Lymphocyte Function-Associated Antigen-1-Dependent Inhibition of Corneal Wound Healing

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Abrasion of murine corneal epithelium induces neutrophil emigration through limbal vessels into the avascular corneal stroma, peaking within 12 to 18 hours after wounding. A central corneal wound closes within 24 hours by epithelial cell migration and division, and during wound closure corneal epithelial cells express intercellular adhesion molecule (ICAM)-1 (CD54). We investigated the contributions of lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) by analyzing wound closure in mice with targeted deletions of CD11a (CD11a^{-/-}) or CD11b (CD11b^{-/-}). In contrast to CD11a^{-/-} mice, CD11b deficiency revealed a much greater delay in epithelial wound closure with >90% inhibition of epithelial cell division at a time when neutrophil accumulation in the cornea was approximately threefold higher than normal. Treating CD11b^{-/-} mice with anti-CD11a monoclonal antibody at the time of epithelial abrasion resulted in significant reductions in neutrophils and significant increases in corneal epithelial cell division and migration. Treating CD11b^{-/-} mice with anti-ICAM-1 significantly increased measures of healing but marginally reduced neutrophil influx. In conclusion, wound healing after corneal epithelial abrasion is disrupted by the absence of CD11b. The disruption is apparently linked to excessive neutrophil accumulation at a time when epithelial division is essential to wound repair, and neutrophils appear to be detrimental through processes involving LFA-1 and ICAM-1. (*Am J Pathol* 2006, 169:1590–1600; DOI: 10.2353/ajpath.2006.060415)

stroma of the cornea. These leukocytes emigrate from limbal vessels^{1,2} at the periphery of the cornea and migrate through the avascular stroma to the region of the wound.³ Keratocytes beneath the wounded epithelium rapidly undergo apoptosis,⁴ but in the absence of stromal injury, there is little if any fibrotic response.⁵ Under normal circumstances, re-epithelialization progresses rapidly,⁶ the numbers of infiltrating leukocytes return to baseline presumably as a result of apoptosis,⁷ and keratocytes repopulate the stroma beneath the repair.⁸

In earlier studies we demonstrated that central corneal epithelial abrasion in C57BL/6 mice resulted in two peaks of neutrophil infiltration, one at 12 to 18 hours after injury and the second at 30 to 36 hours after injury.⁹ The adhesion molecules required for these two peaks of infiltration appeared to differ because mice deficient in both P-selectin and E-selectin (P/E^{-/-}) were profoundly deficient in neutrophil influx, whereas mice deficient in CD18 (CD18^{-/-}) exhibited a single peak of emigration corresponding quantitatively and temporally to the second peak in wild-type mice. Closure of a central corneal epithelial wound in C57BL/6 wild-type mice was found to be complete within 24 hours. In CD18^{-/-} and P/E^{-/-} mice epithelial wound closure was delayed by ~12 to 24 hours. Given these observations and our data that neutropenic mice also exhibited delayed wound healing, it appears that early neutrophil infiltration facilitates corneal re-epithelialization.

Two members of the CD18 (β 2) integrin family, lymphocyte function-associated antigen (LFA)-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), are of principal importance in most functions of neutrophils.¹⁰ LFA-1-dependent adhesion is necessary for efficient emigration of neutrophils at sites of inflammation,^{11–14} and Mac-1-dependent adhesion enhances phagocytosis,^{15,16} exocytosis,¹⁷ and reactive oxygen production¹⁸ as well as

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Superficial wounds in corneal epithelium result in leukocyte infiltration into the avascular connective tissue

influences apoptotic pathways.^{19–22} The contributions of each integrin have been investigated with blocking antibodies, specific inhibitors, or in mice with targeted deletions of either integrin. In acute models of neutrophil-dependent tissue injury, removal or blocking of Mac-1 has been effective in reducing tissue damage,^{14,23–27} whereas in more complex models involving other leukocytes and adaptive immune processes, removal or blocking LFA-1 has been effective in reducing tissue pathology.^{14,28–30} In addition to the varying contributions of these two integrins in models with different pathological etiologies, differences in vascular beds appear as well. Given this perspective, we have been investigating the contributions of LFA-1 and Mac-1 in various models of disease. In this report we focus on wound healing in the cornea and provide evidence that in contrast to other models, removal of Mac-1 significantly delays wound healing through processes dependent on LFA-1 and intercellular adhesion molecule (ICAM)-1.

Materials and Methods

Animals

C57BL/6 mice were purchased from Harlan (Indianapolis, IN). CD11b^{−/−} mice,¹¹ CD11a^{−/−} mice,¹² and CD18^{−/−} mice⁹ were backcrossed as previously described at least 10 generations with C57BL/6 mice (Harlan). All mice used in this study were 6 to 8 weeks old, weighed 18 to 20 g, and were treated according to the Association for Research in Vision and Ophthalmology statement for the use of animals as well as institutional and federal guidelines.

Wound Model

The central corneal wound was performed as previously described.^{9,31} In brief, mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and using aseptic technique 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. Assessment of wound closure used fluorescein staining (sterile fluorescein solution followed by rinsing with sterile saline solution) of the ocular surface and digital analysis of stained area. Mice were anesthetized for the assessment of wound closure. Some mice were treated with anti-ICAM-1 monoclonal antibody (mAb) YN1 [American Type Culture Collection (Rockville, MD) number CRL-1878³²] or anti-CD11a mAb KBA as previously described¹¹ before corneal abrasion. At various times cornea tissues including the limbus were excised and processed for immunohistology, electron microscopy, and myeloperoxidase determination. Myeloperoxidase activity was measured in some mice at 24 hours after injury. Six corneas from WT mice and six from

CD11b^{−/−} mice were homogenized and sonicated in 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. After centrifugation at 14,000 × *g* for 5 minutes at 4°C, the supernatant fluids containing myeloperoxidase were incubated in a 50 mmol/L potassium phosphate buffer containing the substrate, H₂O₂ (1.5 mol/L), and *o*-dianisidine dihydrochloride (167 μg/ml; Sigma Aldrich, St. Louis, MO). The enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm.

Immunohistology

Wounded corneas were dissected (taking care to include the limbus), fixed, permeabilized, and incubated with labeled mAb as described.³¹ Anti-Gr-1-fluorescein isothiocyanate and anti-CD31/PECAM-1-phycoerythrin (PharMingen, La Jolla, CA) were selected for neutrophils and limbal vessels, respectively. Radial cuts were made in the cornea so that it could be flattened by a coverslip, and it was mounted in Airvol (Celanese Chemicals, Dallas, TX) containing 1 μmol/L 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical, St. Louis, MO) to assess nuclear morphology. Epithelial cell division was determined as previously described.^{9,31} Digital images were captured and saved for digital analysis (DeltaVision; Applied Precision, Issaquah, WA). To compare the relative level of neutrophils in the different areas from the limbus to the central cornea, each cornea was counted separately. 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 sclera as the most peripheral field for microscopic analysis (see Figures 1 and 2). A standard pattern for morphometric analysis was used throughout the study. Whole mounts were evaluated using a ×40 oil immersion lens to assess nine fields of view across the cornea from limbus to limbus. For neutrophils, a central frame covering 8% of the field of view was counted including all neutrophils throughout the depth of the cornea from the epithelial to endothelial surfaces (a range of ~90 μm). For epithelial cell division and epithelial cell density, cells per entire field of view were counted at the focal plane of the basal cell layer of the epithelium.

Electron Microscopy

Excised corneas were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (1 hour) followed by 1% tannic acid (5 minutes). Postfixation in 1% osmium tetroxide (1 hour) was followed by *en bloc* fixation in aqueous uranyl acetate. The samples were subsequently dehydrated in a graded ethanol series and embedded in LX 112 resin (Polysciences, Warrington, PA), and ultrathin transverse sections (80 nm) were obtained using an ultramicrotome (RMC 7000; RMC, Tucson, AZ) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before viewing with a JEOL 200CX electron microscope (Tokyo, Japan).

Figure 1. Murine cornea after central epithelial abrasion. **A:** Cross-section of limbal region showing vessels and infiltrating leukocytes at 2 hours after central epithelial abrasion. The region shown in this micrograph has not been directly injured. **B:** Limbal vessels with infiltrating neutrophils in whole mount preparations of cornea labeled with phycoerythrin anti-CD31 to identify the endothelium of the vessels and fluorescein isothiocyanate anti-Gr-1 to identify the leukocytes (>95% are neutrophils based on high-power examination of nuclear morphology seen by DAPI staining). Image captured using the DeltaVision deconvolution system (Applied Precision, Issaquah, WA). **C:** The stages of re-epithelialization revealed by fluorescein staining at 12, 18, and 24 hours after central epithelial abrasion. Note that by 24 hours wound closure appears complete in wild-type mice.

Statistical Analysis

Data analysis was performed using two-way repeated measures analysis of variance and pairwise multiple comparisons using Tukey's test. A *P* value of <0.05 was considered significant. Data are expressed as mean $\pm$ SEM.

Results

Central Corneal Epithelial Wound Closure in Wild-Type Mice Occurs within 24 Hours and Epithelial Cell Division Is Prominent in the Unwounded Epithelium between 18 and 36 Hours

Within 6 hours after wounding, neutrophils were evident in the extravascular spaces of the limbus (Figure 1, A and B). Fluorescein staining of the ocular surface demonstrated that re-epithelialization was evident within 24 hours (Figure 1C). For morphometric analysis of neutrophil migration and epithelial response to injury, we followed a pattern illustrated in Figure 2A. Whole mounts of the corneas were examined microscopically and neutrophils, basal endothelial cell density, and dividing epithelial cells were determined in the numbered regions. The epithelial basal cell nuclei (DAPI-stained) were visible in a uniform focal plain immediately above the stroma. Region 1 in Figure 2 indicates the limbus-containing blood vessels (the other regions are avascular corneal tissue), and regions 4 and 5 encompass the area within which the epithelium was removed. Region 3 represents a border

region between the wounded and unwounded tissue. The rate of wound closure was analyzed by two distinct approaches. The first, as shown in Figure 1C, was fluorescein staining of the corneal surface at repeated intervals until closure was evident. Figure 2B indicates the rate of closure. Because this analysis involves repeated anesthesia at 6-hour intervals there was concern regarding effects on the rate of closure. A second analysis of healing involved determination of epithelial basal cell density in region 4 in separate excised corneas prepared as whole mounts at different times after epithelial abrasion from animals not exposed to repeated anesthesia. As shown in Figure 2B, the increasing number of cells migrating into the wound reaches near maximum at 24 hours, consistent with the observations using fluorescein staining of the ocular surface. Epithelial division was most pronounced at 18 to 36 hours in the regions of the cornea that were not directly wounded (regions 1 to 3), and lower levels of epithelial division were observed in regions 4 and 5 after epithelial wound closure (Figure 2C). Thus, the observations in this murine model are consistent with current understanding of corneal epithelial wound healing observed in this and other species.^{33–35} Epithelial division occurs primarily in the peripheral regions of the cornea, and epithelial cells migrate to fill the abraded region.

Corneal Wound Healing Is Delayed in CD11a^{-/-} Mice

As indicators of re-epithelialization, fluorescein staining of the corneal surface and microscopic analysis of basal cell density were determined at various times after injury (Figure 3). In wild-type mice, the wound area was evident at 6 hours as indicated by the lack of epithelial cells in regions 4 and 5, the area of original abrasion. However, by 12 hours migrating epithelial cells were beginning to fill in the wound, and by 96 hours a relatively uniform density of epithelial cells was evident across the cornea. In wild-type mice 96 hours was sufficient time for epithelial density to return to unwounded levels. In contrast, wounds in CD11a^{-/-} mice exhibited delayed healing. At 12 hours in these mice, the wounded regions (regions 4 and 5) were still devoid of epithelium. By 24 hours, the wound areas contained epithelial cells, but their density was below that of wild-type mice at this time, and the fluorescein staining of the corneal surface was evident (Figure 3B). At 96 hours, basal cell density in CD11a^{-/-} mice lagged behind that of wild-type mice.

The Wound Healing Deficit in CD11b^{-/-} Mice Is Markedly Greater than in CD11a^{-/-} Mice

As shown in Figure 3, epithelial cells in CD11b^{-/-} corneas had not returned to region 5 (the center of the original wound) by 24 hours, and the density of epithelial cells in region 4 was significantly below the same region in CD11a^{-/-} mice. When individual mice were followed throughout several days by analyzing wound closure us-

Figure 2. Temporal relationship between epithelial wound closure and epithelial cell division in regions of the cornea. **A:** The schematic shows the pattern used in the morphometric analysis of whole mount corneas. The numbered areas indicate regions encompassed within a $\times 40$ microscopic field. **Region 1** includes the limbal vessels as seen in Figure 1, A and B. **Region 3** is an area of transition with varying degrees of wounding depending on the position of the trephine used to demarcate the area to be abraded. **Regions 1 to 3** include the corneal epithelium that is not directly wounded, and **regions 4 and 5** indicate the area where epithelium is removed by abrasion. **B:** This graph plots wound closure as percent open wound revealed by fluorescein staining of the ocular surface and the number of basal epithelial nuclei per $\times 40$ field of view in **region 4** at different times after central corneal abrasion. **C:** This graph plots epithelial cell division at different times after central corneal abrasion, distinguishing **regions 1 to 3** (epithelium not directly injured) and **regions 4 and 5** (where epithelium was initially removed by abrasion). The values given are the sum of dividing cells in regions. Some cell division is seen in the epithelium in **regions 4 and 5** after wound closure occurs. The **inset** shows images of dividing epithelial nuclei (DAPI stain, **arrows** indicate two such cells) in **region 2** at 18 hours after wounding.

ing fluorescein staining, $CD11a^{-/-}$ mice appeared to heal within ~ 30 to 36 hours. In contrast, $CD11b^{-/-}$ mice revealed markedly prolonged open wounds. In more than 50% of the mice, wounds appeared to begin closing around 42 hours after wounding but opened again and remained open for the duration of the observation (72 hours) (Figure 3). This was not seen in wild-type mice, $CD11a^{-/-}$ mice, or the other knockouts studied.⁹ This failure of wound closure is seen graphically in Figure 4A, where the density of epithelial cells in region 4 was plotted throughout time. It is evident that wound closure in $CD11b^{-/-}$ mice was disrupted between 18 and 24 hours after wounding. As will be shown in the next section, this is the period of time when neutrophil influx markedly increased in the $CD11b^{-/-}$ mice compared with either the wild-type or $CD11a^{-/-}$ mice.

Neutrophil Migration into the $CD11a^{-/-}$ Corneas Differs Significantly from that in $CD11b^{-/-}$ Corneas

Analysis of neutrophils arriving in the wound (ie, regions 4 and 5 in Figure 2A) revealed significant differences

among the three strains of mice studied (Figure 4B). In wild-type mice accumulation of neutrophils in the wound peaked within 12 to 18 hours, and in $CD11a^{-/-}$ mice neutrophil accumulation was significantly reduced and delayed, peaking at 30 hours. In contrast to $CD11a^{-/-}$ mice, $CD11b^{-/-}$ mice had neutrophil accumulation at the wound site equivalent to or exceeding that of wild-type mice, but in contrast to wild-type mice the peak accumulation was delayed until 24 to 30 hours after wounding (Figure 4B). In addition, accumulation of neutrophils within the stroma beneath the epithelial regions not directly wounded (regions 1 to 3) was greater than that of wild-type or $CD11a^{-/-}$ mice. This was most evident at 24 hours after wounding (Figure 5, A and B), a time at which re-epithelialization was reduced in the $CD11b^{-/-}$ mice (Figures 3A and 4A).

Peak Neutrophil Accumulation in $CD11b^{-/-}$ Corneas Corresponds Temporally with Depressed Epithelial Cell Division

Epithelial cell division was maximal in the wild-type cornea between 18 and 30 hours after wounding and was most

Figure 3. Corneal epithelial wound healing. **A:** Basal epithelial cell density across the cornea after central epithelial abrasion is shown in the panel of graphs for wild-type (WT), $CD11a^{-/-}$, and $CD11b^{-/-}$ mice. Analysis of the cell density in nine regions across the diameter of the cornea (see Figure 2A), with abrasion occurring in **regions 4 and 5**. Cell densities ($\pm$ SEM) are plotted for cornea without wounding (normal) and at different times after wounding up to 96 hours. The patterns of cell density across the cornea for $CD11a^{-/-}$ were significantly different from wild-type mice at 12 and 96 hours ($P < 0.01$, $n = 4$). The density patterns for $CD11b^{-/-}$ were significantly different from wild-type mice at 12, 24, and 96 hours ($P < 0.01$, $n = 4$) and significantly different from $CD11a^{-/-}$ at 12 hours ($P < 0.01$, $n = 4$). At 96 hours after wounding, basal epithelial cell density in the WT mice was not different from unwounded values. However, in both $CD11a^{-/-}$ and $CD11b^{-/-}$ mice, basal epithelial cell density was significantly ($P < 0.01$, $n = 4$) less than that of unwounded levels. To simplify the visual presentation of data, the variance was not indicated on the plots. **B:** Photographic images of ocular surfaces stained with fluorescein indicate re-epithelialization. Wound closure in $CD11a^{-/-}$ corneas ($n = 8$) was complete in all mice by 36 hours. The top two rows are typical examples from these animals. Wound closure in $CD11b^{-/-}$ mice ($n = 8$) was delayed beyond 48 hours, with 50% of the corneas beginning to close at ~42 to 48 hours but reopening for longer than 72 hours. The bottom three rows are representative of this pattern in the $CD11b^{-/-}$ mice. Each row represents a single mouse photographed at the times indicated.

prominent in the regions of the epithelium that were not wounded. Delayed wound healing in the $CD11b^{-/-}$ mice appeared to be reflected in reduced epithelial cell division in these regions of the cornea. A drop of greater than 80% occurred in epithelial cell division at 24 and 30 hours in the $CD11b^{-/-}$ mice (Figure 5C). This reduction in cell division corresponded to an approximate threefold rise (Figure 5A) during this time in the number of neutrophils (compared with neutrophil levels in wild-type mice) in the stroma beneath the epithelium in regions 1 to 3 (Figure 5, A and C) as determined by counting Gr-1⁺ neutrophils. An alternative assessment of total neutrophils in the entire cornea was reflected in the ratio of myeloperoxidase activity in $CD11b^{-/-}$ corneas to that in WT mice; this ratio was 2.36 (Figure 5B). $CD11b^{-/-}$ mice exhibited reciprocal changes in the number of neutrophils and epithelial cell division between 18 hours (when neutrophil influx was low and cell division was high) and 30 hours (when neutrophil influx was significantly higher and epithelial cell division was significantly lower) (Figure 5D).

Do LFA-1 and ICAM-1 Contribute to the Delayed Wound Healing in $CD11b^{-/-}$ Mice?

Because $CD11b^{-/-}$ neutrophils express functional CD11a/CD18 (LFA-1),¹¹ its possible contribution to delayed healing in the $CD11b^{-/-}$ mice was assessed. ICAM-1, a principal ligand for CD11a/CD18 (LFA-1) was examined in wild-type mice, and as reported by others,^{3,36,37} ICAM-1 was expressed on the limbal vessels. In addition, it was expressed by the corneal epithelial cells only after injury, initially in the limbus (Figure 6A) by 2 to 4 hours and then throughout the basal epithelium by 18 hours (Figure 6B). Figure 6, C and D, shows ICAM-1 staining of epithelial cells at the wound edge at 12 hours with adjacent neutrophils. In the absence of wounding, corneal epithelium was negative for binding of phycoerythrin-labeled anti-ICAM-1 mAb 3E2.³⁸ Neutrophils were abundant near the epithelial cell ICAM-1 (Figure 6D) and were observed in electron micrographs adjacent to

Figure 4. Kinetics of healing and neutrophil emigration. **A:** Changes in the density of basal epithelial cells in region 4 of the wounded cornea. This region is the area of the wound adjacent to unwounded epithelium. The value at 24 hours for CD11b^{-/-} mice compared with either wild-type or CD11a^{-/-} mice was significantly lower ($P < 0.01$). **B:** Neutrophils in the stroma beneath the area of the original wound (region 4 and 5). * $P < 0.01$ for the 24- and 30-hour time points for CD11b^{-/-} compared with either WT or CD11a^{-/-}; # $P < 0.01$ for 12- and 18-hour time points compared with either CD11b^{-/-} and CD11a^{-/-}.

epithelial cells on the stromal side and within the stratified epithelium (Figure 6E).

To directly assess possible contributions of CD11a and ICAM-1 to the delayed healing in the CD11b^{-/-} mice, CD11b^{-/-} mice were treated with mAb KBA (anti-CD11a) or mAb YN1 (anti-ICAM-1) immediately before epithelial abrasion, as we reported previously.¹¹ Corneas were evaluated at 24 hours, the time when epithelial cell division in regions 1 to 3 was significantly depressed in the CD11b^{-/-} mice (Figure 5C). Treatment with KBA resulted in a 64% reduction ($P < 0.01$) in neutrophil emigration (Figure 7A), whereas YN1 was without significant effect. However, both antibodies resulted in significant increases in the number of dividing epithelial cells at 24 hours after epithelial wounding (Figure 7A). KBA treatment increased epithelial division by 312% ($P < 0.01$), and YN1 increased division by 210% ($P < 0.01$). Additional studies were done with CD18^{-/-} mice and CD11a^{-/-} mice. As shown, neutrophil counts and epithelial cell division were not significantly different from CD11b^{-/-} mice treated with KBA, but they were significantly different from CD11b^{-/-} mice. Antibodies KBA and YN1 also increased the number of epithelial cells migrated into the wounded corneas at 24 hours (Figure 7B). KBA treatment increased epithelial density in region 4 by 310% ($P < 0.01$); YN1 increased density by 275% ($P < 0.01$). Thus, at 24 hours after wounding, YN1 treatment resulted in increases in both parameters of healing without significantly reducing neutrophils.

As we reported previously, CD18^{-/-} mice exhibited a pronounced emigration of neutrophils into the corneal stroma at 30 hours after wounding. As shown in Figure 8A, neutrophil levels in these corneas reached those of CD11b^{-/-} mice at 30 hours, although at 24 hours after wounding, CD11b^{-/-} corneas had approximately three-fold more neutrophils than CD18^{-/-} corneas. Thus, there

was approximately a 6-hour delay in the emigration of neutrophils in the CD18^{-/-} mice compared with the CD11b^{-/-} mice. Determination of epithelial cell division at 30 hours demonstrated a significant difference between CD11b^{-/-} and CD18^{-/-} mice (Figure 8B). Although neutrophil levels were comparable at 30 hours, epithelial cell division, in contrast to CD11b^{-/-} mice, was not significantly depressed. By 30 hours after wounding, YN1 treatment significantly reduced both neutrophils and parameters of healing, although less efficiently than KBA (Figure 8B).

These results are consistent with the conclusion that LFA-1 contributes to the delayed wound closure in CD11b^{-/-} mice. In addition, a principal ligand for LFA-1, ICAM-1, also contributes to the delayed wound closure in the CD11b^{-/-} mice. The principal effect of anti-LFA-1 seems to be the inhibition of neutrophil influx whereas that of anti-ICAM-1 seems to be protection of epithelial cell migration and division. Although CD18^{-/-} neutrophils can migrate into the corneal stroma in excess of wild-type neutrophils, epithelial cell division was not reduced.

Discussion

Our earlier studies of corneal wound healing in response to epithelial abrasion supported the conclusion that neutrophil emigration was required for efficient re-epithelialization.⁹ Inhibition of neutrophil migration into the cornea extended the healing time by 12 to 24 hours.⁹ The specific experiments included antibody-dependent depletion of circulating neutrophils in wild-type mice as well as analysis of healing in knockout mice (E-selectin- and P-selectin-deficient mice (E/P-sel^{-/-}) and CD18-deficient mice (CD18^{-/-}). All three experimental conditions

Figure 5. Kinetic patterns of neutrophil infiltration and epithelial cell division. **A:** Neutrophils in corneal regions 1 to 3 were determined in mice at various times after central abrasion, $*P < 0.01$ compared with values in abraded corneas of wild-type mice. **B:** Myeloperoxidase activity in whole corneal (including the limbus) extracts from wild-type (WT) and CD11b^{-/-} mice collected at 24 hours after central corneal epithelial abrasion ($n = 6$, $*P < 0.01$). **C:** Epithelial cell division was evaluated in regions 1 to 3 (unwounded area) in corneas from CD11b^{-/-} at various times after central abrasion, $*P < 0.01$ compared with values in abraded corneas of wild-type mice. **D:** Changes in neutrophil infiltration in regions 1 to 3 and epithelial cell division in regions 1 to 3 between 18 (open symbol) and 24 hours (closed symbol) after corneal abrasion in knockout mice, CD11b^{-/-}. For each time point, mean $\pm$ SEM of each parameter is plotted. The increases in neutrophils between 18 and 24 hours were significant ($P < 0.01$). The decreases in epithelial cell division were significant ($P < 0.01$).

resulted in profound reductions in neutrophil emigration within the first 24 hours after corneal wounding. Transfer of wild-type neutrophils into CD18^{-/-} mice significantly improved re-epithelialization and increased neutrophil

accumulation after corneal abrasion. These observations were consistent with studies in rabbits in which re-epithelialization of wounded rabbit corneas was also significantly delayed when leukocyte emigration was inhibit-

Figure 6. ICAM-1 and corneal epithelial wounds. Images of corneal epithelial wounds showing that corneal epithelial cells express ICAM-1. **A:** A low-magnification view of regions 1 to 3 (see Figure 2A) of a cornea 4 hours after central corneal abrasion. The whole mount preparation was labeled with phycoerythrin anti-ICAM-1 (clone 3E2) showing staining over the limbal region (left side of the photomicrograph) but not epithelium in regions 2 and 3 (see Figure 2A). **B:** Low-magnification view of regions 2 to 4 of a cornea at 18 hours after central corneal abrasion. **C:** The epithelial wound edge at 12 hours is shown with neutrophils in the open wound area. This focal plane reveals binding of phycoerythrin-labeled anti-ICAM-1 (red punctuate pattern on epithelial cells) and fluorescein isothiocyanate-labeled anti-Gr-1 (green staining of neutrophils). DAPI-stained (blue) epithelial nuclei are out of focus. **D:** In regions of the intact epithelium 12 hours after wounding, neutrophils (green) appear adjacent to the basal epithelial ICAM-1 (red) (Z-plane view). DAPI (blue) epithelial nuclei are visible. **E:** Transmission electron microscopic image of neutrophils adjacent to or within corneal epithelium 12 hours after epithelial abrasion injury. **Arrowheads** and **dotted line** indicate junction of stroma and basal epithelial cells. Scale bars in panels **A-D** = 15 μ m.

Figure 7. Effects of anti-ICAM-1 and anti-CD11a antibodies on re-epithelialization 24 hours after central epithelial abrasion. **A:** The graphs plot total neutrophil counts across the cornea and epithelial cell division in regions 1 to 3 at 24 hours after central corneal abrasion. CD11b^{-/-} mice were treated with either YN1 (anti-ICAM-1) or KBA (anti-CD11a) mAb, as indicated by the +YN1 and +KBA labels in this figure, at the time of central corneal abrasion. These parameters were also evaluated in CD18^{-/-} and CD11a^{-/-} mice. NS, not significant; **P* < 0.01. **B:** CD11b^{-/-} mice were treated with either YN1 (anti-ICAM-1) or KBA (anti-CD11a) mAb at the time of central corneal abrasion, and epithelial basal cell density in region 4 was assessed at 24 hours after wounding. **Closed diamond**, data from untreated CD11b^{-/-} corneas; **open circle**, data from CD11b^{-/-} mice treated with anti-ICAM-1 monoclonal antibody YN1; **open diamond**, data from CD11b^{-/-} mice treated with anti-CD11a monoclonal antibody KBA. Mean ± SEM. Anti-CD11a (KBA) treatment significantly affected both neutrophil numbers and epithelial cell density when compared with CD11b^{-/-} (*P* < 0.01); anti-ICAM-1 (YN1) significantly affected only the epithelial cell density. NS, not significant.

ed.³⁹ Our results in the current report indicate that LFA-1 (CD11a/CD18) is required for normal early kinetics of neutrophil emigration into the cornea, and, consistent with other conditions that reduce neutrophil emigration, corneal re-epithelialization was delayed by 12 to 24 hours. Thus, several distinct experimental conditions reveal delayed corneal epithelial wound closure when neutrophils fail to emigrate within the first 12 to 18 hours after abrasion.

The contributions of neutrophils to healing are unknown at present, but some possible issues are of interest. Neutrophils arrive at the open wound before epithelial cell migration into the wound and may participate in debridement of the stromal surface, thereby facilitating epithelial adhesion. Corneal epithelium is normally renewed by division in the basal cell layer⁴⁰ or from stem cells within the region of the limbus.^{41–43} The stem cells in the limbus appear to be sensitive to injury and provide expansion of basal epithelial cells necessary for wound coverage. Various endogenous growth factors appear to be involved,^{2,40} including transforming growth factor- β , hepatocyte growth factor, insulin-like growth factor-1, insulin-like growth factor-2, epidermal growth factor, and platelet-derived growth factor.^{44–49} One possible mechanism by which the accumulating leukocytes contribute to re-epithelialization is the delivery of growth factors to the limbus, the site of important stem cells thought to be important for healing. Grenier and colleagues⁵⁰ reported

that hepatocyte growth factor is stored in the secretory and secondary granules of the neutrophils and released in an active form on neutrophil degranulation. Exocytosis of these classes of neutrophil granules occurs rapidly after activation by chemokines⁵¹ shown to be present in wounded corneas within the time frame of leukocyte accumulation.⁵² Another neutrophil-derived factor of potential importance is CAP37, which was shown by Pereira and colleagues⁵³ to augment corneal epithelial cell migration and proliferation. Thus, it is possible that the leukocyte functions extend beyond scavenging.

Although delayed or absent neutrophil emigration is associated with delayed healing, wound closure occurred, extending the time for re-epithelialization by ~50 to 100%. In contrast, observations in the CD11b^{-/-}-deficient mice indicate that altered kinetics of neutrophil emigration may produce more extended disruptions in corneal wound closure. It is important to emphasize that CD11b^{-/-} mice exhibited differences from the knockout of all members of the CD18 (β 2) integrin family.⁹ Possible explanations may be derived from the following observations. In the CD11b^{-/-} mice, 1) neutrophil accumulation in the corneal tissue greatly exceeded that of wild-type mice at a time when epithelial division in response to the abrasion was normally most active (18 to 30 hours), and this increase in neutrophils coincided with a marked drop in epithelial cell division; 2) anti-CD11a monoclonal antibody KBA administered before corneal abrasion pre-

Figure 8. Effects of anti-ICAM-1 and anti-CD11a antibodies on re-epithelialization 30 hours after central epithelial abrasion. **A:** Neutrophils in the corneal tissues of wild-type, CD11b^{-/-} mice, and CD18^{-/-} mice. The values are the sum of counts in nine microscopic fields across the cornea as illustrated in Figure 2. *n* = 4. **P* < 0.01, CD11b^{-/-} compared with either wild-type or CD18^{-/-} mice at this time. All values of CD11b^{-/-} and CD18^{-/-} from 24 hours to 48 hours were significantly greater (*P* < 0.01) than wild type. **B:** CD11b^{-/-} mice were treated with either YN1 (anti-ICAM-1) or KBA (anti-CD11a) mAb, as indicated by the +YN1 and +KBA labels in this figure, at the time of central corneal abrasion. Neutrophil levels and epithelial cell division (the sum of counts in nine microscopic fields across the cornea as illustrated in Figure 2) were assessed at 30 hours after wounding. The values given for the CD18^{-/-} mice were also collected at 30 hours after wounding. **P* < 0.01 compared with CD11b^{-/-} +KBA but not significantly different from CD11b^{-/-}. ***P* < 0.01 compared with CD11b^{-/-} but not significantly different from CD11b^{-/-} +KBA.

vented the excessive accumulation of neutrophils in the CD11b^{-/-} mice; 3) ICAM-1 (a primary ligand for LFA-1) was expressed on both limbal endothelium and corneal basal epithelial cells; and 4) administration of either anti-CD11a or anti-ICAM-1 monoclonal antibodies significantly enhanced epithelial cell migration into the wound space and epithelial cell division.

There seems to be two broad functional contributions of LFA-1 to the extended delay of wound closure in the CD11b^{-/-} mice. The first is that LFA-1 is necessary for CD11b^{-/-} neutrophil migration into and through the corneal tissue. This function is consistent with our previous observations of peritoneal inflammation in the CD11b^{-/-} mice.¹¹ In that model, neutrophil emigration into the peritoneal cavity of CD11b^{-/-} mice occurred at a rate equivalent to wild-type mice, and anti-CD11a antibody KBA was significantly inhibitory. Studies in CD11a^{-/-} mice are also consistent with a major role for LFA-1 in the emigration of neutrophils.^{12,13} Our observations in CD18^{-/-} mice are of potential interest because there appears to be inconsistency with the results of treating CD11b^{-/-} mice with KBA (anti-CD11a). At 30 hours after wounding, KBA resulted in marked reductions of neutrophils in CD11b^{-/-} mice while neutrophil emigration in the CD18^{-/-} mice at this time was equivalent to the levels in CD11b^{-/-} without KBA treatment. Others have observed

CD18-independent extravasation of neutrophils in CD18^{-/-} mice,^{54,55} suggesting that these animals may have developed compensatory mechanisms or that the marked leukocytosis of these animals permits significant leukocyte accumulation via residual migratory mechanisms of low efficiency. Assuming that LFA-1-dependent adhesion is a primary mechanism for neutrophil extravasation, Mac-1 deficiency may contribute to extended accumulation of neutrophils in tissue by delaying apoptosis.^{14,20} We observed an approximately threefold increase in neutrophils at a time and in an area of the cornea (regions 1 to 3) where epithelial cell division is necessary for effective wound closure. This is of interest in our effort to understand the mechanisms of delayed healing in the CD11b^{-/-} mice. Migration of neutrophils to the region of the cornea beneath the wound was significantly reduced in the CD11a^{-/-} (LFA-1-deficient) mice, and anti-CD11a monoclonal antibody KBA significantly reduced the numbers of stromal neutrophils in the CD11b^{-/-} mice. The data indicate a role for LFA-1 in the migration of neutrophils into the corneal tissue.

A second functional contribution is that LFA-1 allows adhesion of neutrophils to ICAM-1-expressing epithelial cells. Support for possible LFA-1-dependent adhesion to epithelial cells is that anti-ICAM-1 was more effective in enhancing the parameters of healing (ie, epithelial cell

division and migration) than it was in inhibiting the emigration of neutrophils (most clearly seen in Figure 7). Neutrophil emigration through vascular endothelium is known to be supported by LFA-1,¹¹ but LFA-1 interactions with ligands other than ICAM-1 can support emigration (eg, JAM-A^{56,57} and ICAM-2⁵⁸). We have found (unpublished data) that limbal vessels express both JAM-A and ICAM-2. ICAM-2 may contribute to leukocyte infiltration into the cornea.⁵⁹ Basal corneal epithelial cells not directly damaged by the central corneal abrasion express ICAM-1 and may be susceptible to direct neutrophil adhesive interactions with LFA-1. LFA-1 on neutrophils has been shown to support reactive oxygen production by binding to ICAM-1.⁶⁰ Also possibly supportive of this role for LFA-1 in the corneal tissue is our finding of high neutrophil counts in the tissues of CD11b^{-/-} mice at 30 hours associated with high levels of epithelial cell division. This contrasts with observations in the CD11b^{-/-} mice in which LFA-1 was still active and epithelial cell division was low but is consistent with the observations that epithelial cell division was high with antibody KBA treatment (Figure 8B).

Our results in this and a previous report are consistent with the following model of neutrophil involvement in corneal wound healing after epithelial abrasion. Neutrophil emigration within the first 12 to 18 hours after corneal epithelial injury is necessary for efficient re-epithelialization. This peak of emigration precedes the wave of epithelial division that is necessary for wound coverage. Because neutrophils can release growth factors to which epithelium is known to respond, it is interesting to speculate that this delivery of growth factors promotes healing. In contrast, when leukocyte accumulation is dysregulated, as is the case in the absence of CD11b (ie, the absence of the adhesion molecule Mac-1), excessive accumulation of neutrophils in a region where epithelial division and migration are essential is detrimental to epithelial repair through processes involving LFA-1 and ICAM-1. Given the unique environment (ie, avascular cornea) in which neutrophil emigration and wound healing are occurring, this model is unlikely to reflect a general paradigm for other tissues.

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