$\gamma\delta$ T Cells Are Necessary for Platelet and Neutrophil Accumulation in Limbal Vessels and Efficient Epithelial Repair after Corneal Abrasion

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Corneal epithelial abrasion in C57BL/6 mice induces an inflammatory response with peak accumulation of neutrophils in the corneal stroma within 12 hours. Platelets localize in the limbal vessels throughout the same time course as neutrophils and contribute to wound healing because antibody-dependent depletion of platelets retards epithelial division and wound closure. In the present study, T cells in the limbal epithelium were found to predominantly express the $\gamma\delta$ T-cell receptor (TCR). Corneal abrasion in wild-type, CD11a^{-/-}, and P-sel^{-/-} mice increased the numbers of $\gamma\delta$ T cells in the limbal and peripheral corneal epithelium and in the corneal stroma adjacent to the limbal blood vessels. Intercellular adhesion molecule (ICAM)-1^{-/-} mice exhibited a reduction in $\gamma\delta$ T-cell accumulation. TCR δ ^{-/-} mice exhibited reduced inflammation and delayed epithelial wound healing as evidenced by delayed wound closure, reduced epithelial cell division, reduced neutrophil infiltration, and reduced epithelial cell density at 96 hours after wounding. TCR δ ^{-/-} mice also exhibited >60% reduction in platelet localization in the limbus despite similar platelet counts and platelet function assessed with an *in vivo* thrombosis model. These results are consistent with the conclusion that $\gamma\delta$ T cells are necessary for efficient inflammation, platelet localization in the limbus, and epithelial wound healing after corneal abrasion. (Am J Pathol 2007, 171:838–845; DOI: 10.2353/ajpath.2007.070008)

Superficial wounds in corneal epithelium evoke leukocyte emigration through limbal vessels.^{1–3} Under normal circumstances, re-epithelialization progresses rapidly,⁴ and the infiltrating leukocytes return to pre-injury levels presumably as a result of apoptosis.⁵ We recently observed that healing of corneal epithelial wounds is significantly delayed in P-selectin-deficient (P-sel^{-/-}) mice and in wild-type mice after antibody-dependent depletion of either circulating neutrophils or platelets.⁶ Passive transfer of normal platelets into P-sel^{-/-} mice restored epithelial healing to within 80% of wild-type levels.⁶ In this murine model, central corneal epithelial abrasion induces a substantial neutrophil infiltrate within 6 hours that peaks in 12 to 18 hours⁷ and a coordinated accumulation of platelets localizing in the limbal vessels.⁶ P-sel^{-/-} mice have profoundly diminished platelet and neutrophil accumulation, and as with epithelial wound healing, passive intravenous transfer of freshly isolated wild-type platelets results in significant platelet and neutrophil localization in limbal vessels of P-sel^{-/-} mice. A relationship between neutrophil accumulation at inflammatory sites and platelets has become apparent in several tissues^{8–11} including other ocular tissues.^{12,13} We found that antibody-induced depletion of circulating neutrophils significantly reduced platelet localization in the limbal vessels, and depletion of blood platelets reduced neutrophil accumulation.⁶

In the current study, we investigate the contributions of T cells to the healing and inflammatory events after corneal epithelial abrasion. Our focus is on $\gamma\delta$ T cells because we observed that >90% of the T cells resident in the limbal epithelium were positive for antibody GL3, a

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pan- $\gamma\delta$ T-cell marker,¹⁴ and because $\gamma\delta$ T cells resident in the epidermis apparently contribute to wound healing.^{15–17} Our results reveal a dependence of neutrophil and platelet localization in limbal vessels on $\gamma\delta$ T cells.

Materials and Methods

Animals

TCR $\delta^{-/-}$ mice on the C57BL/6 background and C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). Intercellular adhesion molecule (ICAM)-1 $^{-/-}$,¹⁸ P-selectin $^{-/-}$,¹⁹ and CD11a $^{-/-}$ ²⁰ mice were backcrossed as previously described at least 10 generations with C57BL/6 mice. All animals were bred and housed in our facility²¹ 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 used at 10 to 14 weeks of age.

Corneal Epithelial Wounding Model

The central corneal wound was performed as previously described.²² In brief, mice were anesthetized by intraperitoneal 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. Wound closure was assessed using fluorescein staining of the ocular surface and digital analysis of the stained area. At various times corneal tissues including the limbus were excised and processed for immunohistology. Some mice were depleted of $\gamma\delta$ T cells as previously described²³ by an intraperitoneal injection of 500 μ g of hamster anti-T-cell receptor (TCR) $\gamma\delta$ monoclonal antibody (mAb) (clone GL3¹⁴; BD Pharmingen, La Jolla, CA) in a volume of 0.30 ml of phosphate-buffered saline before corneal abrasion. Sham depletion was accomplished with hamster immunoglobulin (The Jackson Laboratory).

Immunohistology

Taking care to include the limbus, wounded corneas were dissected, fixed, permeabilized, and incubated with the following labeled monoclonal antibodies as described^{6,7,22,24}: anti-Gr-1-fluorescein isothiocyanate (FITC) (clone RB6-8C5), anti-TCR δ -phycoerythrin (PE) (clone GL3), anti-CD41-PE (clone MWReg30), and anti-CD31-FITC (clone MEC 13.3) (BD Pharmingen), which were selected for staining neutrophils, $\gamma\delta$ T cells, platelets, and endothelial cells of the limbal vessels, 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 (DAPI;

Sigma Chemical, St. Louis, MO) to assess nuclear morphology. Cell division was assessed as previously described.⁶ 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 (shown in Figure 3, inset).^{6,7,22,24} Whole mounts were evaluated using a $\times 40$ oil immersion lens to assess each field of view across the cornea from limbus to limbus. For neutrophils, a central frame covering 8% of the field of view was used to count neutrophils throughout the depth of the cornea from the epithelial to endothelial surfaces (a range of ~ 90 μ m). To compare the relative level of neutrophils in the different areas from the limbus to the central cornea, each cornea was counted separately. To compare the relative level of platelet accumulation in the limbal areas, each cornea was counted in eight random fields along limbal vessels separately. For epithelial cell division, cells per entire field of view were counted at the focal plane of the basal cell layer of the epithelium. For epithelial density, a central frame 162 $\times$ 102 μ m in the field of view was counted at the focal plane of the basal cell layer of the epithelium. At least four corneas were examined for immunohistology, and four quadrants were analyzed for each to obtain the average number per field. The limbus was defined as the intervening zone between the cornea and conjunctiva as the most peripheral field.

Histological Assessment of Corneal Thickness

Enucleated eyes were fixed overnight at 4°C in 0.1 mol/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) using a $\times 20$ objective, transverse measurements of the central epithelial thickness were made using the calibrated linear measurement tool contained in the supplied imaging software (Applied Precision SoftWorx).

In Vivo Thrombosis Model

Platelet function *in vivo* was assessed with a light/dye-induced model of platelet thrombus formation in cremaster venules of pentobarbital-anesthetized mice, as described previously.^{11,25,26} In brief, platelet thrombi were induced by epi-illumination of FITC-dextran (150 kd, 10 ml/kg of a 5% solution), injected via a jugular venous line. Thrombosis kinetics were assessed by determining the time of onset of platelet aggregates and time of thrombotic occlusion, by an investigator blinded to the mouse genotype. In some experiments,

Figure 1. Immunofluorescence studies of murine corneal T cells. **a:** Unrounded corneal epithelium from a wild-type C57BL/6 mouse stained with PE-labeled anti-TCR δ , GL3. **b:** FITC-labeled anti-CD3 showing four cells that are positive for both antibodies. Nuclei are stained with DAPI. Unrounded corneal epithelium from TCR $\delta^{-/-}$ mice stained with FITC-labeled anti-CD3 (**c**) and PE-labeled anti-TCR δ , GL3 (**d**). The **arrows** indicate the positions of the four CD3 $^{+}$ cells demonstrating the absence of TCR δ expression. **e:** Wild-type C57BL/6 mouse cornea at 18 hours after central corneal abrasion. The field of view is within limbal epithelium (see region 1 of Figure 3) showing PE-labeled anti-TCR δ (GL3)-stained cells in the region of dividing cells (**arrows**). Nuclei and mitotic figures stained with DAPI. **f:** Unrounded limbal and peripheral corneal epithelium from a wild-type mouse stained with PE-labeled anti-TCR δ , GL3 showing the restricted distribution of the GL3 $^{+}$ cells to the limbus. **g:** Limbal and peripheral corneal epithelium 18 hours after central corneal abrasion stained with PE-labeled anti-TCR δ (GL3) showing an increased number of GL3 $^{+}$ cells and expanded distribution of the GL3 $^{+}$ cells into the cornea. Scale bars: 10 μ m (**a** and **b**); 20 μ m (**c** and **d**); 40 μ m (**f** and **g**).

mice were pretreated 1 hour earlier with 0.2 mg i.v. of monoclonal antibodies against glycoprotein Ib α (Emfret Analytics, Würzburg, Germany), used by us previously to induce platelet depletion *in vivo*.⁶ In antibody-treated mice, venules that failed to develop thrombotic occlusion after 60 minutes of epi-illumination were assigned a value of 60 minutes.

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

Cells of leukocytic origin (ie, CD45 $^{+}$) are resident in limbal epithelium and express CD11b, CD11c, CD3, and F4/80.^{24,27} CD11c $^{+}$ cells are dendritic²⁸ and within 6 hours after central epithelial abrasion their numbers drop by ~50%.²⁴ In the current study >90% of resident CD3 $^{+}$ cells were positive for antibody GL3, a marker of $\gamma\delta$ T cells (Figure 1, a and b).¹⁴ CD3 $^{+}$ cells in corneal epithelium of TCR $\delta^{-/-}$ mice were negative for GL3 (Figure 1, c and d). In contrast to CD11c $^{+}$ cells,²⁴ the GL3 $^{+}$ cells

increased in number after central corneal epithelial abrasion (Figure 2) and by 18 hours were evident in both limbus and peripheral cornea (Figure 1, f and g). GL3 $^{+}$ cells increased approximately threefold in the limbal stroma by 12 hours after epithelial abrasion, and by 18 hours 84% of the total GL3 $^{+}$ cells were in the same regions of the epithelium as dividing epithelial cells (Figure 1e).^{6,29}

Figure 2. Changes in GL3 $^{+}$ cells after central corneal epithelial abrasion. **A:** Temporal changes in the number of GL3 $^{+}$ cells after corneal abrasion in C57BL/6 wild-type mice. The values plotted at each time point are the sum of GL3 $^{+}$ cells in the epithelium and stroma in nine $\times 40$ fields of view across the diameter of the cornea (*n* = 4). **P* < 0.01 compared with unwounded corneas. **B:** The distribution of GL3 $^{+}$ cells at 18 hours after abrasion is plotted as cells per $\times 40$ fields across the diameter of the cornea (limbus to limbus).

Figure 3. Epithelial response to central corneal epithelial abrasion in C57BL/6 mice—morphometric analysis. **Inset:** Schematic representation of whole mount excised cornea showing regions for microscopic analysis from the limbus (region 1) to the original wound area (regions 4 and 5). The width of each region is encompassed by the field of view of $\times 40$ objective (~ 0.53 mm). Basal epithelial cell division is the total number of dividing cells counted in three $\times 40$ fields (regions 1, 2, and 3) across the cornea in the epithelium not directly injured during the central abrasion. Basal epithelial cell density is the number of cells per $\times 40$ field in region 4 (the peripheral region abraded) and in region 5 (the center of the abraded area). * $P < 0.01$ compared with the level of cell division in uninjured corneas; ** $P < 0.01$ compared with the density immediately after abrasion, $n = 6$.

Corneal Epithelial Healing

Because $\gamma\delta$ T cells may participate in epidermal wound healing,¹⁵ corneal epithelial healing in the TCR $\delta^{-/-}$ mice was assessed. Central epithelial abrasion without stromal injury results in three general phases of healing.^{22,24,30,31} The initial lag phase lasts ~ 6 hours. The migration phase in which epithelial cells (basal and suprabasal) crawl over the provisional matrix on the stromal surface to close the wound lasts ~ 24 hours. This is evident as increasing epithelial cell density in the abraded area (Figure 3), first seen in region 4 between 6 and 12 hours and region 5 at 18 to 24 hours after injury. The third phase, epithelial division, is initially prominent at 18 hours (Figure 3). Dividing cells in the limbal epithelium increased from 1.9 ± 0.36 per field at 12 hours to 16.9 ± 1.6 per field ($n = 8$, $P < 0.01$) at 18 hours.

TCR $\delta^{-/-}$ mice exhibited marked depression of epithelial division at 18 hours (Figure 4), and pretreatment of mice with antibody GL3 prevented the initiation of epithelial division at 18 hours. The migration phase of wound closure was significantly delayed as indicated by the numbers of epithelial cells migrating into the abraded area (Figure 5A). The epithelial basal cell density and the thickness of the restored epithelium at 96 hours after

Figure 4. Corneal response to central corneal epithelial abrasion in TCR $\delta^{-/-}$ mice. Dividing basal epithelial cells were evaluated at 6-hour intervals after central corneal abrasion in wild-type mice and TCR $\delta^{-/-}$ mice (A), and wild-type mice treated before wounding with either GL3 antibody or hamster IgG (B). The values for the dividing epithelial cells are sums of six $\times 40$ fields of view (regions 1, 2, and 3 shown in the schema in Figure 3). The lower number of dividing cells in controls on B compared with the wild type in A reflects mouse strain differences. These antibody-blocking studies were done with C57BL/6 mice from Harlan (Indianapolis, IN). The studies in A were with C57BL/6 mice from The Jackson Laboratory, the appropriate controls for the TCR $\delta^{-/-}$ mice. Statistical analysis indicated the values for the TCR $\delta^{-/-}$ mice ($n = 4$) at 12, 18, 24, and 30 hours after wounding were significantly less than wild type ($P < 0.01$), and that for animals ($n = 4$) treated with GL3 (anti-TCR δ), values at 18, 24, 30, and 48 hours after wounding were significantly less than ($P < 0.01$) the hamster IgG controls.

wounding was significantly less than in wild-type mice (Figure 5, B and C). Thus, the migratory and proliferative phases of epithelial healing were significantly delayed in the TCR $\delta^{-/-}$ mice.

ICAM-1 but Not LFA-1 Contributes to $\gamma\delta$ T-Cell Accumulation and Epithelial Cell Division in Response to Epithelial Abrasion

Two important adhesion molecules involved in leukocyte migration are CD11a/CD18 (LFA-1), a member of the $\beta 2$ integrin family expressed on lymphocytes,³² and ICAM-1 (CD54), expressed on limbal vessel endothelial cells and corneal epithelium during the time period (ie, 18 hours after wounding) in which $\gamma\delta$ T-cell numbers increase.²² Accumulation of $\gamma\delta$ T cells was analyzed in two strains of C57BL/6 mice with targeted deletions. CD11a $^{-/-}$ mice exhibited no significant reduction at 18 hours in GL3 $^{+}$ cell accumulation after central corneal abrasion (Figure 6A). Animals deficient in ICAM-1 exhibited significant reductions in the GL3 $^{+}$ cell accumulation at 18 and 24 hours after corneal abrasion (Figure 6A). Epithelial basal cell

Figure 5. Corneal epithelial healing after central abrasion in wild-type and TCRδ^{-/-} mice. **A:** Basal epithelial density was determined in region 4 shown in the schema in Figure 3, ie, the periphery of the epithelial wound. **P* < 0.01, *n* = 4. **B:** Basal cell density was determined at 96 hours after central abrasion. Values from ×40 fields across the diameter of the cornea are shown. **P* < 0.01. **C:** Analysis of epithelial thickness in the center of the cornea at 96 hours after abrasion shown in photomicrographs of representative wild-type and TCRδ^{-/-} epithelium. Plots of epithelial thickness in region 5 measured from toluidine blue-stained plastic sections of wild-type corneas (1), unwounded; wild-type corneas at 96 hours after abrasion (2); TCRδ^{-/-} corneas, unwounded (3); and TCRδ^{-/-} corneas at 96 hours after abrasion (4), **P* < 0.01, *n* = 4.

division was delayed in ICAM-1^{-/-} mice and its duration was shortened compared with that in wild-type and CD11a-deficient mice (Figure 6B). Consistent with observations in the wild-type mice, treatment of the CD11a^{-/-} mice with GL3 before corneal abrasion significantly inhibited cell division at 18 hours (Figure 7).

Leukocyte Migration

Experimental conditions preventing neutrophil emigration (eg, CD18 deficiency or neutropenia) reduce wound closure and early onset of epithelial division.⁷ To determine whether reduced healing in the TCRδ^{-/-} mice is possibly linked to reduced neutrophil emigration, neutrophil accumulation was analyzed throughout the first 48 hours after

Figure 6. Response to corneal abrasion in knockout mice. **A:** Mice with targeted deletions of CD11a or ICAM-1 were evaluated for GL3⁺ cell accumulation (expressed as sums of cells in nine ×40 fields of view across a diameter of the cornea) after central corneal epithelial abrasion. CD11a^{-/-} mice were very similar to wild-type mice in this parameter. ICAM-1^{-/-} mice exhibited significant reductions in GL3⁺ cell accumulation at 18 and 24 hours after wounding when compared with wild-type mice (**P* < 0.001, *n* = 4). **B:** Mice with targeted deletions of CD11a or ICAM-1 were evaluated for epithelial cell division (sums of three ×40 fields of view, regions 1, 2, and 3 shown in the schema in Figure 3) after central corneal epithelial abrasion. CD11a^{-/-} mice were similar to wild-type mice in this parameter. ICAM-1^{-/-} mice exhibited significant reductions in epithelial cell division at 18, 30, and 36 hours after wounding (**P* < 0.001, *n* = 4).

Figure 7. Effect of GL3 on epithelial division in CD11a^{-/-} mice. CD11a^{-/-} mice were treated with antibody GL3 or hamster IgG before central corneal epithelial abrasion. At 18 hours after wounding, corneas were collected and evaluated for epithelial cell division (*n* = 4).

abrasion. Neutrophils were significantly reduced in the TCRδ^{-/-} mice and in wild-type mice pretreated with antibody GL3 (Figure 8). Antibody GL3 interrupted neutrophil influx if given at 18 hours after abrasion in wild-type mice (Figure 8C). Blood neutrophil counts in TCRδ^{-/-} mice were not different from wild type ($6.3 \times 10^2/\mu\text{l}$ and $8.6 \times 10^2/\mu\text{l}$, respectively, laboratory normal range 5 to $20 \times 10^2/\mu\text{l}$). Blood lymphocyte, monocyte, and eosinophil counts also did not differ.

Platelet Accumulation in Limbal Vessels

In published studies⁶ we have shown that platelet accumulation contributes to the early aspects of healing after central corneal abrasion. Platelet accumulation in the limbus is initially coincident with that of neutrophils, peaking within the first 12 hours after injury, and platelet ac-

Figure 8. Leukocyte emigration after central corneal abrasion. **A:** Neutrophil accumulation kinetics throughout a 48-hour observation time based on analysis of corneas collected at 6-hour intervals from wild-type mice, TCRδ^{-/-} mice, and wild-type mice treated before wounding with either GL3 antibody or hamster IgG. Plotted is the sum of the number of neutrophils in nine fields of view across a diameter of the cornea, see Figure 3. **P* < 0.01 for both TCRδ^{-/-} mice and wild-type mice treated with GL3 compared with appropriate controls at 12, 18, 30, 36, and 42 hours after wounding, *n* = 4. **B:** Neutrophil (per nine fields of view) distributions across the corneas collected at 12 hours after wounding. All values for TCRδ^{-/-} mice and wild-type mice treated with GL3 are significantly lower (*P* < 0.01, *n* = 4) compared with appropriate controls, except in region 5. **C:** Wild-type mice were given antibody GL3 at 18 hours after central corneal epithelial abrasion. Corneas were collected at 30 hours after abrasion and evaluated for neutrophil emigration (expressed as the sum of neutrophils counted in nine fields of view across a diameter of the cornea, *n* = 4).

Figure 9. Platelet accumulation in TCR $\delta^{-/-}$ mice after corneal abrasion. **A:** Corneas were evaluated for platelet accumulation (expressed as the sum of platelets in eight fields of view in the limbal region) at 6-hour intervals after central corneal epithelial abrasion (* $P < 0.05$, ** $P < 0.01$; $n = 4$). **B:** Photomicrographs of the limbus showing wild-type and TCR $\delta^{-/-}$ mice 12 hours after epithelial abrasion stained with FITC-labeled anti-CD31 (labels endothelial junctions and some neutrophils) and PE-labeled anti-CD41 (to identify platelets). Scale bar, 25 μ m.

cumulation is markedly reduced in neutropenic mice. In the current studies, TCR $\delta^{-/-}$ mice also revealed significant reduction in platelet accumulation in the limbus (Figure 9, A and B). The reduced platelet accumulation in limbus of TCR $\delta^{-/-}$ mice was not a result of lower circulating platelets, because these mice had comparable blood platelet counts compared with wild-type mice (Figure 10A). Likewise, platelet function *in vivo* of TCR $\delta^{-/-}$ mice, assessed with a light/dye-induced thrombosis model,^{11,25,26} did not differ from that of wild-type mice. The TCR $\delta^{-/-}$ mice had similar time of onset of platelet aggregates as wild-type mice (18 ± 1 versus 22 ± 2 seconds, respectively; N.S.) and similar time to thrombotic occlusion (Figure 10A). In contrast, anti-GPIIb α antibodies, which reduced platelet counts in wild-type mice by $\sim 85\%$, induced a marked delay in microvascular thrombosis.

Figure 10. **A:** Evaluation of platelet function in TCR $\delta^{-/-}$ mice. Light dye-induced thrombotic venous occlusion was induced in wild-type (WT) and TCR $\delta^{-/-}$ mice. The time from vessel injury until blood flow stopped is plotted. As a control setting, WT mice received anti-GPIIb α to induce thrombocytopenia, as shown by the platelet counts (asterisk). $n = 4$. **B:** Accumulation of GL3 $^{+}$ cells in P-selectin-deficient mice. Because P-selectin-deficient (P-sel $^{-/-}$) mice exhibit marked reductions in platelet and neutrophil accumulation in the limbal vessels after corneal abrasion, the effects of P-selectin deficiency on GL3 $^{+}$ cell accumulation in the epithelium and stroma (expressed as sums of cells in nine $\times 40$ fields of view across a diameter of the cornea) were evaluated at 6-hour intervals. Statistical analysis revealed no difference in these curves, $n = 4$.

P-selectin-deficient mice exhibit significant reductions in neutrophil and platelet accumulation after corneal abrasion.⁶ The possibility that this outcome is attributable to inability of GL3 $^{+}$ cells to accumulate in the corneal tissue was tested by assessing the levels of GL3 $^{+}$ cells in the P-sel $^{-/-}$ mouse corneas. As shown in Figure 10B, P-sel $^{-/-}$ mice were indistinguishable from wild-type mice with regard to this parameter. This observation along with the previous studies showing that passive transfer of wild-type platelets into P-sel $^{-/-}$ mice primarily restores the healing defect⁶ is consistent with the interpretation that $\gamma\delta$ T cells are insufficient to promote healing in the absence of platelets.

Discussion

The conclusions that $\gamma\delta$ T cells are necessary for the early localization of neutrophils and platelets in the limbus and initiation of epithelial cell division within 18 hours after central abrasion are supported by the following evidence: i) Antibody GL3 (anti-TCR δ) bound to CD3 $^{+}$ cells in the limbal epithelium, and TCR $\delta^{-/-}$ mice lacked binding of this antibody to CD3 $^{+}$ cells in the limbus. ii) TCR $\delta^{-/-}$ mice exhibited marked reductions in epithelial cell division as well as neutrophil and platelet accumulation after epithelial abrasion. iii) Administration of antibody GL3 before corneal abrasion reproduced the pattern of epithelial, neutrophil, and platelet response in wild-type mice that occurred in TCR $\delta^{-/-}$ mice. iv) Mice with a targeted deletion of ICAM-1 had reduced accumulation of GL3 $^{+}$ cells (also neutrophils and platelets as seen in our earlier study⁶) and significantly delayed epithelial cell division.

The conclusion that platelets form a mechanistic link between $\gamma\delta$ T cells and early epithelial cell division is supported by the following observations: i) anti-GPIIb α -induced depletion of circulating platelets significantly delays epithelial cell division and wound closure.⁶ ii) Mice with targeted deletion of P-selectin (P-sel $^{-/-}$) have $>80\%$ reduction in platelet accumulation in the limbus after central corneal abrasion.⁶ iii) P-sel $^{-/-}$ mice exhibit reduced wound healing evidenced by reduced epithelial cell division and delayed

wound closure.⁶ iv) Passive intravenous transfer of wild-type platelets in P-sel^{-/-} mice promotes epithelial cell recovery from abrasion (evidenced by increased cell division and migration of epithelial cells into the wounded area⁶). v) $\gamma\delta$ T cells were insufficient to promote normal epithelial cell division because resident levels and the increases in numbers of $\gamma\delta$ T cells in the cornea after injury were unaffected in the P-sel^{-/-} mice.

Our interest in this previously unreported finding that $\gamma\delta$ T cells are necessary for platelet and neutrophil localization in the limbus arises from the current understanding that $\gamma\delta$ T cells are an important part of the populations of innate T lymphocytes, and they have been reported to influence epithelial cell growth and participate in epithelial repair in other organs.^{16,33} $\gamma\delta$ TCRs vary with the roles they play in different tissues³⁴ and are considered important to the maintenance of tissue homeostasis,³⁵ response to injury,¹⁵ or resistance to infection.^{36–38} Different populations of $\gamma\delta$ T cells with distinct TCRs migrate from the thymus to various organs during ontogeny.^{39,40} For example, V γ 5 V δ 1 TCR-expressing cells (also known as V γ 3 V δ 1) migrate to the skin, V γ 4 V δ 1 TCR-expressing cells migrate to the reproductive tract, and V γ 1 TCR-expressing cells migrate to the lung. Consistent with other organs, the $\gamma\delta$ T cells resident in the peripheral corneal and limbal surface are within the epithelium and, like those in the skin,¹⁵ have a dendritic morphology (see Figure 1a). There is convincing evidence that the $\gamma\delta$ T cells in the skin, also called dendritic epidermal T cells, play a critical role in wound healing^{15,41} and in the maintenance of epidermal integrity.^{35,42,43} Dendritic epidermal T cells apparently recognize endogenous factors released or expressed on stressed or damaged epidermal cells^{42,44} and are activated through ligation of the TCR. Activation may result in release of proinflammatory chemokines such as XCL1, CXCL1, CCL3, CCL4, and CCL5⁴⁵ that are capable of attracting other leukocytes and releasing growth factors such as FGF-7 and FGF-10⁴¹ that influence the division and migration of keratinocytes. In addition, some $\gamma\delta$ T-cell subsets limit inflammation or assist in the resolution of inflammation by direct cytotoxic interactions on activated macrophages^{46,47} through a mechanism that involves FasL expression on $\gamma\delta$ T cells (eg, those expressing V γ 1 TCR) interacting with Fas expressed on target cells.⁴⁶ There is evidence for anti-inflammatory activity through the production and release of thymosin- β 4,⁴⁰ of possible interest in the cornea given published data for an anti-inflammatory and beneficial wound-healing effect of thymosin- β 4 in the cornea.^{48,49}

The finding that ICAM-1^{-/-} mice exhibited reduced accumulation of $\gamma\delta$ T cells and delayed, abbreviated epithelial basal cell division remains to be explained, especially because the CD11a^{-/-} mice were not significantly different from wild type. ICAM-1 is known to be a dominant ligand for CD11a/CD18 (LFA-1), but in this specific inflammatory condition, it seems to be functioning in some other capacity. It is unlikely to be functioning as a ligand for CD11b/CD18 (Mac-1) in this setting because CD11b^{-/-} mice exhibit exaggerated leukocyte accumulation in the cornea after epithelial abrasion.²²

Comparison of our results with recently published reports on wound healing in the skin reveals possibly distinct features of the corneal response. Jameson and colleagues⁴¹ reported that dermal wound healing (excisional wounds) was characterized by significantly delayed re-epithelialization, reduced keratinocyte cell division, and reduced macrophage infiltration, but neutrophil accumulation was not different from that of wild-type mice. Alexander and colleagues¹⁷ reported that postburn wound healing in the skin of $\gamma\delta$ T-cell-deficient mice was also significantly altered, but neutrophil accumulation in response to the burn injury was not diminished. Our results demonstrate that neutrophil influx and platelet accumulation are significantly depressed in the $\gamma\delta$ T-cell-deficient mice after corneal epithelial wounding compared with wild-type mice, an apparent difference between skin and cornea. Because platelet depletion significantly delays corneal healing it seems that $\gamma\delta$ T cells may contribute to corneal healing in part by promoting platelet accumulation. The mechanistic links between $\gamma\delta$ T cells and platelets are likely complex, but our previous studies indicate that neutrophil localization is necessary for platelet accumulation in the limbus.⁶ We have determined that CXCL1 and CXCL5²⁴ and XCL1 (unpublished) are elevated in wild-type corneas after epithelial abrasion and coordinated with neutrophil influx. XCL1, reported to be released from some $\gamma\delta$ T cells,⁴⁵ has been shown to be an attractant for neutrophils that express the receptor XCR1.⁵⁰ We assume, though, that the mechanisms for platelet accumulation are multifactorial, but that P-selectin-dependent adhesion of platelets to neutrophils forms a necessary link.⁶

In summary, these observations support a conceptual model whereby $\gamma\delta$ T cells are necessary for platelet and neutrophil localization in the limbal vessels within 12 to 18 hours after epithelial abrasion, and platelet and neutrophil accumulation are necessary for early initiation of epithelial cell division.^{6,7} Adhesion molecules distinguish aspects of these events. P-selectin is necessary for platelet localization in the limbal vessels.⁶ ICAM-1, but not LFA-1 or P-selectin, participates in $\gamma\delta$ T-cell accumulation after abrasion.

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