$ 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.

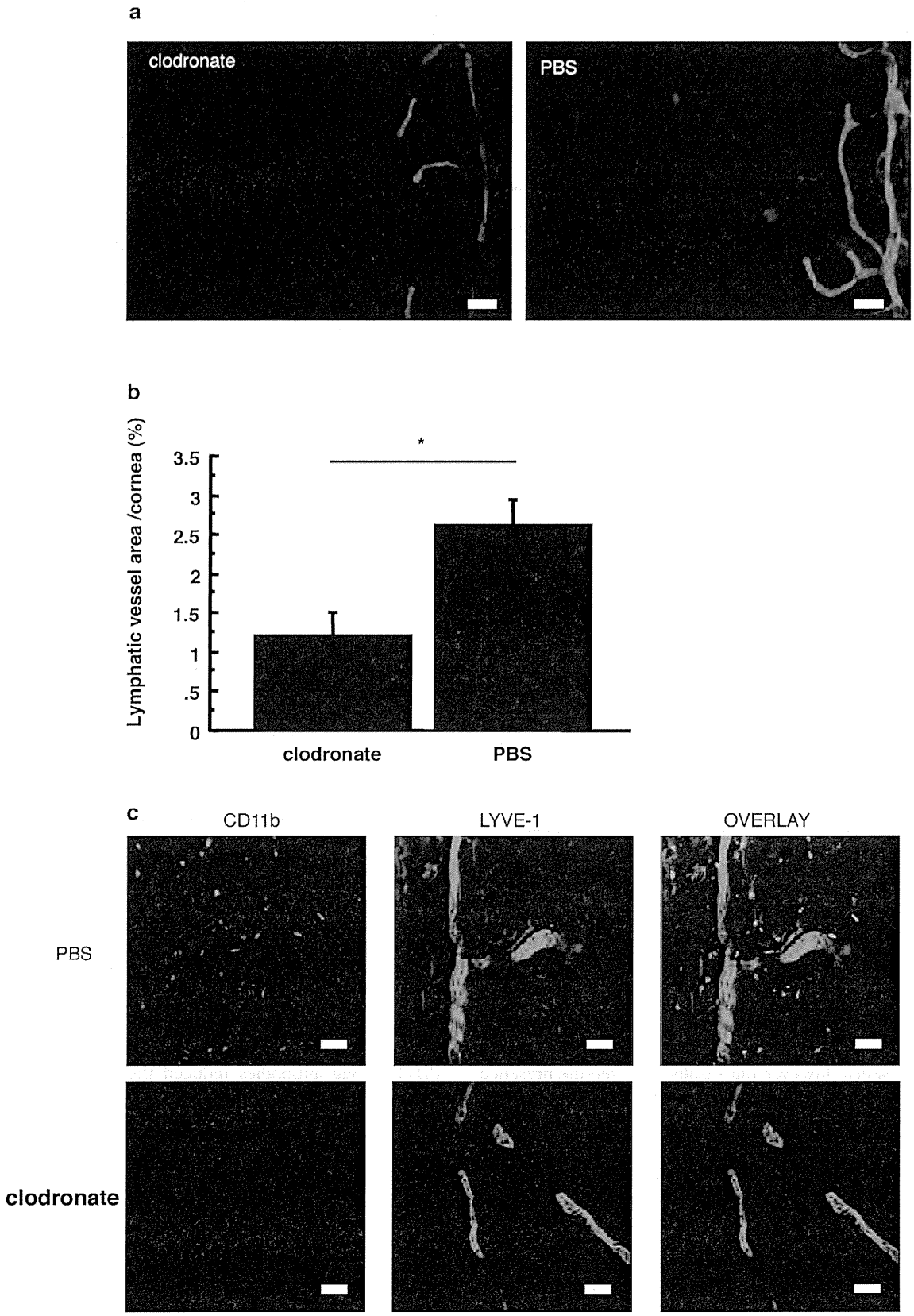

FIGURE 4. The effect of macrophage depletion on lymphangiogenesis in C57BL/6 mouse cornea. (**a**) Immunofluorescence of C57BL/6 mouse corneal flat mounts following clodronate liposome treatment and PBS liposome treatment. (**b**) Comparison of lymphatic vessel area in corneal limbus following clodronate 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 μm and (**c**) 40 μm.

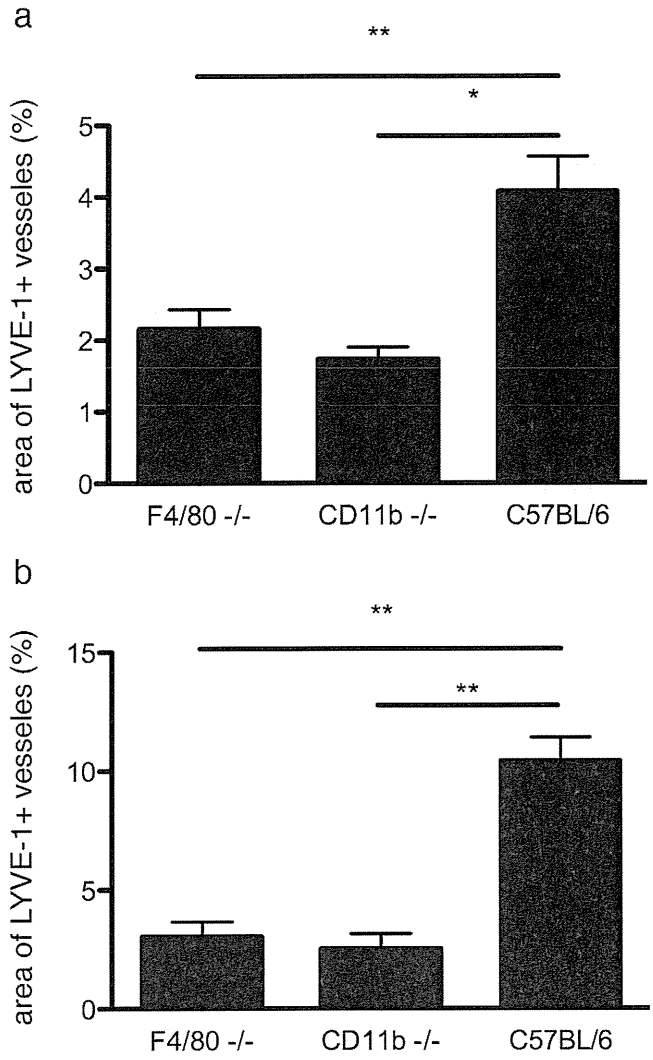

FIGURE 5. Endogenous lymphatic vessels and lymphangiogenesis in F4/80$^{-/-}$ and CD11b$^{-/-}$ mouse corneas. Quantification of (**a**) spontaneous lymphatic vessels $^{*}P = 0.0061$, $^{**}P = 0.0051$, no significant difference between F4/80$^{-/-}$ and CD11b$^{-/-}$, $n = 5$. (**b**) Lymphangiogenesis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

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His-Hsien Lin provided the F4/80$^{-/-}$ mice that were critical to our studies.

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Prostaglandin E2 Suppresses Poly I:C-Stimulated Cytokine Production Via EP2 and EP3 in Immortalized Human Corneal Epithelial Cells

Mayumi Ueta, MD, PhD,† Toshiyuki Matsuoka, MD, PhD,‡ Chie Sotozono, MD, PhD,* and Shigeru Kinoshita, MD, PhD**

Purpose: We previously reported that prostaglandin (PG) E_2 acts as a ligand for prostaglandin E receptor 3 (EP3) in conjunctival epithelial cells, that it downregulates the progression of experimental murine allergic conjunctivitis, and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via prostaglandin E receptor 2 (EP2) and EP3, suggesting that PGE_2 might have important roles in ocular surface inflammation such as allergic conjunctivitis. Here, we investigated whether PGE_2 also downregulates polyI:C-induced cytokine production in human corneal epithelial cells.

Methods: We used enzyme-linked immunosorbent assay and quantitative reverse transcription–polymerase chain reaction to examine the effects of PGE_2 on polyI:C-induced cytokine expression by immortalized human corneal-limbal epithelial cells (HCLE). Using reverse transcription–polymerase chain reaction, we examined the messenger RNA (mRNA) expression of the PGE_2 receptor, EP1–4.

Results: PGE_2 significantly attenuated the expression of CC chemokine ligand (CCL)5 ($P < 0.0005$), CCL20 ($P < 0.0005$), C-X-C chemokine (CXCL)10 ($P < 0.0005$), CXCL11 ($P < 0.05$), and interleukin (IL)-6 ($P < 0.005$) in human corneal-limbal epithelial cells. Human corneal epithelial cells manifested the mRNA expression of EP2, EP3, and EP4, but not EP1. The EP2 agonist significantly suppressed the polyI:C-induced expression of CCL5 ($P < 0.005$), CXCL10 ($P < 0.0005$), and CXCL11 ($P < 0.05$) but not of CCL20 and IL-6. The EP3 agonist significantly suppressed the expression of CCL5 ($P < 0.05$), CCL20 ($P < 0.005$), CXCL10 ($P < 0.0005$), CXCL11 ($P < 0.0005$), and IL-6 ($P < 0.005$). The EP4 agonist failed to suppress cytokine production induced by polyI:C stimulation.

Conclusions: Our results show that in human corneal epithelial cells, PGE_2 attenuated the mRNA expression and production of CCL5, CXCL10, and CXCL11 via both EP2 and EP3, and that the mRNA expression and production of CCL20 and IL-6 was attenuated only by EP3.

Key Words: prostaglandin E_2 (PGE_2), human corneal epithelial cells, prostaglandin E receptor 3, prostaglandin E receptor 2

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Prostanoids are a group of lipid mediators that form in response to various stimuli. They include prostaglandin (PG)D_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane (TX)A_2. They are released extracellularly immediately after their synthesis, and they act by binding to a G protein–coupled rhodopsin-type receptor on the surface of target cells. There are 8 types of prostanoid receptors: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP).[1]

PolyI:C, a synthetic double-stranded (ds)RNA, which mimics viral dsRNA, is the well-known ligand of Toll-like receptor 3.[2] We have reported that polyI:C stimulation induces the secretion of inflammatory cytokines such as interleukin (IL)-6, IL-8, type I interferon (IFN) such as IFN-β, IFN-inducible proteins such as C-X-C chemokine (CXCL)10 and CXCL11, and allergy-related proteins such as CC chemokine ligand (CCL)5 and thymic stromal lymphopoietin in human ocular surface epithelium, both corneal and conjunctival.[3–5] Moreover, we also reported that not only Toll-like receptor 3, but also cytoplasmic helicase proteins, RIG-I (retinoic acid–inducible protein I) and MDA5 (melanoma differentiation–associated gene 5) contribute to polyI:C-inducible responses in conjunctival epithelium.[6]

We previously reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells, that it downregulates the

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From the *Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; †Department of Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan; and ‡Department of Ophthalmology, Tenri Hospital, Nara, Japan.

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Reprints: Mayumi Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan (e-mail: mueta@koto.kpu-m.ac.jp).

progression of experimental murine allergic conjunctivitis,[7] and that in human conjunctival epithelial cells it modulates the expression of polyI:C-induced proinflammatory genes via not only EP3 but also EP2,[8] suggesting that PGE_2 might have important roles in the ocular surface inflammation such as allergic conjunctivitis.

PGE_2 was reported to be produced during inflammatory responses and to suppress the production of cytokines and chemokines induced by lipopolysaccharide (LPS) stimulation in macrophages[9,10] and dendritic cells.[11] Elsewhere, we documented that human corneal and conjunctival epithelial cells produce cytokines such as IL-6, IL-8, and IFN-β in response to stimulation with polyI:C but not LPS.[3,12,13] In this study, we examined the expression of the PGE_2 receptors, EP1, EP2, EP3, and EP4, in human corneal epithelial cells and investigated whether polyI:C-induced cytokine production is downregulated by PGE_2 in these cells.

MATERIALS AND METHODS

Human Corneal Epithelial Cells

This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the tenets set forth in the Declaration of Helsinki.

For reverse transcription–polymerase chain reaction (RT-PCR) assay, we obtained human corneal epithelial cells from corneal grafts of patients who had undergone corneal transplantation for bullous keratopathy. Immortalized human corneal-limbal epithelial cells (HCLE), a gift from Dr Irene K. Gipson, were cultured in low calcium–defined keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) with defined growth-promoting additives that included insulin, epidermal and fibroblast growth factors, and 1% antibiotic–antimycotic solution. The cells were used after reaching 80% confluence.[7]

Reverse Transcription–Polymerase Chain Reaction

RT-PCR assay was as previously described.[7] Briefly, total RNA was isolated from HCLE and human corneal epithelium using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript Preamplification kit (Invitrogen). Amplification was with DNA polymerase (Takara, Shiga, Japan) for 38 cycles at 94°C for 1 minute, annealing for 1 minute, and 72°C for 1 minute on a commercial PCR machine (GeneAmp; PE Applied Biosystems). The primers were as previously reported.[7] RNA integrity was assessed by electrophoresis in ethidium bromide–stained 1.5% agarose gels. We performed 2 separate experiments.

Enzyme-Linked Immunosorbent Assay

Protein production was confirmed by enzyme-linked immunosorbent assay (ELISA). The amount of IL-6, CCL5, CCL20, CXCL11, and CXCL10 released into the culture supernatant was determined by ELISA using the human CCL5, CCL20, CXCL11, CXCL10 DuoSet (R&D Systems Inc, Minneapolis, MN) or the OptEIA IL-6 set (BD Pharmingen, San Diego, CA).[4,7,14]

We performed 3 separate experiments, each being carried out in 6 wells per group.

Quantitative RT-PCR

Total RNA was isolated from HCLE using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RT reaction was with the SuperScript Preamplification kit (Invitrogen). Quantitative RT-PCR was on an ABI-prism 7700 instrument (Applied Biosystems, Foster City, CA) using a previously described protocol.[4,7,14] The primers and probes were from Applied Biosystems [assay ID: CCL5 (Hs00174575), CCL20 (Hs01011368), CXCL10 (Hs00171042), CXCL11 (Hs00171138), IL-6 (Hs00174131), and human GAPDH (Hs 4326317E)]. For complementary DNA (cDNA) amplification, we performed PCR in a 25 μl total volume that contained a 1-μl cDNA template in 2× TaqMan universal PCR master mix (Applied Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed with sequence detection software (Applied Biosystems). The quantification data were normalized to the expression of the housekeeping gene *GAPDH*. We performed 3 separate experiments, each being carried out in 6 wells per group.

Data Analysis

Data are expressed as the mean ± SEM and were evaluated by Student *t* test using the Microsoft Excel software program.

RESULTS

PGE2 Downregulated the Production of Cytokines Induced by Poly I:C Stimulation

Using HCLE and ELISA, we examined whether PGE_2 downregulated the production of IL-6, IL-8, CCL5, CCL20, CXCL10, and CXCL11 induced by polyI:C stimulation in human corneal epithelial cells. HCLE were exposed to 10 μg/mL polyI:C and 100 μg/mL PGE_2 for 24 hours (ELISA) or 6 hours (quantitative RT-PCR). We found that PGE_2 significantly attenuated the production of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (all, $P < 0.0005$) (Fig. 1A). Quantitative RT-PCR assay confirmed that the messenger RNA (mRNA) expression of CCL5, CCL20, CXCL10, CXCL11, and IL-6 (respectively, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$, $P < 0.05$ and $P < 0.005$) was significantly downregulated by PGE_2 (Fig. 1B).

Human Corneal Epithelial Cells Expressed EP2-, EP3-, and EP4-Specific mRNA

We then performed RT-PCR to assay the mRNA expression of the PGE_2 receptors, EP1, EP2, EP3, and EP4, in human corneal epithelial cells. PCR products of expected