Cell Injury, Repair, Aging, and Apoptosis

NK Cells Modulate the Inflammatory Response to Corneal Epithelial Abrasion and Thereby Support Wound Healing

Qiong Liu,* C. Wayne Smith,* Wanyu Zhang,[†]
Alan R. Burns,*[†] and Zhijie Li*[‡]

From the Section of Leukocyte Biology, Department of Pediatrics,*
Children's Nutrition Research Center, Baylor College of Medicine,
Houston, Texas; the College of Optometry,[†] University of Houston,
Houston, Texas; and the Key Laboratory for Regenerative
Medicine,[‡] Ministry of Education, and Department of
Ophthalmology, Jinan University, Guangzhou, China

Natural killer (NK) cells are lymphocytes of the innate immune system that have crucial cytotoxic and regulatory roles in adaptive immunity and inflammation. Herein, we consider a role for these cells in corneal wound healing. After a 2-mm central epithelial abrasion of the mouse cornea, a subset of classic NK cells migrated into the limbus and corneal stroma, peaking at 24 hours with an eightfold increase over baseline. Depletion of $\gamma\delta$ T cells significantly reduced NK cell accumulation (>70%; $P < 0.01$); however, in neutrophil-depleted animals, NK cell influx was normal. Isolated spleen NK cells migrated to the wounded cornea, and this migration was reduced by greater than 60% ($P < 0.01$) by *ex vivo* antibody blocking of NK cell CXCR3 or CCR2. Antibody-induced depletion of NK cells significantly altered the inflammatory reaction to corneal wounding, as evidenced by a 114% increase ($P < 0.01$) in neutrophil influx at a time when acute inflammation is normally waning. Functional blocking of NKG2D, an activating receptor for NK cell cytotoxicity and cytokine secretion, did not inhibit NK cell immigration, but significantly increased neutrophil influx. Consistent with excessive neutrophil accumulation, NK depletion and blocking of NKG2D also inhibited corneal nerve regeneration and epithelial healing ($P < 0.01$). Findings of this study suggest that NK cells are actively involved in corneal healing by limiting the innate acute inflammatory reaction to corneal wounding. *(Am J Pathol 2012, 181:452–462; http://dx.doi.org/10.1016/j.ajpath.2012.04.010)*

Corneal epithelium is critically important to the integrity of the cornea, serving as a physical and chemical barrier against infection and providing physiologic and biochemical mechanisms necessary for sustained visual clarity. The cornea contains resident leukocytes[1–9] of the innate immune system prominently in the peripheral regions of the cornea,[4,7,10] and an abundance of sensory nerves arrayed in a dense plexus just beneath the basal epithelial layer.[11–13] Because superficial corneal wounds can readily damage the epithelium and the subbasal nerves, complete healing depends not only on re-epithelialization but on regeneration of the subbasal nerve plexus. Animal models of epithelial abrasion reveal an acute inflammatory response of the limbal blood vessels and substantial accumulation of leukocytes within the avascular dense connective tissue stroma and the stratified epithelium. Neutrophils migrate to corneal wounds predominantly within the anterior stroma,[3,7] whereas lymphocytes, macrophages, and dendritic cells migrate within both stroma and stratified epithelium.[7,10,14,15] Platelets also accumulate in the limbus within and outside of limbal vessels.[16,17]

Current evidence indicates that neutrophils are necessary for efficient epithelial healing and sensory nerve regeneration. Experimental interventions that markedly reduce neutrophil influx significantly delay epithelial wound closure and the recovery of subbasal nerves.[16–18] One direct contribution of neutrophils and platelets to wound healing seems to be the release of vascular endothelial growth factor A, a trophic factor for neurite generation,[19–24] in the anterior stroma adjacent to the healing tissues.[17] Vascular endothelial growth factor A and other potentially important growth factors are packaged in neutrophil granules[25–29] available for rapid release in the

tissue. In contrast to a direct role of neutrophils in healing, dysregulation of neutrophil influx with prolonged and increased presence in the tissue has a negative influence on corneal wound healing,[30] consistent with the well-known ability of neutrophils to induce direct tissue injury. Herein, we analyze a mechanism that apparently limits the tenure of neutrophils in the cornea, possibly tipping the balance of inflammation in favor of wound healing. Regarding adaptive immune responses, NK cells have important regulatory roles; however, a role for NK cells in resolution of innate acute inflammation has not been defined. We provide evidence that after epithelial abrasion, a subset of classic NK cells migrates into the corneal stroma in response to locally generated chemokines, and serves to limit the acute inflammatory process.

Materials and Methods

Animals

Female C57BL/6 and TCRδ$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Icam-1*$^{-/-}$, *Cd11b*$^{-/-}$, and *Cd11a*$^{-/-}$ mice were backcrossed, as previously described, at least 10 generations with C57BL/6 mice.[30,31] All animals used in the present study were aged 8 to 12 weeks, and 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 the Baylor College of Medicine Animal Care and Use Committee policy.

Antibodies

The following antibodies were used in the present study: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-NKp46 (Rat IgG2A, clone 29A1.4), Alexa Fluor 700-conjugated anti-CXCR3, purified anti-NKG2D (Rat IgG2b, clone 191004), and anti-β-III tubulin (Mouse IgG2a, clone TuJ-1) (all from R&D Systems, Inc., Minneapolis, MN); purified anti-CXCR3 (Rat IgG2a, clone CXCR3-173; Abcam, Inc., Cambridge, MA); PE-conjugated anti-CD94 (Rat IgG2a, clone 18D2), purified anti-NK1.1 (Mouse IgG2a, clone PK136), and PE-conjugated anti-RORγt (Rat IgG, clone B2D clone) (all from eBioscience, Inc., San Diego, CA); allophycocyanin (APC)-conjugated anti-CD31 (Rat IgG2a, clone MEC 13.3), FITC-conjugated anti-GL3 (Hamster IgG2), PE-conjugated anti-NK1.1, PE-conjugated anti-eomesodermin (EOMES), PE-conjugated anti-CD127, APC-conjugated anti-NKG2D, APC-conjugated anti-CD3, purified anti-Ly6G (Rat IgG2a, clone 1A8), purified anti-TCRδ (Hamster IgG2, clone GL3), purified anti-MCP-1 (Rat IgG2b, clone 123616), and purified anti-IP-10 (Rat IgG2a, clone 134013) (all from BD Pharmingen, Franklin Lakes, NJ); and purified anti-asialo GM1 (Rabbit IgG, IgA, and IgM; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Corneal Wound Healing Model

Central corneal abrasion was performed as previously described.[18] In brief, animals were anesthetized using sodium phenobarbital (Nembutal, 40 mg/kg), and the central epithelium was demarcated using a 2-mm trephine and removed using a golf club spud for refractive surgery (Accutome, Inc., Malvern, PA) under a dissecting microscope. Some animals received topical anti–MCP-1 [100 μg/mL (10 μL)] or anti–IP-10 [100 μg/mL (10 μL)] dissolved in lubricating artificial tears (Advanced Medical Optics, Inc., Santa Ana, CA) once every 4 hours for 24 hours. To inhibit neutrophil and γδ T-cell influx, at 24 hours before epithelial abrasion, some animals received a single i.p. injection of anti-Ly6G (1 mg) or anti-GL3 (1 mg) prepared in 1 mL PBS. At 1 hour before epithelial abrasion, one group of animals received an i.p. injection of rIL-17A (1 mg; R&D Systems). Wound healing rate was measured using two methods: counting of basal epithelial cell division, as previously described,[15,16] and photographing of the ocular surface at a known scale and calculating the surface area of the wound at each time point, as previously described.[32] At various times after injury, the animals were sacrificed, and the anterior segment of the eye was excised. Then the iris and lens were removed, and the cornea was processed for immunohistologic analysis, enzyme-linked immunosorbent assay (ELISA), or proteomics array. Image analysis and quantification of wounded corneas were performed using a DeltaVision microscope (Applied Precision, Inc., Issaquah, WA), as previously described.[17] For documentation of corneal nerve regeneration, subbasal nerve density was assessed as previously described.[18] In brief, whole mounts were evaluated using a ×40 oil immersion lens to assess each field of view (150 × 150 μm) in the paralimbal region and wound margin. Each field was digitally captured for analysis as a projected image of a 30-μm deep deconvolved Z-stack (0.3-μm increments) encompassing the corneal epithelium, the subbasal nerve plexus, and an adjacent portion of the corneal stroma. Systematic uniform random sampling was used to prevent observer sampling bias. Digital images of tubulin III-positive nerve fibers within the field were analyzed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) on a standard point-counting grid to obtain estimates of subbasal nerve density.

Adoptive Transfer of NK Cells

To assess the factors involved in NK cell migration into the cornea, NK cells were first isolated from the spleen of wild-type or *Cd11a*$^{-/-}$ mice. The spleen was teased into pieces using forceps, minced, and filtered through a 40-μm pore cell strainer (BD Falcon; BD Bioscience, Inc., San Diego, CA). NK cells were first negatively isolated using an NK cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), then incubated for 30 minutes at 37°C in serum-free DMEM (Dulbecco's modified Eagle's medium) with anti-mouse CXCR3 (10 μg/mL), anti-mouse NKG2D-blocking antibodies (10 μg/mL), or IgG control (rat IgG, 10 μg/mL) and labeled with PKH67 (5 μmol/L;

Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's instructions, followed by washing twice with DMEM supplemented with 10% fetal calf serum antibiotics (Gibco penicillin-streptomycin; Invitrogen Corp., Carlsbad, CA). The NKG2D antibody serves two distinct experimental roles: it represents a binding antibody because NKG2D should have no role in the emigration of NK cells into tissue, and it serves as a blocking antibody to inhibit the cytotoxic role of the NKG2D receptor.[33] At 24 hours after NK cell depletion with anti-asialo GM1, corneal abrasion was performed in the recipient mice, followed by reconstitution of NK cells (i.v.) using approximately 2×10^6 freshly isolated donor NK cells. The number of PKH67 and NKp46$^+$ cells in the cornea was recorded at 24 to 30 hours after epithelial wounding.

Flow Cytometric Analysis

Freshly isolated NK cell preparations were incubated with fluorescent-labeled antibodies on ice for 20 minutes, washed twice with cold PBS, and resuspended in lysis buffer with fixative (Becton-Dickinson, Franklin Lakes, NJ). Fluorescence of surface markers was recorded from 30,000 events occurring within standardized flow and fluorescence parameters using a FACS LSRII (Becton Dickinson), and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Cell viability was assessed using 7-AAD (eBioscience), and labeled, nonbinding, isotype-matched antibodies served as controls.

Immunofluorescence and Deconvolution Imaging

Immunostaining was performed on whole mount corneas as described previously.[17,18] In brief, normal or wounded corneas with the complete limbus were dissected, fixed with 2% paraformaldehyde, permeabilized using 0.1% Triton X-100/1% bovine serum albumin for 20 minutes, and incubated with the fluorescent-labeled antibodies overnight at 4°C. Each step was followed by three washes with PBS. Radial cuts were made in the cornea so that it could be flattened under a coverslip, and the cornea was mounted in Airvol 205 (Celenase Corp., Dallas, TX), containing 1 μmol/L DAPI (Sigma-Aldrich Corp.) for nuclear staining. Image analysis and quantification of wounded corneas were performed using a DeltaVision microscope (Applied Precision) as described previously.[17,18] Controls using isotype- and species-matched antibodies were in all cases negative.

Proteomics Array and ELISA

Normal or wounded corneas with limbus intact were collected at 18 hours after injury and cultured with serum-free DMEM for 12 hours. The culture supernatants were collected and stored at −80°C until use. The proteomics array was performed using a Proteome Profiler Array panel (R&D Systems) following the manufacturer's instructions.

To confirm the results of the proteomics array, the corneas were collected at the end of culturing, and were digested using 500 μL radioimmunoprecipitation buffer containing DNase and protease inhibitor cocktail. The samples were stored at −80°C until use. The concentrations of IP-10 and CCL7 (both from eBioscience) and MCP-1 (R&D Systems) from corneal lysates were measured using an ELISA following the manufacturer's instructions. Four corneas were pooled in 0.5 mL buffer. All samples were homogenized for 30 seconds at 250 Hz. All homogenates were frozen at −80°C until assayed.

Data Analysis

Data analysis was performed using analysis of variance and pairwise multiple comparisons using the Bonferroni test. $P < 0.05$ was considered significant. Data are given as means ± SD.

Results

NK Cells in the Cornea

The epithelial abrasion model used in the present study induces an acute inflammatory response at the ocular surface that involves recruitment of neutrophils, platelets, $\gamma\delta$ T cells, and alterations in inflammatory cytokines.[7,15,16] Tissues were analyzed for additional cell types that appear or change in response to injury. Using a defined morphometric approach to analyzing cells in the cornea, corneal whole mounts were stained with anti-CD31 to identify the limbal vessel endothelium, DAPI to enable recognition of the basal epithelial layer, and specific antibodies for distinct leukocyte subsets. At various times after central epithelial abrasion, corneas were labeled with anti-NKp46, a marker selective for NK cells in mice,[34–36] and were microscopically assessed for positive cells in the epithelium and stroma within the limbus, paralimbus, parawound, and center of the cornea. NKp46$^+$ cells were rarely found in the epithelium at all times examined up to 48 hours after wounding. NKp46$^+$ cells were also rare in the stromal limbus of unwounded corneas, but were markedly increased at later times, prominently around the limbal vessels (Figure 1A). The peak accumulation of these cells occurred at 24 hours after wounding (Figure 2A), and they extended in the stroma from the limbus to the center of the cornea (Figure 2B).

Other potential markers were used to evaluate the phenotype of corneal NKp46$^+$ cells. To differentiate NK T cells from NK cells, corneas were co-stained with anti-NKp46 and anti-CD3, a pan T-cell marker. Double-positive cells were rare (<3%), indicating that the immigrant cells were likely NK cells (Figure 1B), further supported by the finding that they were positive for EOMES (Figure 1C), a transcription factor that is prominently expressed in NK cells.[37] To determine whether these cells were similar to a subset of NK-like innate lymphoid cells found in some mucous membranes and the intestinal lamina propria, NKp46$^+$ cells were co-stained for RORγt (a transcription factor) and IL-22.[38–40] They were negative for these factors (Figure 1, D and E). We have previously
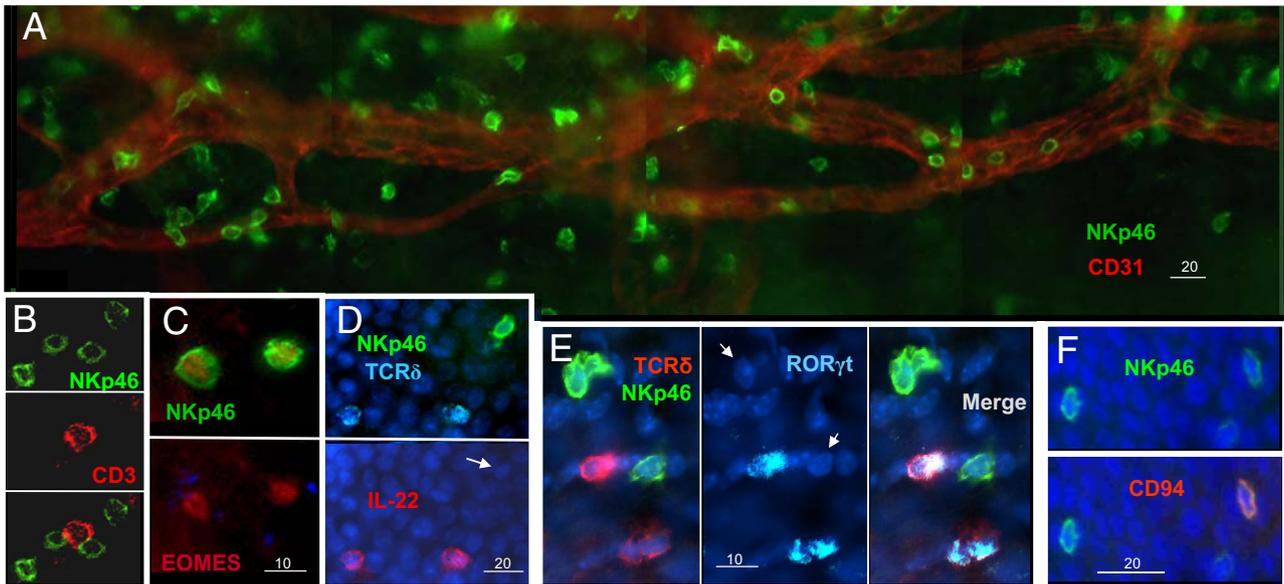
Figure 1. NKp46$^+$ cells in the mouse cornea. **A:** At 24 hours after central corneal epithelial abrasion, corneal whole mounts were prepared for microscopic analysis using a DeltaVision microscope (Applied Precision). A montage of images (30-μm deep Z-stack, 0.3-μm increments) showing limbal vessels stained with anti-CD31-APC and cells positive for anti-NKp46-FITC. The NKp46$^+$ cells were negative for anti-CD3-PE (**B**), positive for anti-EOMES-PE (**C**), and negative for anti-IL-22-PE (**D, arrow**). The NKp46$^+$ cells were negative for anti-RORγt-APC (**E, arrows**), although cells stained with GL3 (**D** and **E**) (ie, $\gamma\delta$ T cells) in the same tissue were positive for IL-22, RORγt, and CD94 (**F**). In normal corneas, the NKp46$^+$ cells were occasionally positive for anti-CD94.

reported that the only cells in the cornea within 48 hours after epithelial abrasion positive for RORγt and IL-22 are $\gamma\delta$ T cells.[7,31] CD127, IL-7 receptor-α (rIL-7A), was negative in corneal NKp46$^+$ cells (data not shown), with or without injury, which suggests that corneal NK cells were not developmentally derived from the thymus.[41] NK cells in the uninjured cornea included both CD94$^+$ and CD94$-$ subtypes (Figure 1F), indicative of functionally distinct mouse subsets.[42] CD94 is a protein associated with the NK cell inhibitory receptor NKG2A.[43] However, at 24 hours after injury, greater than 90% of the NKp46$^+$ cells were positive for CD94. The NKp46$^+$ cells were consistently positive for NKG2D, an activating NK cell receptor of the NKG2 family,[44] and NK1.1 (NKRP1C), an activating NK cell receptor of the NKRP1 family,[45] receptors known to signal enhanced NK cell-dependent cytotoxicity and cytokine release. Thus, the NKp46$^+$ cells that accumulate in the cornea within 24 hours after epithelial abrasion are apparently classic NK cells[34–36] of the CD94$^+$ subset.[42]

Immigration of NK Cells Is Inhibited by Blocking $\gamma\delta$ T Cells and Adhesion Molecules

After corneal abrasion, $\gamma\delta$ T cells rapidly migrate into the stroma and epithelium, and significantly contribute to the inflammatory response.[7] Mice deficient in $\gamma\delta$ T cells have substantially reduced neutrophil influx and wound healing.[7,18] We assessed their possible influence on NK cell accumulation using two models, TCR$\delta^{-/-}$ mice and wild-type mice treated systemically with $\gamma\delta$ T-cell receptor blocking antibody GL3. In TCR$\delta^{-/-}$ mice, the number of NK cells in the cornea did not change significantly with injury, whereas in the control wild-type mice, the number of NK cells increased significantly (Figure 2, C and D). Antibody GL3 treatment before epithelial abrasion significantly inhib-

ited greater than 90% of the increase in NK cell accumulation, and greater than 50% if administered 14 hours after abrasion (Figure 2C). These results indicate that $\gamma\delta$ T cells in the early phase of the inflammatory response are necessary for the increase in NK cells (Figure 2D). Conversely, depleting NK cells by systemic administration of anti-NK1.1 did not affect the influx of $\gamma\delta$ T cells into the cornea (Figure 2E), indicating that NK cells are not necessary for trafficking of $\gamma\delta$ T cells in this tissue.

Neutrophil influx into the corneal stroma peaks 12 hours earlier than the NK cells.[16,30] We sought to determine whether neutrophil influx was a necessary condition for the increase in NK cells. Anti-Ly6G depletes neutrophils for approximately 3 days,[17] and was given i.p. at 24 hours before corneal abrasion. This treatment did not significantly inhibit the increase in NK cells (Figure 2F). Inducing an inflammatory response in the corneas via topical administration of rIL-17A to TCR$\delta^{-/-}$ mice failed to increase NK cells, although this treatment significantly increases the number of neutrophils immigrating after corneal injury.[17]

To assess possible mechanisms for extravasation of NK cells into the corneal stroma, we analyzed contributions of β2 integrin leukocyte adhesion molecules, given that the NK cells in the stroma after corneal abrasion were positive for both CD11a and CD11b (data not shown). Corneas from mice deficient in ICAM-1 (*Icam-1$^{-/-}$*), LFA-1 (CD11a$^{-/-}$), and Mac-1 (*Cd11b$^{-/-}$*) were evaluated at 24 hours after corneal abrasion. The total number of corneal NK cells was significantly lower than in wild-type mice (Figure 2F), which suggests that ICAM-1 (CD54), LFA-1 (CD11a/CD18), and Mac-1 (CD11b/CD18) expression is essential for NK cell migration into the cornea. The contribution of LFA-1 on NK cells was further confirmed using an adoptive transfer experiment in which NK cells were isolated from the spleen of CD11a$^{-/-}$ mice. The adop-
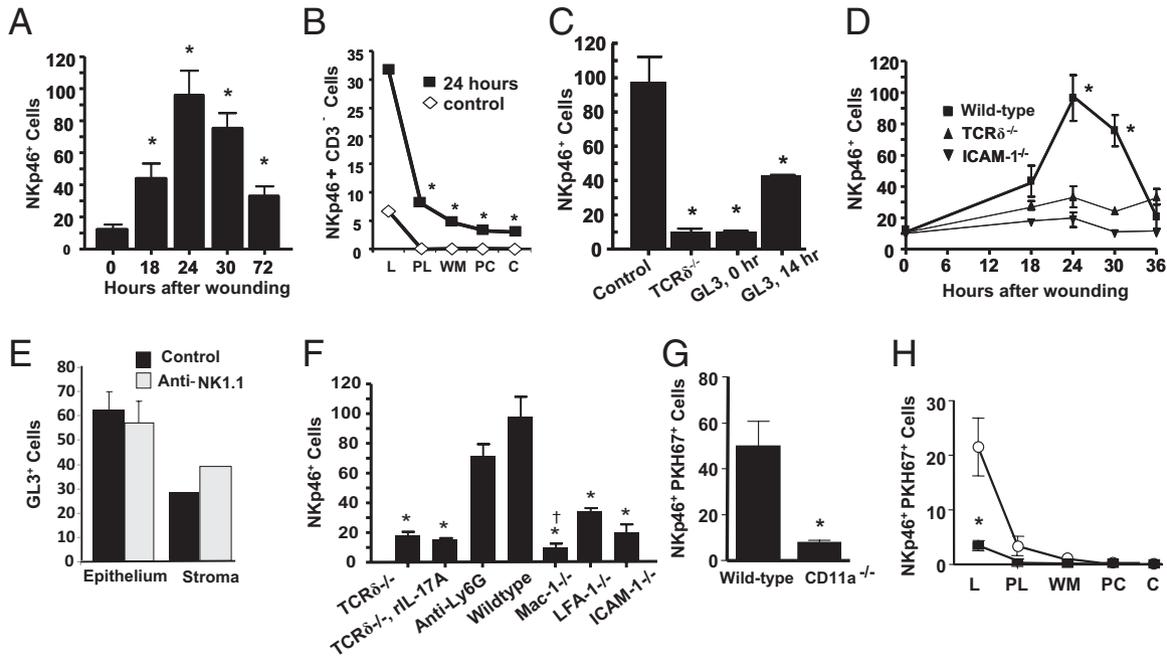
Figure 2. Factors that affect NK cell accumulation in corneas after central corneal epithelial abrasion. **A:** Time course of NKp46$^+$ cell accumulation, plotting total cells counted in the stroma in nine 150 × 150-μm microscopic fields across the cornea. **B:** Distribution of NKp46$^+$ cells in the corneal stroma at 24 hours after injury. C, center; L, limbus; PC, paracenter; PL, paralimbus; WM, wound margin. **C:** TCR$\delta^{-/-}$ mice had significantly fewer NKp46$^+$ cells at 24 hours after injury than did control wild-type mice. Treatment of wild-type mice with antibody GL3 before epithelial abrasion also inhibited accumulation of these cells. Treatment of wild-type mice with GL3 at 14 hours after injury was less inhibitory. **D:** TCR$\delta^{-/-}$ and *Icam-1$^{-/-}$* mice failed to accumulate NKp46$^+$ cells within the time frame of influx of these cells in wild-type mice. **E:** NKp46$^+$ cell influx was counted at 24 hours after epithelial abrasion. Depletion of neutrophils with anti-Ly6G failed to prevent NKp46$^+$ cell influx, and systemic treatment of TCR$\delta^{-/-}$ mice with rIL-17A failed to increase NKp46$^+$ cells, although this cytokine has previously been reported to significantly increase neutrophils. NKp46$^+$ cell influx was significantly reduced in mice deficient in CD11b (*Mac-1$^{-/-}$*), CD11a (CD11a$^{-/-}$), and ICAM-1 (*ICAM-1$^{-/-}$*). **F:** Anti-NK1.1 was used to treat mice before epithelial abrasion. At 24 hours after abrasion, corneas were labeled with antibody GL3 to detect $\gamma\delta$ T cells. GL3$^+$ cells were not statistically different from those in control IgG-treated mice. **G:** The number of adoptively transferred NKp46$^+$PKH67$^+$ cells from LFA-1$^{-/-}$ (*Cd11a$^{-/-}$*) mice that migrated into the corneal stroma at 30 hours after epithelial abrasion was significantly decreased when compared with adoptively transferred wild-type NK cells. **H:** Distribution in the stroma at 30 hours after epithelial abrasion from limbus to the corneal center in wild-type mice of adoptively transferred NKp46+PKH67+ spleen NK cells from wild-type and *Cd11a$^{-/-}$* mice. *$P < 0.01$ versus wild-type control mice; $^\dagger P < 0.01$ versus LFA-1$^{-/-}$ mice; $n = 4$ to 6 per condition; mean ± SD.

tive transfer experiment assessed the effect of LFA-1 on NK cells more selectively because LFA-1 is universally expressed on leukocytes.[46] Consistent with the experiment in CD11a$^{-/-}$ mice, LFA-1–deficient NK cells also showed diminished migration at 30 hours after injury (Figure 2, G and H).

Migration of NK Cells Depends on CCR2 and CXCR3

To begin analyzing chemokine influences on NK cell migration into the cornea, a profile of chemokines released by injured corneas was studied using an inflammatory cytokine array. In this experiment, the corneas were collected at 18 hours after injury, then cultured for 12 hours to enable sufficient release of chemokines into the culture supernatant. The chemokine concentrations in the culture supernatant were then assessed using a proteomics profile assay, and selected chemokines were measured using an ELISA. Abraded cornea released several chemokines of potential influence on mouse NK cells because NK cells express CCR5, CCR2, CXCR4, and CXCR3.[34] These included MIP-1, MIP-2, MCP-1, and IP-10 (Figure 3A). ELISA of corneal lysates revealed significantly increased levels of MCP-1 (CCL2), IP-10 (CXCL10), and MCP-3 (CCL-7) when compared with uninjured controls (Figure 3B).

In as much as individual chemokines may influence several different subsets of cells, blocking individual chemokines may be difficult to interpret with regard to NK cell migration. However, we chose to initially study *in vivo* two chemokines recognized by CCR2 and CXCR3 that were significantly increased in the explant cultures. MCP-1 (CCL2) and IP-10 (CXCL10) were assessed via topical application of MCP-1 or IP-10 blocking antibodies at 4-hour intervals during 18 hours after epithelial abrasion. Compared with the untreated and IgG controls, anti-IP-10 significantly inhibited the migration of NK cells into the stroma at 24 hours after injury (Figure 3C). Migration of $\gamma\delta$ T cells was partially inhibited in the epithelium, but not the stroma, by IP-10 blocking antibody (Figure 3C). Neutrophil influx, however, was not affected by topical anti-IP-10 (Figure 3C), revealing some selectivity for NK cells and $\gamma\delta$ T cells. In contrast, NK cells, $\gamma\delta$ T cells, and neutrophils were significantly inhibited by MCP-1 blocking antibody (data not shown).

Because blocking individual chemokines proved to be insufficiently selective for NK cells, we focused on the response of NK cells in adoptive transfer experiments, blocking chemokine receptors known to recognize the abrasion-induced chemokines. In these experiments, isolated spleen NK cells were pre-treated with blocking antibodies against CXCR3, an IP-10 receptor, and CCR2, an
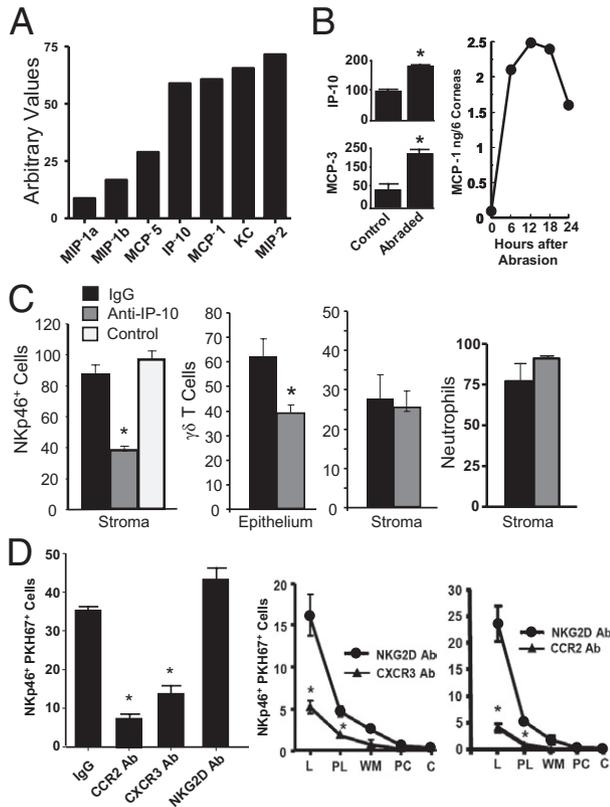
Figure 3. Migration of NK cells into corneal stroma was affected by chemokines. **A:** Protein antibody array, culture supernatant from six pooled corneas collected 18 hours after central epithelial abrasion and cultured with serum-free medium for 12 hours. **B:** Topical administration of anti-IP10 every 4 hours after corneal abrasion. NKp46$^+$ cells, GL3$^+$ cells ($\gamma\delta$ T cells), and Ly6G$^+$ cells (neutrophils) were counted in the corneal limbus at 24 hours after corneal injury. **C:** ELISA results showing IP-10 (CXCL10, pg/mL) and MCP-3 (CCL7, pg/mL) in lysates of pooled corneas at 18 hours with and without (Control) central epithelial abrasion (mean ± SD; $n = 3$; *$P < 0.01$). ELISA results showing MCP-1 (CCL2, ng/6 corneas) from corneal lysates at different times with and without central epithelial abrasion. **D:** Adoptive transfer of spleen NK cells with *ex vivo* blocking using anti-CXCR3, anti-CCR2, or anti-NKG2D. NKp46$^+$ cell counts in the cornea at 30 hours after corneal injury (mean ± SD; *$P < 0.01$ versus wild-type IgG-treated control mice, $n = 4$ to 6 per condition). Distribution of NKp46$^+$ cells within the corneal stroma is plotted in the accompanying graphs (mean ± SD; *$P < 0.01$ versus cells treated *ex vivo* with anti-NKG2D; $n = 4$ to 6 per condition).

MCP-1 and MCP-3 receptor. Consistent with published data,[33,42] the isolated spleen NK cells were consistently positive for these two receptors, and were split approximately equally between CD94$^+$ and CD94$^-$ cells when analyzed at flow cytometry (data not shown). In control mice, adoptive transfer resulted in localization predominantly of the CD94$^+$ subset, consistent with the natural inflammatory response described above. The blocking antibody against NKG2D, an NK cell activation receptor, was used as an antibody-binding control.[47,48] This antibody blocks activation of NK cells through the NKG2D receptor,[44] but did not affect their migration to the cornea. Compared with the NKG2D antibody, blocking either CXCR3 or CCR2 significantly reduced the migration of NK cells into the stroma, most evident in the limbus (Figure 3D).

NK Cells, Corneal Inflammation, and Wound Healing

To analyze contributions of NK cells to inflammation and wound healing, mice were injected i.p. with anti-NK1.1 at 24 hours before epithelial abrasion. Corneas analyzed at 24 and 30 hours after abrasion revealed no NKp46$^+$ cells in the stroma or epithelium. There were, however, increased numbers of neutrophils in the anterior stroma under the region of the original wound margin at 24 and 30 hours after wounding (Figure 4A). Because the typical inflammatory response exhibits waning of neutrophil accumulation at these times,[15] it seems that NK cells may participate in limiting or resolving acute inflammation. Another intervention affecting NK cell numbers that also increases neutrophil accumulation at these times after epithelial abrasion is Mac-1 deletion in the *Cd11b*$^{-/-}$ mice.[30] Confirming our previously published observations in these mice, we found that neutrophil accumulation was also significantly increased at 24 and 30 hours after epithelial abrasion in *Cd11b*$^{-/-}$ mice (Figure 4A) to a similar degree as NK cell depletion. Previous interpretations of this phenomenon (eg, that neutrophil survival is prolonged in the absence of CD11b)[49,50] did not include the possibility that CD11b deficiency limits NK cell immigration, with the secondary effect of prolonged neutrophil accumulation. CD11a$^{-/-}$ mice also have markedly reduced NK cell accumulation, but do not exhibit the same phenomenon as *Cd11b*$^{-/-}$ mice, most likely because LFA-1, unlike Mac-1,[51] is critical for neutrophil migration from blood.

Wound healing was also delayed by NK cell depletion. The epithelial wound was closed in control wild-type mice by 24 hours, consistent with previous studies in this model[30]; however, 15% to 30% of the wound area remained open in NK cell-depleted mice, as indicated by fluorescein staining of the corneal surface. A representative image of the wound area at 24 hours in NK cell-depleted mice is shown in Figure 4B, inset. Epithelial abrasion also induces a wave of basal epithelial cell division, with a sustained peak in the peripheral cornea and limbus between 18 and 30 hours.[16] Delayed epithelial healing was revealed in the NK cell-depleted mice by significantly reduced numbers of dividing basal epithelial cells analyzed at 24 hours after injury (Figure 4C). An additional parameter of wound healing includes subbasal nerve fiber density within the abraded area. Minimal nerve recovery is normally evident until 24 hours after injury, and progresses to about 20% of the uninjured density by 96 hours after abrasion.[17] Subbasal nerve recovery was significantly reduced in NK cell-depleted mice at 96 hours after epithelial abrasion (Figure 4D), indicating a prolonged effect of NK cell depletion.

Effect of NKG2D Blocking on Corneal Wound Healing

Additional studies of wound healing and neutrophil inflammation used adoptive transfer of anti-NKG2D-treated NK cells. In this experiment, wild-type NK cells were preincubated with NKG2D blocking antibody to inhibit activation of NK cells through this receptor after recon-
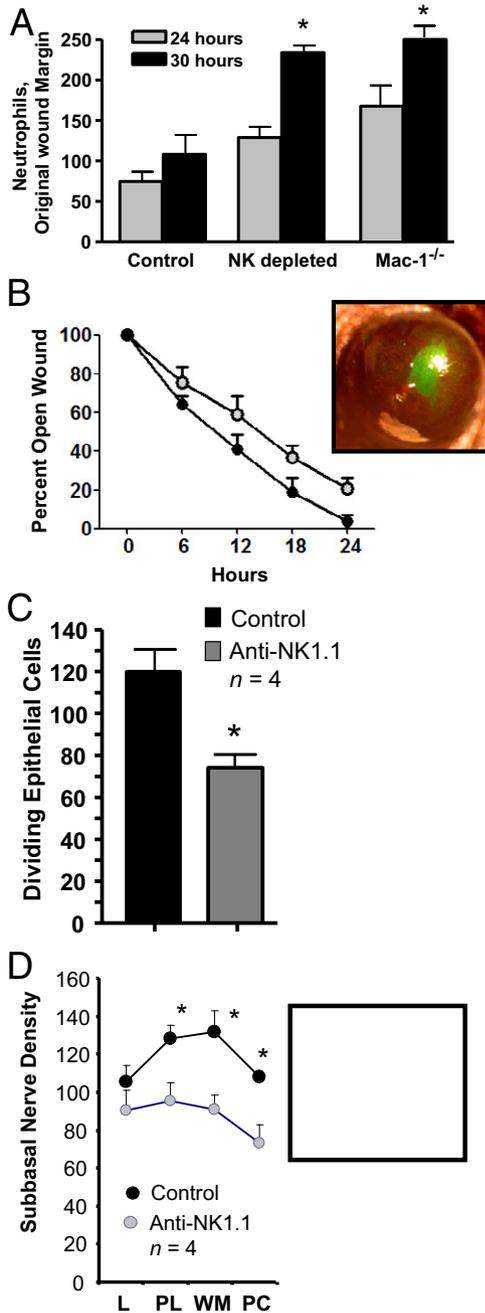
Figure 4. Effects of NK cell depletion. **A:** Neutrophil counts in the region of the original wound margin at 24 or 30 hours after central corneal epithelial abrasion in mice either depleted of NK cells via systemic injection of anti-NK1.1 before injury or in mice with engineered deletion of CD11b (mean ± SD; *P < 0.01 versus 30 hours in control mice). **B:** Epithelial wound closure over time after NK depletion (mean ± SD; *P < 0.01 for the 18- and 24-hour measurements, *n* = 4). **C:** Epithelial cell division counted in the basal layer of the epithelium at 24 hours after abrasion, comparing IgG-treated control mice with those treated with anti-NK1.1 at 24 hours before injury to deplete NK cells. **Inset:** Representative image of the ocular surface at 24 hours after abrasion in an NK cell-depleted mouse showing fluorescein staining of the open epithelial wound. **D:** Corneal subbasal nerve density at 96 hours after epithelial abrasion comparing IgG-treated control mice with those depleted of NK cells with anti-NK1.1 (mean ± SD; *P < 0.versus IgG-treated control mice; *n* = 4 to 6 per condition). **Inset** shows typical subbasal nerve density in the IgG-treated control mice.

stitution into recipient mice. NKG2D blocking antibody did not significantly affect migration of NK cells into the cornea after injury (Figure 3D). Consistent with results after NK cell depletion, neutrophil counts in the corneas

reconstituted with NKG2D-blocked NK cells were significantly increased compared with the corneas with IgG-treated NK cells (Figure 5A). NK cells treated *ex vivo* with anti-CXCR3 to reduce migration of the cells into the cornea exhibited some increase in neutrophils, but failed to reach statistical significance. Wound healing was similarly reduced, as indicated by reduction in dividing basal epithelial cells, systemic NK cell depletion, and reconstitution of NKG2D antibody-pretreated NK cells (Figure 5B). Subbasal nerve recovery in the wound margin was also significantly decreased when the recipient animals were given NKG2D-blocked NK cells, in contrast to IgG-treated NK cells (Figure 5C).

Discussion

In the present study, we provide evidence that NK cells migrate into the corneal stroma as a component of the acute inflammatory response to epithelial abrasion. These NK cells are phenotypically NKp46^{+}, NK1.1^{+}, NKG2D^{+}, and EOMES^{+}, and CD3^{−}, CD94^{−}, RORγt^{−}, IL-22^{−}, and CD127^{−}, consistent with a subset of classic NK cells.[34,38–40,42,52,53] Their peak accumulation in the corneal stroma occurred at 24 to 30 hours after injury, in contrast to neutrophils, which peak at 12 to 18 hours and are waning at 24 hours.[16,30] Their distribution was predominately in the stroma of the corneal limbus, with some migration into the center of the cornea, a pattern consistent with that of neutrophils.[17,30] Their accumulation was dependent on leukocyte adhesion molecules LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and ICAM-1 (CD54), as well as chemokine receptors CXCR3 and CCR2, consistent with directed migration into the cornea.[34,36,54–56] Selective neutrophil depletion did not significantly reduce the accumulation of NK cells, and topical application of anti-CXCL10 inhibited NK cell accumulation without reducing neutrophils, consistent with distinct phlogistic mechanisms for these cell types. Recruitment of both cell types was dependent on γδ T cells, which contribute to the acute inflammatory response to corneal abrasion.[7,17]

Data from the present study support a possible modulatory role for NK cells during the inflammatory response to corneal epithelial abrasion. Systemic administration of anti-NK1.1 depleted the corneal tissue of NKp46^{+} cells without reducing the number of γδ T cells within the first 24 hours after epithelial abrasion. However, anti-NK1.1 significantly increased neutrophils at 30 hours after abrasion. Systemic administration of anti-asialo GM1 also depleted NKp46^{+} cells, and increased neutrophils after epithelial abrasion. Adoptive transfer of NK cells isolated from the spleen of wild-type mice into wild-type mice depleted of NK cells (via anti-asialo GM1) reduced neutrophil accumulation when compared with adoptive transfer of NK cells blocked *ex vivo* with an antibody against NKG2D, an activating receptor for NK cell-dependent cytotoxicity and cytokine production.[36,56–58] Inasmuch as both blocked and unblocked NK cells migrated into the corneal stroma, the differences in neutrophil accumulation are consistent with
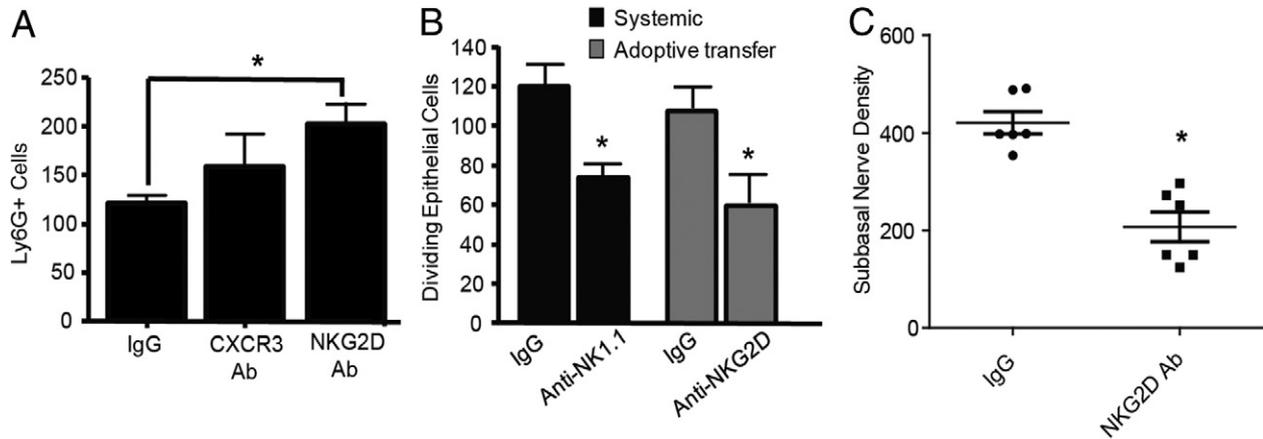
Figure 5. Effects of adoptive transfer spleen NK cells after blocking NKG2D. **A:** Neutrophil counts in the region of the original wound margin at 24 hours after central corneal epithelial abrasion in mice after adoptive transfer of IgG and anti-NKG2D-blocked spleen NK cells. **B:** Epithelial cell divisions counted in the basal layer of the epithelium at 24 hours after abrasion after adoptive transfer of IgG and anti-NKG2D-blocked spleen NK cells compared with systemic anti-NK1.1 (ie, NK depletion). **C:** Data from 96 hours subbasal nerve healing after adoptive transfer of IgG and anti-NKG2D-blocked spleen NK cells. Subbasal nerve density in regions of the original wound margin is plotted for each cornea. Data plotted as mean ± SD; *$P < 0.01$ versus IgG-treated control mice; $n = 4$ to 6 per condition.

an NKG2D-dependent modulatory effect on an innate acute inflammatory process.

Haworth et al[33] have published evidence that NK cells are important for resolution of adaptive inflammation involving eosinophil and activated T-cell infiltration into the lung that developed over 2 to 3 weeks. In their model, resolution of the inflammatory response after cessation of antigen exposure was significantly delayed by depletion of the NK cells that normally accumulated in lung and draining lymph nodes during peak antigen-induced inflammation. Most important, they found that blocking the NKG2D receptor on NK cells impaired resolution of lung inflammation and the clearance of antigen-responsive T cells from the lung and draining lymph nodes. Although inflammation in our model of corneal injury depends on a different time frame and cascade of events involving $\gamma\delta$ T cells and neutrophils in an innate acute inflammatory response, we have evidence that indicates a similar important role for the NKG2D receptor in limiting acute inflammation developing during the first 24 hours. A possible mechanism for such function of NK cells could involve ligands for the NKG2D receptor up-regulated by cellular stress and inflammation.[57,58] Regarding adaptive immune responses, NK cells promote maturation and activation of dendritic cells, macrophages, and T cells, or, conversely, kill these cells (a process involving NKG2D), depending on the cytokine milieu and stage of cell activation.[36] A role for NK cells in resolution of innate acute inflammation has not been defined, but seems to be evident in the inflammatory response to corneal epithelial abrasion, possibly through cytokine production or cytotoxicity toward key inflammatory cells.

Inflammation is clearly important to healing. We observed in earlier studies that altered kinetics of neutrophil emigration produces profound disruption in corneal wound closure.[30] In $Cd11b^{-/-}$ mice, neutrophil accumulation at inflammatory sites is excessive, proposed to be a result of delayed apoptosis.[49,50] After corneal abrasion in these mice, accumulation of neutrophils exceeds that in wild-type mice (confirmed in the present study) at a

time when epithelial division in response to the abrasion is normally most active (18 to 30 hours).[30] The use of anti-adhesion antibodies (anti-CD11a and anti-ICAM-1) to reduce neutrophil accumulation in $Cd11b^{-/-}$ mice prevented the profound delay in wound healing.[30] Regenerating corneal epithelial cells express ICAM-1,[31] and may be susceptible to direct neutrophil adhesive interactions with LFA-1 on leukocytes.[59] LFA-1 supports reactive oxygen production by binding to ICAM-1.[60] Thus, excessive prolonged accumulation of neutrophils in the cornea is apparently destructive.

Findings of the present study indicated that NK cell-dependent modulation of the developing inflammatory process may enable positive contributions of neutrophils to wound healing. Our earlier studies provided evidence that neutrophil emigration in the abrasion model is required for efficient re-epithelialization[15] and subbasal nerve regeneration.[17] In four distinct experimental conditions that profoundly reduce neutrophil influx, epithelial healing was prolonged by 12 to 24 hours,[15] and nerve regeneration at 4 days after injury was significantly reduced.[17] Further, transfer of wild-type neutrophils into $CD18^{-/-}$ mice significantly improved re-epithelialization, and adoptive transfer of normal platelets into P-selectin-deficient mice restored neutrophil emigration and epithelial healing.[16] These observations are consistent with studies in rabbits in which re-epithelialization of abraded rabbit corneas was also significantly delayed when leukocyte emigration was inhibited.[61] Such studies show significant delays in corneal epithelial and nerve recovery when neutrophils fail to emigrate within the first 12 to 18 hours after abrasion.

The contributions of neutrophils to healing may involve two general mechanisms, resistance to infection and direct release of growth factors. Corneal epithelium is normally renewed by division in the basal cell layer[62] or from stem cells within the region of the limbus.[63–65] The stem cells in the limbus seem to be sensitive to corneal injury and to provide expansion of basal epithelial cells necessary for wound coverage. Various endogenous growth

factors seem to be involved.[62,66] Of particular interest are transforming growth factor-β, hepatocyte growth factor, insulin-like growth factors 1 and 2, epidermal growth factor, and platelet-derived growth factor.[67–72] One possible mechanism by which the accumulating leukocytes contribute to re-epithelialization is the delivery of growth factors to the limbus, the site of stem cells thought to be important for healing. Grenier et al[73] reported that hepatocyte growth factor is stored in the secretory and secondary granules of the neutrophils, and is released in an active form on neutrophil degranulation. Exocytosis of these classes of neutrophil granules occurs rapidly after activation by chemokines[74] present in wounded corneas within the time frame of leukocyte accumulation.[75] Another neutrophil-derived factor of potential importance is CAP37, reported by Pereira et al[76] to augment corneal epithelial cell migration and proliferation. We have recently provided evidence that neutrophils deliver vascular endothelial growth factor A (a trophic factor for neurite growth that is stored in secondary granules of neutrophils) to the anterior stroma adjacent to the regenerating corneal nerve plexus.[17] Thus, it is likely that emigrated neutrophils function well beyond simple host resistance to infection.

Consistent with this paradigm, we observed in the present study that either depletion of NK cells or blocking of the NKG2D receptor resulted in significantly increased neutrophil accumulation, with reduced epithelial healing and subbasal nerve regeneration. The effects of NK cell depletion may suggest that NK cells directly contribute to epithelial healing and nerve regeneration; however, our NKG2D blocking data do not seem to be consistent with this possibility because blocking of NKG2D did not inhibit NK cell accumulation in the tissue. Furthermore, because depletion of neutrophils significantly reduced corneal healing without reducing NK cell accumulation, it seems that NK cells are insufficient to directly support corneal healing. Thus, our present conceptual model attributes a measured modulatory role to the NK cells that prevents excessive accumulation and tissue injuring activity of neutrophils, thereby enabling predominately tissue healing functions of neutrophils.

Acknowledgments

References

1. Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, Rudolph R, Jameson J, Havran WL: A role for human skin-resident T cells in wound healing. J Exp Med 2009, 206:743–750
2. Havran WL, Jameson JM: Epidermal T cells and wound healing. J Immunol 2010, 184:5423–5428
3. Yamagami S, Hamrah P, Miyamoto K, Miyazaki D, Dekaris I, Dawson T, Lu B, Gerard C, Dana MR: CCR5 chemokine receptor mediates recruitment of MHC class II-positive Langerhans cells in the mouse corneal epithelium. Invest Ophthalmol Vis Sci 2005, 46:1201–1207
4. Hamrah P, Huq SO, Liu Y, Zhang Q, Dana MR: Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. J Leukoc Biol 2003, 74:172–178
5. Knickelbein JE, Watkins SC, McMenamin PG, Hendricks RL: Stratification of antigen-presenting cells within the normal cornea. Ophthalmol Eye Dis 2009, 1:45–54
6. Chinnery HR, Ruitenberg MJ, Plant GW, Pearlman E, Jung S, McMenamin PG: The chemokine receptor CX3CR1 mediates homing of MHC class II-positive cells to the normal mouse corneal epithelium. Invest Ophthalmol Vis Sci 2007, 48:1568–1574
7. Li Z, Burns AR, Rumbaut RE, Smith CW: gamma delta T cells are necessary for platelet and neutrophil accumulation in limbal vessels and efficient epithelial repair after corneal abrasion. Am J Pathol 2007, 171:838–845
8. Shirane J, Nakayama T, Nagakubo D, Izawa D, Hieshima K, Shimomura Y, Yoshie O: Corneal epithelial cells and stromal keratocytes efficiently produce CC chemokine-ligand 20 (CCL20) and attract cells expressing its receptor CCR6 in mouse herpetic stromal keratitis. Curr Eye Res 2004, 28:297–306
9. Huang LC, Jean D, Proske RJ, Reins RY, McDermott AM: Ocular surface expression and in vitro activity of antimicrobial peptides. Curr Eye Res 2007, 32:595–609
10. Lee EJ, Rosenbaum JT, Planck SR: Epifluorescence intravital microscopy of murine corneal dendritic cells. Invest Ophthalmol Vis Sci 2010, 51:2101–2108
11. Muller LJ, Marfurt CF, Kruse F, Tervo TM: Corneal nerves: structure, contents and function. Exp Eye Res 2003, 76:521–542
12. Kubilus JK, Linsenmayer TF: Developmental corneal innervation: interactions between nerves and specialized apical corneal epithelial cells. Invest Ophthalmol Vis Sci 2010, 51:782–789
13. Leiper LJ, Ou J, Walczysko P, Kucerova R, Lavery DN, West JD, Collinson JM: Control of patterns of corneal innervation by Pax6. Invest Ophthalmol Vis Sci 2009, 50:1122–1128
14. Jin Y, Shen L, Chong EM, Hamrah P, Zhang Q, Chen L, Dana MR: The chemokine receptor CCR7 mediates corneal antigen-presenting cell trafficking. Mol Vis 2007, 13:626–634
15. Li Z, Burns AR, Smith CW: Two waves of neutrophil emigration in response to corneal epithelial abrasion: distinct adhesion molecule requirements. Invest Ophthalmol Vis Sci 2006, 47:1947–1955
16. Li Z, Rumbaut RE, Burns AR, Smith CW: Platelet response to corneal abrasion is necessary for acute inflammation and efficient re-epithelialization. Invest Ophthalmol Vis Sci 2006, 47:4794–4802
17. Li Z, Burns AR, Han L, Rumbaut RE, Smith CW: IL-17 and VEGF are necessary for efficient corneal nerve regeneration. Am J Pathol 2011, 178:1106–1116
18. Li Z, Burns AR, Byeseda MS, Smith CW: CCL20, $\gamma\delta$ T cells, and IL-22 in corneal epithelial healing. FASEB J 2011, 25:2659–2668
19. Sondell M, Lundborg G, Kanje M: Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci 1999, 19:5731–5740
20. Sondell M, Sundler F, Kanje M: Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. Eur J Neurosci 2000, 12:4243–4254
21. Zhu Y, Jin K, Mao XO, Greenberg DA: Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. FASEB J 2003, 17:186–193
22. Liu Y, Figley S, Spratt SK, Lee G, Ando D, Surosky R, Fehlings MG: An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. Neurobiol Dis 2010, 37:384–393
23. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA: Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci USA 2002, 99:11946–11950
24. Yu CQ, Zhang M, Matis KI, Kim C, Rosenblatt MI: Vascular endothelial growth factor mediates corneal nerve repair. Invest Ophthalmol Vis Sci 2008, 49:3870–3878
25. Taichman NS, Young S, Cruchley AT, Taylor P, Paleolog E: Human neutrophils secrete vascular endothelial growth factor. J Leukoc Biol 1997, 62:397–400
26. Scapini P, Morini M, Tecchio C, Minghelli S, Di Carlo E, Tanghetti E, Albini A, Lowell C, Berton G, Noonan DM, Cassatella MA: CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. J Immunol 2004, 172:5034–5040

27. Scapini P, Calzetti F, Cassatella MA: On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). J Immunol Methods 1999, 232:121–129

28. Gaudry M, Bregerie O, Andrieu V, El Benna J, Pocidalo MA, Hakim J: Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood 1997, 90:4153–4161

29. Gong Y, Koh DR: Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model. Cell Tissue Res 2010, 339:437–448

30. Li Z, Burns AR, Smith CW: Lymphocyte function-associated antigen-1-dependent inhibition of corneal wound healing. Am J Pathol 2006, 169:1590–1600

31. Byeseda SE, Burns AR, Dieffenbaugher S, Rumbaut RE, Smith CW, Li Z: ICAM-1 is necessary for epithelial recruitment of gammadelta T cells and efficient corneal wound healing. Am J Pathol 2009, 175:571–579

32. Li Z, Rivera CA, Burns AR, Smith CW: Hindlimb unloading depresses corneal epithelial wound healing in mice. J Appl Physiol 2004, 97:641–647

33. Haworth O, Cernadas M, Levy BD: NK cells are effectors for resolvin E1 in the timely resolution of allergic airway inflammation. J Immunol 2011, 186:6129–6135

34. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, Walzer T: The trafficking of natural killer cells. Immunol Rev 2007, 220:169–182

35. Schwacha MG: Gammadelta T-cells: potential regulators of the post-burn inflammatory response. Burns 2009, 35:318–326

36. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S: Functions of natural killer cells. Nat Immunol 2008, 9:503–510

37. Tayade C, Fang Y, Black GP, Paffaro AP Jr, Erlebacher A, Croy BA: Differential transcription of Eomes and T-bet during maturation of mouse uterine natural killer cells. J Leukoc Biol 2005, 78:1347–1355

38. Colonna M: Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. Immunity 2009, 31:15–23

39. Sanos SL, Vonarbourg C, Mortha A, Diefenbach A: Control of epithelial cell function by interleukin-22-producing RORγt+ innate lymphoid cells. Immunology 2011, 132:453–465

40. Vivier E, Spits H, Cupedo T: Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? Nat Rev Immunol 2009, 9:229–234

41. Luther C, Warner K, Takei F: Unique progenitors in mouse lymph node develop into CD127+ NK cells: thymus-dependent and thymus-independent pathways. Blood 2011, 117:4012–4021

42. Yu J, Wei M, Mao H, Zhang J, Hughes T, Mitsui T, Park IK, Hwang C, Liu S, Marcucci G, Trotta R, Benson DM Jr, Caligiuri MA: CD94 defines phenotypically and functionally distinct mouse NK cell subsets. J Immunol 2009, 183:4968–4974

43. Braud VM, McMichael AJ: Regulation of NK cell functions through interaction of the CD94/NKG2 receptors with the nonclassical class I molecule HLA-E. Curr Top Microbiol Immunol 1999, 244:85–95

44. Gilfillan S, Ho EL, Cella M, Yokoyama WM, Colonna M: NKG2D recruits two distinct adapters to trigger NK cell activation and co-stimulation. Nat Immunol 2002, 3:1150–1155

45. Aust JG, Gays F, Mickiewicz KM, Buchanan E, Brooks CG: The expression and function of the NKRP1 receptor family in C57BL/6 mice. J Immunol 2009, 183:106–116

46. Cornwell RD, Gollahon KA, Hickstein DD: Description of the leukocyte function-associated antigen 1 (LFA-1 or CD11a) promoter. Proc Natl Acad Sci USA 1993, 90:4221–4225

47. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T: Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999, 285:727–729

48. Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH: Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat Immunol 2000, 1:119–126

49. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD: Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. Cell 1993, 74:541–554

50. Liu Z, Zhao M, Li N, Diaz LA, Mayadas TN: Differential roles for beta2 integrins in experimental autoimmune bullous pemphigoid. Blood 2006, 107:1063–1069

51. Jiang Y, Beller DI, Frendl G, Graves DT: Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. J Immunol 1992, 148:2423–2428

52. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, Pasqualetto V, Enault L, Richard-Le Goff O, Corcuff E, Guy-Grand D, Rocha B, Cumano A, Rogge L, Ezine S, Di Santo JP: A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. Nat Immunol 2006, 7:1217–1224

53. Ribeiro VS, Hasan M, Wilson A, Boucontet L, Pereira P, Lesjean-Pottier S, Satoh-Takayama N, Di Santo JP, Vosshenrich CA: Cutting edge: thymic NK cells develop independently from T cell precursors. J Immunol 2010, 185:4993–4997

54. Ley K, Laudanna C, Cybulsky MI, Nourshargh S: Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 2007, 7:678–689

55. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S: Innate or adaptive immunity? the example of natural killer cells. Science 331:44–49, 2011

56. Shi FD, Ljunggren HG, La Cava A, Van Kaer L: Organ-specific features of natural killer cells. Nat Rev Immunol 2011, 11:658–671

57. Zafirova B, Wensveen FM, Gulin M, Polic B: Regulation of immune cell function and differentiation by the NKG2D receptor. Cell Mol Life Sci 2011, 68:3519–3529

58. Raulet DH: Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol 2003, 3:781–790

59. Iwata M, Sawada S, Sawa M, Thoft RA: Mechanisms of lymphocyte adhesion to cultured human corneal epithelial cells. Curr Eye Res 1997, 16:751–760

60. Lu H, Ballantyne CM, Smith CW: LFA-1 (CD11a/CD18) triggers hydrogen peroxide production by canine neutrophils. J Leuk Biol 2000, 68:73–80

61. Gan L, Fagerholm P, Kim HJ: Effect of leukocytes on corneal cellular proliferation and wound healing. Invest Ophthalmol Vis Sci 1999, 40:575–581

62. Lu L, Reinach PS, Kao WW: Corneal epithelial wound healing. Exp Biol Med (Maywood) 2001, 226:653–664

63. Lehrer MS, Sun TT, Lavker RM: Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. J Cell Sci 1998, 111(Pt 19):2867–2875

64. Schlotzer-Schrehardt U, Kruse FE: Identification and characterization of limbal stem cells. Exp Eye Res 2005, 81:247–264

65. Park KS, Lim CH, Min BM, Lee JL, Chung HY, Joo CK, Park CW, Son Y: The side population cells in the rabbit limbus sensitively increased in response to the central cornea wounding. Invest Ophthalmol Vis Sci 2006, 47:892–900

66. Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong J, Lee J: The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. Prog Retin Eye Res 2001, 20:625–637

67. Hayashida-Hibino S, Watanabe H, Nishida K, Tsujikawa M, Tanaka T, Hori Y, Saishin Y, Tano Y: The effect of TGF-beta1 on differential gene expression profiles in human corneal epithelium studied by cDNA expression array. Invest Ophthalmol Vis Sci 2001, 42:1691–1697

68. Lee JS, Liu JJ, Hong JW, Wilson SE: Differential expression analysis by gene array of cell cycle modulators in human corneal epithelial cells stimulated with epidermal growth factor (EGF), hepatocyte growth factor (HGF), or keratinocyte growth factor (KGF). Curr Eye Res 2001, 23:69–76

69. Yanai R, Yamada N, Inui M, Nishida T: Correlation of proliferative and anti-apoptotic effects of HGF, insulin, IGF-1, IGF-2, and EGF in SV40-transformed human corneal epithelial cells. Exp Eye Res 2006, 83:76–83

70. Haber M, Cao Z, Panjwani N, Bedenice D, Li WW, Provost PJ: Effects of growth factors (EGF. PDGF-BB and TGF-beta 1) on cultured equine epithelial cells and keratocytes: implications for wound healing Vet Ophthalmol 2003, 6:211–217

71. Hoppenreijs VP, Pels E, Vrensen GF, Felten PC, Treffers WF: Platelet-derived growth factor: receptor expression in corneas and effects on corneal cells. Invest Ophthalmol Vis Sci 1993, 34:637–649

72. Valeri CR, Saleem B, Ragno G: Release of platelet-derived growth factors and proliferation of fibroblasts in the releasates from platelets stored in the liquid state at 22 degrees C after stimulation with agonists. Transfusion 2006, 46:225–229

73. Grenier A, Chollet-Martin S, Crestani B, Delarche C, El Benna J, Boutten A, Andrieu V, Durand G, Gougerot-Pocidalo MA, Aubier M, Dehoux M: Presence of a mobilizable intracellular pool of hepatocyte

growth factor in human polymorphonuclear neutrophils. Blood 2002, 99:2997–3004

74. Borregaard N, Cowland JB: Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997, 89:3503–3521

75. Spandau UH, Toksoy A, Verhaart S, Gillitzer R, Kruse FE: High expression of chemokines Gro-alpha (CXCL-1). IL-8 (CXCL-8), and

MCP-1 (CCL-2) in inflamed human corneas in vivo. Arch Ophthalmol 2003, 121:825–831

76. Pereira HA, Ruan X, Gonzalez ML, Tsyshevskaya-Hoover I, Chodosh J: Modulation of corneal epithelial cell functions by the neutrophil-derived inflammatory mediator CAP37. Invest Ophthalmol Vis Sci 2004, 45:4284–4292