ICAM-1 Is Necessary for Epithelial Recruitment of $\gamma\delta$ T Cells and Efficient Corneal Wound Healing

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Wound healing and inflammation are both significantly reduced in mice that lack $\gamma\delta$ T cells. Here, the role of epithelial intercellular adhesion molecule-1 (ICAM-1) in $\gamma\delta$ T cell migration in corneal wound healing was assessed. Wild-type mice had an approximate fivefold increase in epithelial $\gamma\delta$ T cells at 24 hours after epithelial abrasion. ICAM-1^{-/-} mice had 50.9% ($P < 0.01$) fewer $\gamma\delta$ T cells resident in unwounded corneal epithelium, which failed to increase in response to epithelial abrasion. Anti-ICAM-1 blocking antibody in wild-type mice reduced epithelial $\gamma\delta$ T cells to a number comparable to that of ICAM-1^{-/-} mice, and mice deficient in lymphocyte function-associated antigen-1 (CD11a/CD18), a principal leukocyte receptor for ICAM-1, exhibited a 48% reduction ($P < 0.01$) in peak epithelial $\gamma\delta$ T cells. Re-epithelialization and epithelial cell division were both significantly reduced (~50% at 18 hours, $P < 0.01$) after abrasion in ICAM-1^{-/-} mice versus wild-type, and at 96 hours, recovery of epithelial thickness was only 66% ($P < 0.01$) of wild-type. ICAM-1 expression by corneal epithelium in response to epithelial abrasion appears to be critical for accumulation of $\gamma\delta$ T cells in the epithelium, and deficiency of ICAM-1 significantly delays wound healing. Since $\gamma\delta$ T cells are necessary for efficient epithelial wound healing, ICAM-1 may contribute to wound healing by facilitating $\gamma\delta$ T cell migration into the corneal epithelium. (*Am J Pathol* 2009, 175:571–579; DOI: 10.2353/ajpath.2009.090112)

family² and is expressed by many cell types in response to stimuli such as cytokines,^{3,4} and oxidative and physical stress.^{5,6} It has been extensively studied in the context of adhesion and transmigration of leukocytes through endothelium⁷ and epithelium,^{8,9} and it also serves as an adhesive ligand for leukocyte-mediated cytotoxic activity.^{9–11} ICAM-1 is recognized by members of the $\beta 2$ (CD18) integrin family, especially lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18),¹² and this adhesion is critical to many of the migratory and cytotoxic events in which ICAM-1 participates.^{7,10,11} ICAM-1 also functions as a signaling molecule, dependent on its cytoplasmic tail interacting with cytoskeletal elements.⁷ This capability influences functions such as leukocyte trans-endothelial migration⁷ and vascular permeability.¹³

Of importance to the current study is the fact that ICAM-1 can be expressed by corneal epithelial cells and limbal vessel endothelial cells.^{14–21} It appears to be expressed in conditions associated with inflammation, but its role in this context is poorly understood, especially its expression by the epithelial cells. Using a murine model of central corneal epithelial abrasion, we observed ICAM-1 on corneal epithelial cells in the periphery of the cornea, a region not directly injured by the abrasion.¹⁴ Since migration and division of these cells account for wound closure and re-establishment of full thickness epithelium necessary for healing,^{22,23} it was of interest to determine whether ICAM-1 is necessary for these processes. To this end we studied wound healing in mice that do not express ICAM-1.^{24,25}

As a part of this evaluation, we focused attention on $\gamma\delta$ T cells. We observed in earlier studies that epithelial expression of ICAM-1 occurred at a time when $\gamma\delta$ T cells increased within the corneal epithelium,^{14,26} and that $\gamma\delta$ T cell-deficient mice exhibited poor corneal wound healing. Since these leukocytes express LFA-1,²⁷ and LFA-1/ICAM-1 interactions support adhesion of human lympho-

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Intercellular adhesion molecule-1 (ICAM-1, CD54)¹ is a conserved member of the immunoglobulin supergene

cytes to human epithelial cells expressing ICAM-1,^{20,27} it seemed possible that $\gamma\delta$ T cell accumulation in the epithelium after corneal abrasion would be influenced by the absence of ICAM-1.

Materials and Methods

Animals

T cell receptor (TCR) $\delta^{-/-}$ mice on the C57BL/6 background and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). ICAM-1 $^{-/-}$ mice^{24,25} were backcrossed as previously described at least 10 generations with C57BL/6 mice. CD11a $^{-/-}$ and P-selectin-deficient (P-sel $^{-/-}$) mice were prepared as described.²⁸ All animals were bred and housed in our facility according to the guidelines described in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision and Ophthalmic Research and Baylor College of Medicine Animal Care and Use Committee policy. Mice were controlled for sex (female) and age (12 to 15 weeks). To examine the contribution of adhesion molecule ICAM-1 and platelets to $\gamma\delta$ T cell migration to wounded corneas, some mice were injected intraperitoneally with anti-mouse ICAM-1 mAb (YN1 clone; ATCC) or anti-mouse GPIIb α (Emfret Analytics, Würzburg, Germany) with 0.2 mg in 200 μ l PBS 1 or 24 hours before wounding, respectively.^{14,29}

Corneal Epithelial Wounding Model

The central corneal wound was performed as previously described.³⁰ In brief, mice were anesthetized by i.p. injection of pentobarbital (50 mg/kg body weight), and the central corneal epithelium was demarcated with a 2-mm trephine and then removed using a diamond blade for refractive surgery (Accutome, Malvern, PA) under a dissecting microscope. Care was taken to minimize injury to the epithelial basement membrane and stroma. While under anesthesia, ocular surfaces were protected from drying by topical administration of sterile saline immediately following injury, and 0.1 mg/kg subcutaneous buprenorphine was administered at the time of surgery and every 6 to 12 hours thereafter, as needed, for pain control. At various times following injury, corneal tissue including the limbus was excised and processed for immunohistology, or mRNA isolation.

Immunohistology

Taking care to include the limbus, wounded corneas were dissected, fixed, permeabilized, and incubated with the following labeled monoclonal antibodies as described^{14,26,30,31}: anti-TCR δ -phycoerythrin (PE) colors (clone GL3), anti-PECAM-1-fluorescein isothiocyanate, and anti-ICAM-1-PE, which were selected for staining $\gamma\delta$ T cells, endothelial cells of the limbal vessels, and ICAM-1, respectively. Radial cuts were made in the cornea so that it could be flattened by a coverslip and the cornea was mounted in Airvol (Celenase, Ltd., Dallas,

TX), containing 1 μ mol/L 4',6-diamidino-2-phenylindole (Sigma Chemical, St. Louis, MO) to assess nuclear morphology. Digital images were captured and saved for computer analysis (Delta Vision; Applied Precision, Issaquah, WA). A standard pattern for morphometric analysis was used throughout the study as we described before.¹⁴ Whole mounts were evaluated using a $\times 40$ oil immersion lens to assess each field of view across the cornea from limbus to limbus. The limbus was defined as most peripheral field containing limbal vessels. All images selected were representative images of at least six corneas. The graphical values plotted represent the total number of a selected cell type across a diameter of a cornea. This value was obtained by counting the total number of cells within a selected corneal layer in each of nine $\times 40$ fields of view comprising the diameter of a cornea. Four diameters per cornea were counted and averaged. Six corneas were analyzed.

Histological Assessment of Corneal Thickness

Enucleated eyes were fixed overnight at 4°C in 0.1 M/L sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. The cornea was then excised, taking care to include the limbal tissue, and postfixed in 1% osmium tetroxide for 1 hour at room temperature, dehydrated through an ethanol series and embedded in resin (LX 112; Polysciences, Warrington, PA). Thick (0.5 μ m) sections were cut on an ultramicrotome (RMC 7000; Venana Medical Systems, Tucson, AZ) equipped with a diamond knife. Sections were stained with toluidine blue O and viewed on an inverted microscope (DeltaVision Spectris; Applied Precision, Issaquah, WA) using a $\times 20$ objective, and transverse measurements of the central epithelial thickness were made using the calibrated linear measurement tool contained in the supplied imaging software (SoftWorx).

Quantitative Real-Time PCR

Total RNA was isolated from corneal epithelium with the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantity and quality of the extracted RNA were verified using a Nanodrop-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE). Purified RNA was stored at -80°C until analysis. First-strand cDNA synthesis was performed with the TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA) using 2 μ g total RNA, per the manufacturer's recommendations. The resulting cDNA was stored at -20°C until further analysis. For the amplification of target genes, 5 μ l cDNA was added to a corresponding 20 $\times$ TaqMan MGB probe primer set for each message, multiplexed with primers for glyceraldehyde-3-phosphate dehydrogenase and 2 $\times$ TaqMan Universal PCR master mix (Applied Biosystems). PCR was performed in a 7500 real-time PCR system (Applied Biosystems) using the manufacturer's suggested thermal settings. Relative mRNA expression was calculated using the Δ comparative threshold (Ct) method. glyceraldehyde-3-phosphate

Figure 1. **A:** Recovery of epithelial basal cell density was determined microscopically by counting 4',6-diamidino-2-phenylindole-stained nuclei in five fields from limbus to center of the cornea at various times from immediately after wounding (0 hour) to 7 days after a 2 mm diameter central epithelial abrasion. Limbus (L), paralimbus (PL), parawound (PW), wound (W), center wound (CW). **B:** ICAM-1 expression in murine corneal epithelium. Limbal, paralimbal, and parawound epithelium was collected immediately (0 hour) or at 3, 6, or 18 hours after the central epithelial abrasion. Epithelial ICAM-1 mRNA levels were determined by qPCR and expressed relative to glyceraldehyde-3-phosphate dehydrogenase. Wild-type mice revealed significant (* $P < 0.01$, $n = 6-18$) increases in ICAM-1 expression above uninjured epithelium at each time point after abrasion. **C:** ICAM-1 expression in $\text{TCR}\delta^{-/-}$ mice was similar to that of wild-type (C57BL/6) mice ($n = 6$). **D:** ICAM-1 expression in $\text{TCR}\delta^{-/-}$ mice at 12 hours after central corneal epithelial abrasion. **a)** limbal vessel, anti-CD31-PE, showing endothelial cells (red); **b)** merged image with anti-ICAM-1-fluorescein isothiocyanate, showing ICAM-1 expression of cells lining the limbal vessel (arrows); **c)** corneal epithelial basal cells in the limbus are also positive for anti-ICAM-1-PE.

dehydrogenase was used as internal control. Each experiment was repeated three times.

Statistical Analysis

Data analysis was performed using analysis of variance and pairwise multiple comparisons using Tukey's test. A P value of <0.05 was considered significant. Data are expressed as means $\pm$ SEM.

Results

The corneal abrasion re-epithelialized within 24 hours and basal epithelial cell density increased over the next 7 days. Figure 1A shows at time 0 the wound (W) and center wound (CW) fields were within the area of abrasion and devoid of epithelial cells. By 18 hours, epithelial cells were evident within the original wound area, and the density of basal cells in the limbus (L), paralimbus (PL), and parawound (PW) fields was reduced, consistent with previous studies showing that initial wound closure results from centripetal epithelial migration.^{22,23,32} Epithelial density increases thereafter as a result of cell division.¹⁴ Uninjured murine corneal epithelium exhibits little binding of anti-ICAM-1 monoclonal antibody, but staining becomes evident in the unwounded limbal and paralimbal epithelium within 6 hours after central epithelial abrasion.¹⁴ ICAM-1 expression extends throughout the epithelium within 12 to 18 hours of central corneal abrasion.¹⁴ Limbal and paralimbal epithelium revealed a prominent increase in ICAM-1 mRNA levels 3 hours after

central corneal epithelial abrasion (Figure 1B). ICAM-1 was evident in the epithelium and in limbal vessels of $\text{TCR}\delta^{-/-}$ mice (Figures 1, C and D), indicating that $\gamma\delta$ T cells are not necessary for ICAM-1 expression although they are necessary for efficient wound healing of the corneal epithelium.²⁶

ICAM-1 and Epithelial $\gamma\delta$ T cells

GL3⁺ cells are rare in the corneal epithelium of wild-type mice, although they are found in the limbal epithelium²⁶ and adjacent conjunctival epithelium. They increase significantly in the corneal epithelium after central epithelial abrasion. The possible contribution of ICAM-1 to migration of $\gamma\delta$ T cells following corneal abrasion was assessed in ICAM-1^{-/-} mice. ICAM-1^{-/-} mice had fewer GL3⁺ cells in the limbus of uninjured corneas (Figure 2A), as compared with wild-type mice. GL3⁺ cells increased in the limbal and paralimbal epithelium of wild-type mice and reached peak levels at 24 hours, extending their distribution into the corneal epithelium near the original wound edge (Figure 2, A and B). ICAM-1^{-/-} mice failed to show this increase (Figure 2, A and B) in contrast to P-sel^{-/-}, which were not distinguishable from wild-type. GL3⁺ cells remained elevated in the epithelium of wild-type mice for at least 7 days but at significantly lower levels in the ICAM-1^{-/-} mice (Figure 2C). Their distribution in wild-type mice was mostly limited to the limbal and paralimbal regions (Figure 2D).

Wild-type mice receiving anti-ICAM-1 blocking antibody i.p., 30 minutes before central corneal epithelial

Figure 2. Corneal and limbal epithelial $\gamma\delta$ T cells in response to corneal abrasion. **A:** Whole mount corneal preparations were stained with PE-tagged monoclonal antibody GL3 (anti-TCR δ) at various times after central epithelial abrasion. The sum of GL3⁺ cells in the epithelium in nine microscopic fields across the cornea from limbus to limbus are plotted for wild-type, ICAM-1^{-/-}, and P-selectin^{-/-} mice. All values for the wild-type and P-selectin^{-/-} mice are significantly different ($P < 0.01$, $n = 6$) from those of the ICAM-1^{-/-} mice. **B:** Photomontage covering the limbal and paralimbal regions of wild-type (top) and ICAM-1^{-/-} (bottom) corneas at 24 hours after central abrasion showing the density and distribution of GL3⁺ cells. Scale bar = 10 μ m. **C:** GL3⁺ cells remained elevated in the wild-type corneas at 48 and 96 hours and 7 days after injury ($P < 0.01$, $n = 6$) for each time compared with uninjured wild-type corneas. ICAM-1^{-/-} corneas were not significantly different from uninjured ICAM-1^{-/-} corneas, and remained significantly less ($P < 0.01$, $n = 6$) than wild-type after injury at all time points studied. **D:** The distribution of GL3⁺ cells across the corneas of wild-type mice at various times after central epithelial abrasion is plotted ($n = 6$). Limbus (L), paralimbus (PL), parawound (PW), wound (W), center wound (CW). The value at 24 hours in the paralimbal (PL) region was significantly different ($P < 0.05$) from the other times points in the PL region.

injury, had low levels of GL3⁺ cells in the limbal and corneal epithelium (Figure 3A). Given that ICAM-1 is a known ligand for CD11a/CD18 (LFA-1), a member of the $\beta 2$ integrin family, mice deficient in CD11a were analyzed at the peak time of accumulation of these cells in wild-type mice. CD11a^{-/-} mice exhibited significant reductions in GL3⁺ cell numbers (Figure 3A). Immunocytochemistry revealed staining of GL3⁺ cells with anti-CD11a (Figure 3B).

ICAM-1 and Corneal Stromal $\gamma\delta$ T Cells

GL3⁺ cells were sparse in the limbal stroma and absent in the corneal stroma of unwounded wild-type mice. GL3⁺ cells increased at 6 and 12 hours after central epithelial abrasion and decreased somewhat thereafter (Figure 4, A and B). In contrast to the epithelium, stromal GL3⁺ cells in ICAM-1^{-/-} mice increased parallel with wild-type mice in the first 12 hours, and increased significantly beyond that of wild-type mice at 18 and 24 hours (Figures 4B). The accumulation of GL3⁺ cells in the ICAM-1^{-/-} mice was primarily in the limbus (Figure 4C). The apparent compartmentalization of $\gamma\delta$ T cells into epithelium and stroma was evident, though to a lesser

extent, when CD11a^{-/-} mice were compared with wild-type mice. CD11a-deficient GL3⁺ cells accumulated significantly less than wild-type in the epithelium and significantly more in the stroma (Figures 3A and 4D).

ICAM-1 and Chemokine Expression by Corneal Epithelium

Corneal epithelium is known to express a number of chemokines that potentially attract leukocytes in response to injury or infection.³³ To investigate the possibility that ICAM-1 is necessary for expression of chemokines that attract $\gamma\delta$ T cells, we assessed epithelial mRNA levels for an array of chemokines reported to be chemotactic for $\gamma\delta$ T cells. Limbal and paralimbal epithelium (ie, the regions not directly injured during abrasion) was collected from wild-type and ICAM-1^{-/-} mice, either uninjured or 3 hours after central epithelial abrasion. mRNA levels for the chemokines listed in Figure 5 were significantly increased in abraded wild-type and ICAM-1^{-/-} mice compared with unwounded epithelium. Values for CCL3, CCL4, and CXCL10 were significantly higher in ICAM-1^{-/-} mice than in controls. Two pro-inflammatory

Figure 3. Epithelial $\gamma\delta$ T cells in response to corneal abrasion. **A:** Whole mount corneal preparations were stained with PE-tagged monoclonal antibody GL3 (anti-TCR δ) at 24 hours after central epithelial abrasion, the peak accumulation in wild-type mice (see Figure 2). The sum of GL3⁺ cells in the epithelium in nine microscopic fields across the cornea are plotted for wild-type, ICAM-1^{-/-}, CD11a^{-/-}, and P-selectin^{-/-} mice, as well as wild-type mice treated with either anti-ICAM-1 (30 minutes i.p. before abrasion) or anti-GP1b α (24 hours i.p. before abrasion) monoclonal antibodies. * $P < 0.01$, $n = 6$. **B:** Photomicrographs of wild-type corneal parabulbus at 24 hours after injury stained with both anti-TCR δ -fluorescein isothiocyanate and anti-CD11a-PE showing (a) only GL3⁺ cells and (b) merged image with both antibodies. Arrows indicate double positive cells. Scale bar = 10 μ m.

cytokines, tumor necrosis factor and interleukin-1 β , were evaluated as well, and both were significantly increased in wild-type and ICAM-1^{-/-}. In wild-type, interleukin-1 β increased 4.1-fold ($P < 0.004$) and tumor necrosis factor increased 1.5-fold ($P < 0.004$); in ICAM-1^{-/-}, interleukin-1 β increased 6.6-fold ($P < 0.001$), tumor necrosis factor, 2.0-fold ($P < 0.008$). These data contain no obvious deficits in chemokine expression to possibly account for the significantly reduced levels of $\gamma\delta$ T cells in the epithelium of ICAM-1^{-/-} mice.

ICAM-1 and Epithelial Wound Healing

Epithelial wound closure was delayed in the ICAM-1^{-/-} mice. Open wound area was significantly larger at 18 hours after a 2-mm central epithelial abrasion in ICAM-1^{-/-} mice than wild-type mice (Figure 6A). Cell division in the unwounded regions of the epithelium (limbal and parabulbus) reached a peak at 18 hours after central epithelial abrasion in wild-type mice,³⁴ but was significantly de-

layed in ICAM-1^{-/-} mice (Figure 6B). Basal cell density across the uninjured cornea of ICAM-1^{-/-} mice was not different from that of wild-type (Figure 6C), but recovery after injury in the center of the cornea at 48 hours was significantly reduced in the ICAM-1^{-/-} mice (Figure 6D). The height of the uninjured central corneal epithelium was not significantly different among the wild-type, ICAM-1^{-/-}, and TCR δ ^{-/-} mice. Though the wild-type mice recovered normal epithelial thickness within 96 hours following central corneal epithelial abrasion,²⁶ both ICAM-1^{-/-} mice (recovering ~66%, Figure 3A) and TCR δ ^{-/-} mice (recovering ~60%²⁶) had significantly less epithelial height at this time ($P < 0.01$, $n = 6$ corneas from each strain). Epithelial thickness at 96 hours after injury remained significantly less in the ICAM-1^{-/-} than in uninjured corneas in both the central region where epithelium was removed by abrasion and in the parabulbus region that was initially uninjured (Figure 7A). Two weeks after abrasion ICAM-1^{-/-} epithelium attained a thickness equivalent to wild-type ($P > 0.05$, $n = 6$ corneas, Figure 7B), but TCR δ ^{-/-} recovery remained at 60.7% of the uninjured thickness ($P < 0.05$, $n = 6$ corneas, Figure 7B). These results indicate that ICAM-1 deficiency significantly reduces the rate of epithelial recovery after abrasion but is less detrimental than deficiency of $\gamma\delta$ T cells.

Platelet Depletion and Epithelial $\gamma\delta$ T Cells

Wild-type mice treated with anti-GP1b α to deplete blood platelets exhibit delays in corneal epithelial wound healing³¹ comparable with that seen in the ICAM-1^{-/-} mice. In the current study, wild-type mice were given anti-GP1b α to induce prolonged platelet depletion. In contrast to ICAM-1^{-/-} mice where GL3⁺ cells failed to accumulate in the healing epithelium, increases in GL3⁺ cells in the epithelium following platelet depletion were not different from control wild-type mice (Figures 3A and 7C). These observations demonstrate a condition that delays wound healing without significantly reducing $\gamma\delta$ T cell accumulation in the epithelium.

Discussion

ICAM-1-deficient mice and wild-type mice treated with anti-ICAM-1 monoclonal antibody failed to show the expected accumulation of $\gamma\delta$ T cells in the peripheral corneal epithelium following central epithelial abrasion. Since ICAM-1-deficient mice have delayed wound healing, this failure could simply reflect some aspect of wound healing other than a direct dependence on ICAM-1. Against this interpretation are three experimental observations: 1) Epithelial $\gamma\delta$ T cells accumulated equally in wild-type and platelet-depleted mice, a condition that induces delay in wound closure³¹ comparable with ICAM-1-deficient mice. 2) P-selectin-deficient mice have a significant delay in wound healing³¹ but exhibited normal epithelial accumulation of $\gamma\delta$ T cells. 3) Mice deficient in CD11a, the α subunit of LFA-1 and the major leukocyte adhesion molecule recognizing ICAM-1, exhibited significantly reduced accumulation of epithelial $\gamma\delta$ T cells.

Figure 4. Stromal $\gamma\delta$ T cells in response to corneal abrasion. **A:** Limbus of cornea from wild-type and ICAM-1^{-/-} mice 24 hours after central epithelial abrasion stained with anti-TCR δ (green) and anti-CD31 (red). Scale bar = 20 μ m. **B:** Whole mount corneal preparations were stained with antibody GL3 (anti-TCR δ) at various times after central epithelial abrasion. The sum of GL3⁺ cells in the stroma in nine microscopic fields across the cornea are plotted for wild-type, and ICAM-1^{-/-} mice. * P < 0.05, as compared with wild-type at 18 and 24 hours, n = 6. **C:** Distribution of GL3⁺ cells in the stroma of the cornea from limbus to center wound region at 18 hours after wounding wild-type, and ICAM-1^{-/-} mice. * P < 0.05, compared with wild-type limbus (L). **D:** GL3⁺ cells were counted in the stroma of corneas at 24 hours following central corneal abrasion. Numbers of these cells in ICAM-1^{-/-} and CD11a^{-/-} mice were significantly (* P < 0.01, n = 6) greater than wild-type (WT); values for P-Sel^{-/-} and platelet depleted (anti-GP1ba) were not significantly different from wild-type.

These data are consistent with a direct requirement for ICAM-1 in the accumulation of $\gamma\delta$ T cells in the corneal epithelium following central corneal abrasion.

In contrast to the epithelium, $\gamma\delta$ T cells entered the stroma of ICAM-1-deficient mice in response to epithelial abrasion and the initial rate of increase in the area around the limbal vessels was essentially the same as that of wild-type mice. It appears that ICAM-1 is not critical to their migration from the vasculature into the stroma. Corneal epithelial cells and limbal vessel endothelial cells can express ICAM-1^{14–21} in response to inflammatory cytokines^{35,36} such as tumor necrosis factor and interleukin-1 β (both expressed in our model of epithelial abrasion). While ICAM-1 is known to contribute to lymphocyte adhesion and transendothelial migration, other adhesion molecules (eg, VCAM-1) also support lymphocyte transmigration.^{37–40} In contrast to endothelial adhesion, Iwata et al in earlier work²⁰ found that LFA-1/ICAM-1 interaction

is dominant in human lymphocyte adhesion to human corneal epithelial cells expressing ICAM-1 after stimulation with inflammatory cytokines. Our current observations confirm CD11a on wild-type murine $\gamma\delta$ T cells, and CD11a^{-/-} mice (ie, LFA-1-deficient) have significantly reduced $\gamma\delta$ T cell accumulation in the corneal epithelium. Expression of ICAM-1 mRNA and the presence of

Figure 5. Epithelial chemokine expression. Expression analysis of corneal epithelium at 3 hours after central corneal abrasion in wild-type and ICAM-1^{-/-} mice. RNA was isolated from the regions of the cornea not directly injured by abrasion. All chemokines listed were significantly increased (P < 0.05, n = 3 pools of RNA from six mice per pool) over uninjured epithelium of the same region. * P < 0.01.

Figure 6. Corneal epithelial wound healing. **A:** At 18 hours after central epithelial abrasion, the wound area was assessed microscopically in 4',6-diamidino-2-phenylindole-stained whole mount preparations, and the area without epithelial cells was calculated. This area was significantly less (* P < 0.01, n = 5) in wild-type mice than ICAM-1^{-/-}. **B:** Dividing epithelial cells were counted in nine microscopic fields across the corneas from limbus to limbus at various times after central abrasion. ICAM-1^{-/-} mice exhibited significantly fewer (* P < 0.01, n = 6) dividing cells at 12, 18, and 24 hours after injury. **C:** Basal epithelial cell density was analyzed across uninjured corneas of wild-type and ICAM-1^{-/-} mice. **D:** Basal epithelial cell density was significantly less (P < 0.05, n = 6) at 48 hours after abrasion in ICAM-1^{-/-} mice.

Figure 7. Corneal epithelial wound healing. At 96 hours or 2 weeks after central epithelial abrasion, epithelial thickness was measured in fixed toluidine blue stained 0.5-μm thick plastic sections in the central region (ie, healed epithelium) and in the paralimbus (ie, epithelium that was not directly injured). **A:** ICAM-1^{-/-} mice failed to attain uninjured epithelial thickness at 96 hours after injury either in the central or paralimbal regions. This is in contrast to wild-type mice.²⁶ **B:** Photomicrographs of central corneal sections from wild-type, ICAM-1^{-/-} and TCRδ^{-/-} mice 2 weeks after central epithelial abrasion. In contrast to wild-type and ICAM-1^{-/-} mice, TCRδ^{-/-} epithelium was thinner and less cellular. The arrows indicate the measured height of the epithelium from the basement membrane beneath the basal cells to the outer surface of the epithelium. **C:** Blood platelet depletion was induced by i.p. injection of anti-GP1bα monoclonal antibody 24 hours before central corneal abrasion as previously reported.¹⁴ GL3⁺ cells were counted in the epithelium in designated regions of the cornea at 24 hours following abrasion.

ICAM-1 on limbal and paralimbal epithelial cells¹⁴ after central epithelial abrasion coincides temporally with the accumulation of $\gamma\delta$ T cells in those corneal regions. These observations are consistent with the idea that epithelial ICAM-1 serves as a necessary adhesive ligand for LFA-1-dependent migration or retention of $\gamma\delta$ T cells into the corneal epithelium.

The source of $\gamma\delta$ T cells in corneal epithelium is not clear. In wild-type mice, $\gamma\delta$ T cells increased in the stroma near the limbal vessels at 12 hours after injury and then decreased as they increased in the epithelium of the limbal and paralimbal regions over the next 12 hours. A possible route may be blood-derived $\gamma\delta$ T cells migrating

through the stroma into the epithelium. Evidence in the ICAM-1-deficient mice is consistent with this idea since there is increased accumulation of $\gamma\delta$ T cells in the limbal stroma at 18 and 24 hours after injury without appearance in the epithelium, as if entrance into the epithelium requires ICAM-1, but migration from blood into stroma does not. Since the stromal $\gamma\delta$ T cells in the ICAM-1-deficient mice remain essentially localized in the limbus (see Figure 4C), migration through the stroma may be deficient. A possible role for ICAM-1 in the stroma is its expression on the keratocytes^{41,42} serving as an adhesive site for cell locomotion. We published morphometric evidence that neutrophils interact with keratocytes in the corneal stroma and that deficiency of $\beta 2$ (CD18) integrins (including LFA-1) significantly reduces this interaction.⁴³

Locomotion of $\gamma\delta$ T cells is influenced by chemokines, and expression analysis of the epithelium indicates numerous potential chemokines increased within the time frame of $\gamma\delta$ T cell accumulation. CCL3, CCL4, and CCL5 are of potential interest given results with some populations of $\gamma\delta$ T cells shown to express a relevant chemokine receptor CCR5.^{37,44} Another chemokine receptor of possible importance for future studies is CXCR3 given the prominence of CXCL10 in the epithelial expression profile and published evidence for its expression by some populations of $\gamma\delta$ T cells.^{37,45} A third chemokine receptor of possible importance is CCR2, given increased CCL2 in the expression profile and published evidence on some $\gamma\delta$ T cells.⁴⁴ The data on chemokine expression thus far fails to identify a deficiency in the ICAM-1^{-/-} mice that could account for the failure of $\gamma\delta$ T cell migration into the epithelium. Our current hypothesis is that ICAM-1 is functioning as a necessary adhesive ligand for $\gamma\delta$ T cell motility or retention in the epithelium.

Corneal epithelial wound healing is delayed in ICAM-1-deficient mice. The rate of wound closure, the level of epithelial cell division within the first day following injury, the return of epithelial cell density, and thickness of epithelial stratification in the wounded region, were all significantly delayed in ICAM-1^{-/-} mice. ICAM-1 has also been found necessary for efficient epidermal healing,^{46,47} linked to emigration of neutrophils and macrophages, a well-established role for this adhesion molecule at sites of inflammation.^{7,46} In the current study, we raise that possibility that ICAM-1 contribution to corneal epithelial healing may be linked to lymphocytes entering the epithelium. Elevated numbers of $\gamma\delta$ T cells occur during the phase of re-epithelialization and remain in the epithelium for at least 7 days following injury. Their contribution to corneal healing is indicated by the finding that epithelial recovery is significantly delayed at least 2 weeks following injury in TCRδ^{-/-} mice. $\gamma\delta$ T cells have been reported necessary for efficient epidermal healing,^{48,49} and the healing epidermis contains $\gamma\delta$ T cells.⁵⁰ Potential direct contributions of resident epidermal $\gamma\delta$ T cells include the production of keratinocyte growth factor and insulin-like growth factor-1,⁵¹ as well as the induction of hyaluronan.⁴⁸ Whether the $\gamma\delta$ T cells that migrate into the cornea following epithelial wounding also produce these factors remains to be determined, but it cannot be assumed, since we have preliminary evidence (Smith et

al unpublished) that the corneal $\gamma\delta$ T cells are a different subset from the V γ 3V δ 1 dendritic epidermal T cells found in mouse skin.

In summary, we have demonstrated in the current studies that $\gamma\delta$ T cells accumulate in the corneal epithelium within 18 hours following central epithelial abrasion and remain at significantly elevated levels for at least 7 days, a time well past full recovery of epithelial density and thickness. Further, $\gamma\delta$ T cell deficiency results in failure to recover full epithelial density and thickness even 2 weeks after abrasion. ICAM-1 deficiency results in significant delay in corneal epithelial wound healing, and the expected accumulation of $\gamma\delta$ T cells in the epithelium fails to occur. CD11a-deficiency also significantly reduces $\gamma\delta$ T cells in the epithelium suggesting that normal migration of $\gamma\delta$ T cells into the epithelium requires LFA-1 (CD11a/CD18) interactions with ICAM-1.

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