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Expression of Cell Adhesion Molecules on Limbal and Neovascular Endothelium in Corneal Inflammatory Neovascularization

Su-Ning Zhu and M. Reza Dana

PURPOSE. To investigate the expression of cell-adhesion molecules on corneolimbal and neovascular endothelium and the associated leukocyte infiltration in an experimental model of inflammatory corneal neovascularization (NV).

METHODS. Corneal NV was induced in BALB/c mice by placement of nylon sutures. Interleukin-1 receptor antagonist (IL-1ra) was used topically to determine whether suppression of IL-1 could affect adhesion molecule expression and leukocytic infiltration. At set time points, corneal samples were analyzed immunohistochemically for expression of P-selectin, E-selectin, intercellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1, and platelet-endothelial adhesion molecule (PECAM)-1. Leukocytic infiltration at different time points was quantified histologically. In companion experiments mice deficient in ICAM-1 were investigated to determine the functional relevance of this molecule in corneal leukocyte infiltration.

RESULTS. Significant enhanced expression of ICAM-1 was detected on the corneolimbal vascular endothelium as early as 8 hours and on the newly formed corneal NV by day 3, and treatment with IL-1ra led to significant suppression of this expression. IL-1ra-induced suppression of ICAM-1 expression was accompanied by a profound decrease in corneal leukocytic infiltration by 44.6% at day 1 (P < 0.005), 71.8% at day 3 (P < 0.001), 60.1% at day 7 (P < 0.001), and 63.8% at day 14 (P < 0.001), compared with control corneas. Similarly, in ICAM-1 knockout mice, the corneal leukocytic infiltration was 50.3%, 52.9%, and 36.4%, compared with wild-type control animals on day 1 (P < 0.001), day 7 (P < 0.005), and day 14 (P < 0.001), respectively. Expression of PECAM-1 was constitutively present on perilimbal vascular endothelium and had no response to IL-1ra treatment. No significant expression of P-selectin, E-selectin, or VCAM-1 was detected in this experimental model.

CONCLUSIONS. These results suggest that leukocytic infiltration in this model of inflammatory corneal NV is closely associated with ICAM-1 expression, and that topical IL-1ra displays corneal anti-inflammatory effects, largely by suppressing ICAM-1 expression on vascular endothelial cells. (Invest Ophthalmol Vis Sci. 1999;40:1427-1434)

Leukocytic extravasation and migration into tissues is a critical step in the pathologic course of inflammatory and immune disorders. Studies have uncovered this process as a well-regulated cascade of events involving cell adhesion molecules, cytokines, and chemoattractants. Among the cell adhesion molecules, P-selectin and E-selectin initiate the rolling stage, and intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 mediate the firm-adhesion stage of leukocytic adhesion to endothelial cells (ECs). Platelet-endothelial cell adhesion molecule (PECAM)-1, a member of the immunoglobulin superfamily, is expressed constitutively on ECs, and is also thought to be involved in leukocyte transendothelial migration in certain models of inflammation.

Studies in both human and animal models of disease have confirmed that cell adhesion molecules are closely associated with the development of ocular inflammatory disorders such as uveitis, herpetic keratitis, and corneal allograft rejection. Moreover, blocking of cell adhesion molecules with monoclonal antibodies in experimental models of inflammatory eye disease has had some promising results. Recent experimental evidence has revealed that the cornea has the capacity to synthesize proinflammatory and immunoregulatory cytokines including interleukin (IL)-1, tumor necrosis factor-α and others, thereby contributing to regulation of cell adhesion molecular expression under various stimuli that may play an important role in corneal inflammation and neovascularization (NV).

The codependence of corneal inflammation and NV, whereby one process can mediate or upregulate the other, makes corneal NV a good model to study the mechanisms of
leukocyte recruitment in the cornea. Our standard model for induction of inflammatory corneal NV is associated with development of intrastromal vessels in close association with a mixed-cell (primarily neutrophilic) infiltrate. We have been interested in IL-1ra, a naturally produced antagonist to IL-1RI, for its regulatory activity in corneal immune and inflammatory responses. Our studies have shown that IL-1ra can suppress ocular antigen-presenting cell activity and promote survival of orthotopic corneal allografts in a manner that is independent of the degree of postkeratoplasty NV but that is characterized by a significant suppression of corneal inflammation. Hence, we hypothesized that IL-1ra can suppress corneal inflammation in large part by suppressing the vascular EC expression of adhesion molecules. Our results presented herein suggest that ICAM-1 is the predominantly upregulated adhesion molecule whose expression by ECs correlates with early corneal leukocyte infiltration in the cornea, and that the suppressed expression of ICAM-1 by topical IL-1ra is associated with a profound dampening in the recruitment of inflammatory cells in the corneal stroma.

METHODS

Induction of Corneal Neovascularization

All experiments unless specified otherwise were conducted in male BALB/c mice (Taconic Farms, Germantown, NY). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After deeply anesthetizing each animal with an intraperitoneal injection of 3 mg to 4 mg ketamine and 0.1 mg xylazine, intrastromal sutures were placed in the central cornea of one eye of each mouse to induce inflammatory corneal NV, as previously described. Briefly, three pairs of 11-0 nylon sutures (50-μm-diameter needle, Sharpont; Vanguard, Houston, TX) were placed equidistant from the limbus through the central stroma without perforation of the cornea, using an aseptic microsurgical technique and an operating microscope. Immediately after surgery, erythromycin ophthalmic ointment was applied.

Topical IL-1ra Application

One drop (40 μl recombinant human IL-1ra in 0.2% hyaluronic acid vehicle; Aigen, Thousand Oaks, CA) at a dose of 2.0% was applied topically to wild-type or ICAM-1 knockout (ICAM-1−/−) mouse eyes 1 hour before and three times a day after NV induction. Other groups of animals received 0.2% and 0.02% IL-1ra to determine whether decreasing the cytokine dose by 1:10 to 1:100 would appreciably influence the effect of IL-1ra on ICAM-1 expression. All data presented herein relate to 2% IL-1ra unless specified otherwise. Mice receiving topical vehicle alone (0.2% hyaluronate) served as controls. At set time points (2, 4, and 8 hours; and 1, 2, 3, 5, 7, and 14 days; n = 5-10 eyes per time point) eyes were collected and subjected to histochemical analysis for the expression of cell adhesion molecules and leukocyte infiltration, as described below.

Immunohistochemical Analysis of Cell Adhesion Molecules

Eyes were frozen in OCT compound (Tissue-Tek; Miles, Elkhart, IN), and stored at −70°C until ready for sectioning. Monoclonal antibodies against murine P-selectin (RB40.54), E-selectin (10E9.6), ICAM-1 (3E2), VCAM-1 (429), and PECAM-1 (MEC13.3) were purchased from PharMingen (San Diego, CA). Corneal cryosections on gelatin-coated slides were rinsed in phosphate-buffered saline (PBS; pH 7.2-7.4). To block nonspecific staining, slides were incubated in 2% bovine serum albumin (BSA) in PBS. Primary antibody diluted optimally with PBS containing 1% bovine serum albumin was applied to the tissue sections for 30 minutes, followed by 30 minutes' incubation with the secondary antibody, streptavidin-conjugated fluorescein isothiocyanate (FITC), or an FITC-conjugated goat anti-rat IgG antibody, according to the affinity of the individual primary antibody. All staining procedures were performed at room temperature, and each step was followed by three thorough washings in PBS. Finally, sections were covered with mounting medium (Vector, Burlingame, CA), and the slides were analyzed and photographed with a fluorescence and light microscope (BX50, Olympus, Tokyo, Japan). For negative controls, polyclonal hamster IgG or rat IgG2a (R35-59; PharMingen) was used instead of the primary antibody. Positive controls included cryosections from eyes of C3H/HeN mice with endotoxin-induced uveoretinitis induced by injecting 200 μl lipopolysaccharide into the hind footpad, as previously described.

Evaluation of Corneal Leukocyte Infiltration

Mice were killed at the same time points listed above (n = 5-10 per time point) after corneal suturing and the corneal samples collected. Six-micrometer-thick cryosections were prepared extending transversely across the central cornea of each eye. The following endpoints were identified for measuring the degree of corneal inflammation: the total number of leukocytes infiltrating the stroma, and the number of specific cell types (lymphocytes, macrophages, and neutrophils) comprising the infiltrate. Cryosections were stained with respective primary antibodies to identify cell types of interest: FITC-conjugated anti-mouse Mac-1 (M1/70; PharMingen) to identify macrophages and neutrophils, and FITC-conjugated anti-CD3 (145-2C11; PharMingen) for T-lymphocytes. To distinguish macrophages from other Mac-1+ cells, slides were stained with the PE-conjugated antismouse antibody CD74 (T12-3; Serotec, Kidlington, United Kingdom) to exclude neutrophils. Serial sections were analyzed, and five sections through the central cornea of each specimen were studied. The numbers of infiltrating leukocytes within the corneal stroma (limbus to limbus) identified by immunohistochemistry in each of the sections were counted and averaged for each specimen with use of a fluorescence microscope. The total number of infiltrating leukocytes was determined by summing the average number of Mac-1+ and CD3+ cells. All microscopic analyses were performed by the examiner in a masked fashion without knowledge of the specimens' source or experimental protocol.

Use of ICAM-1-Deficient Mice

To examine directly the functional relevance of ICAM-1 in this model, we used C57BL/6 mice homozygous for a gene-targeted mutation of ICAM-1 (Jackson Laboratory, Bar Harbor, ME). The same procedure to induce corneal inflammatory NV was applied to the ICAM-1−/− mice and their wild-type ICAM-1+/+ C57BL/6 matched controls (n = 10 per time point). Random groups of ICAM-1−/− animals (n = 5 per time point) received topical IL-1ra as detailed. At selected time points of 1, 7, and 14 days after stromal suturing, the corneas were harvested and...
TABLE 1. Time Course of Expression of Adhesion Molecules on Corneolimbal and Neovascular Endothelial Cells in Experimental Inflammatory Corneal Neovascularization

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>2 hours</th>
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<th>8 hours</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
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<tbody>
<tr>
<td>P-selectin</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>E-selectin</td>
<td>—</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>ICAM-1</td>
<td>(−)*</td>
<td>(−)*</td>
<td>(−)*</td>
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<td>++</td>
<td>++</td>
<td>++†</td>
<td>++†</td>
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</tr>
<tr>
<td>VCAM-1</td>
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* (−), No staining in majority of sections; trace staining detected in several sections.
† Expression of relevant molecules by corneal neovascular endothelium.

RESULTS

Expression of Cell Adhesion Molecules on Vascular Endothelium after Induction of Corneal Neovascularization

The expression of adhesion molecules on the limbal vessels of normal eyes was determined, as detailed. No staining was observed with monoclonal antibodies directed against P-selectin, E-selectin, or VCAM-1 (Table 1). Similarly, ICAM-1 was either not detected or was detected at trace levels on perilimbal vascular ECs. In contrast, PECAM-1 was found to be constitutively expressed on all limbal vascular ECs. In contrast to the normal eyes, there was intense expression of all adhesion molecules by the uveal and retinal vascular endothelium of positive control mice with endotoxin-induced uveitis.

Similar to our previously published results,20,21 corneal NV in this model could be appreciated biomicroscopically growing centripetally from the limbus as early as 48 to 72 hours after stimulation and reaching a peak NV response between days 10 and 14. However, even after induction of maximal corneal NV, there was no detectable expression of P-selectin, E-selectin, or VCAM-1 on the perlimbal or corneal NV endothelium at any of the time points studied (Table 1). In contrast, as early as 8 hours after induction of the stimulus (almost 2 days before biomicroscopically detectable corneal NV) the expression of ICAM-1 by the limbal ECs was profoundly upregulated and lasted for the entire 14-day period of observation. Similarly, the onset of corneal NV was associated with a profound overexpression of ICAM-1 by the neovascular ECs that lasted for the duration of the experimental protocol (Fig. 1). The constitutive expression of PECAM-1 observed at baseline was retained after NV induction by both the perilimbal vessels and the NV fronds in the cornea (Table 1).

Based on the observation that the induction of corneal NV in this model is associated primarily with upregulation in expression of ICAM-1 and a sustained expression of PECAM-1, we removed the inciting stimuli (corneal sutures) on day 10 after development of a robust NV response. Subsequently, at day 14 corneas were harvested for analysis of ICAM-1 and PECAM-1, as described. ICAM-1 expression by ECs had largely regressed to near basal (absent-to-trace) levels; however, PECAM-1 expression by the regressed NV was unaltered, suggesting that constant inflammatory stimulation is necessary for retention of significant ICAM-1, but not PECAM-1, expression by corneal NV and limbal vascular ECs.

Effect of IL-1ra on Expression of ICAM-1 and PECAM-1 on the Vascular Endothelium and on Leukocyte Infiltration in Sutured Corneas

Application of topical IL-1ra at all three doses tested (0.02%-2.0%) suppressed the upregulation of ICAM-1 by both the limbal vascular and the corneal neovascular endothelium induced by this model (Fig. 1 and Table 2). In vehicle-treated control eyes, application of 0.2% hyaluronate did not affect the expression of ICAM-1, suggesting that the EC expression of ICAM-1 in our model was largely reliant on an IL-1RI-mediated mechanism. In contrast, application of IL-1ra had no effect on the expression of PECAM-1 (Table 2).

Based on our observation that IL-1ra can profoundly suppress the vascular EC expression of ICAM-1, we hypothesized that application of IL-1ra may reduce corneal leukocyte infiltration. In vehicle-treated corneas, infiltrating leukocytes could be visualized in the perilimbal area as early as 4 hours after induction of NV. There was accumulation of cells in the peripheral stroma by 8 hours, and the infiltrating cells reached the corneal center and peaked in number by 24 hours. A second peak of leukocytic infiltration appeared by day 7 (Fig. 2A). In contrast, among corneas treated with IL-1ra, there was a profound reduction in the number of infiltrating leukocytes: suppression of 44.6% at day 1 (P < 0.003), 71.8% at day 3 (P < 0.001), 60.1% at day 7 (P < 0.001), and 63.8% at day 14 (P < 0.001). Analysis of the corneal infiltrates in vehicle-treated controls by cell type revealed that the majority (ranging from 62.1% to 82.5% in various time points) were neutrophilic in origin (Fig. 2B). To determine whether the suppression of leukocyte recruitment in the IL-1ra-treated corneas was selective for any one leukocyte population, corneas were histologically analyzed by cell type. Similar to the vehicle-treated controls, the infiltrate was primarily comprised of neutrophils ranging from 59.6% to 77.1% at various time points (Fig. 2B), suggesting that the anti-inflammatory effect of IL-1ra was not highly selective for a given leukocyte population.

Leukocyte Infiltration in Corneas of ICAM-1-Deficient Mice

ICAM-1−/− animals were used to evaluate the role of ICAM-1 in corneal leukocyte infiltration. The corneal NV response in the ICAM-1−/− animals in this model of corneal angiogenesis was indistinguishable from that in wild-type ICAM-1+/+ controls (data not shown). The overall population of leukocytes infiltr-
trating the corneas of ICAM-1−/− animals was 50.3% (P < 0.001), 52.9% (P < 0.005), and 36.4% (P < 0.001) of that in ICAM-1+/+ mice at day 1, day 7, and day 14, respectively (Fig. 3). In addition, significant margination of leukocytes to blood vessel walls was generally observed in ICAM-1+/+, but rarely seen in ICAM-1−/− eyes (data not shown). Because both IL-1ra treatment and ICAM-1 deficiency were associated with decreased corneal leukocytic infiltration, we treated ICAM-1−/− animals with IL-1ra to determine whether there would be further (ICAM-independent) suppression of leukocyte infiltration. Our data showed an additional 50% to 70% reduction in leukocyte counts in ICAM-1−/− animals treated with IL-1ra compared with ICAM-1−/− animals receiving vehicle alone (Fig. 3).

Finally, to evaluate whether the downmodulation of corneal inflammation in the ICAM-1−/− animals was selective for specific leukocyte populations, the infiltrate was cell typed. Similar to the wild-type ICAM-1+/+ controls, the principal cell type comprising the infiltrate was neutrophilic, 84.3% at day 1 and 48.2% at day 7 (data not shown). Moreover, at all time

TABLE 2. Effect of IL-1ra or Vehicle Treatment on Expression of ICAM-1 and PECAM-1 on Corneal Vascular Endothelium

<table>
<thead>
<tr>
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<th>4 hours</th>
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<tr>
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<td>(−)</td>
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*IL-1ra effect on ICAM-1 expression was tested at doses of 0.02%-2.0% with similar results.

* Expression of relevant molecules by corneal neovascular endothelium.

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FIGURE 2. Suppression of intrastromal leukocytic infiltration by IL-1ra. Eyes of BALB/c mice were treated topically with IL-1ra or vehicle alone after induction of inflammatory NV (n = 5-10 per time point). Identification and enumeration of leukocyte populations was based on immunofluorescent staining with monoclonal antibodies anti-MAC-1, -CD74, and -CD3. Quantification of total intrastromal leukocytic infiltration (mean ± SD) shows that IL-1ra profoundly suppressed leukocyte recruitment (A). Classification of leukocytes by cell type shows that although the principal infiltrating population is neutrophilic, the relative suppression of neutrophil and mononuclear cell infiltration by IL-1ra was comparable (B).
points studied the relative cell types comprising the infiltrate were identical among ICAM-1-deficient and wild-type animals, suggesting that ICAM-1 deficiency appears to affect leukocytic recruitment similarly across different inflammatory cell populations.

**DISCUSSION**

Previous studies have established that differential expression of cell adhesion molecules by ECs is essential for EC-leukocyte signaling and for mediating leukocytic transendothelial migration and tissue infiltration. In fact, the current dominant paradigm of inflammation is largely based on the concept of specific cytokines, depending on the nature of the inflammatory stimulus, affecting the expression of adhesion factors and chemokines that together mediate leukocyte recruitment to extravascular compartments. Because leukocytic infiltration into the corneal stroma is a critical aspect of the immunopathology of corneal inflammatory disease, it is important to characterize further the mechanisms involved in the expression of cell adhesion molecules on the perilimbal and corneal neovascular endothelium.

Using an experimental murine model of corneal inflammatory NV, we were able to show the time course of expression of cell adhesion molecules on the corneal limbal and neovascular ECs, and simultaneously, to correlate this expression with the extent of leukocytic infiltration within the cornea. This model of experimental corneal inflammatory NV led to selective and profound stimulation of ICAM-1 and PECAM-1 coexpression but no appreciable expression of P-selectin, E-selectin, or VCAM-1 on neovascular ECs. Although our data cannot rule out some minute expression of other adhesion molecules below the detection levels of our assay system, the consistent absence of expression of adhesion factors other than ICAM-1 and PECAM-1 using a sensitive detection system suggests that the differential expression of adhesion factors is characteristic of this model system. This should not be surprising per se, because it has been shown that the constitutive and stimulated expression of cell adhesion molecules by EC in different vascular beds and under different stimuli may vary.
significantly.\textsuperscript{26,27} As such, our experimental model contrasts with findings in corneas that undergo allograft rejection that have been found to express E-selectin and VCAM-1\textsuperscript{110} and is more similar to findings in herpetic stromal keratitis in which there is coexpression of ICAM-1 and PECAM-1.\textsuperscript{19}

Our previous work has shown that agonistic IL-1 ligands, IL-1α and IL-1β, are profoundly upregulated in this experimental model of inflammatory corneal NV.\textsuperscript{29} Our data showing suppression of ICAM-1 expression in corneas receiving IL-1ra suggest that blockade at the level of IL-1RI (by IL-1ra) is capable of suppressing ICAM-1 expression in response to the inflammatory cytokines. As such, our results are compatible with those of Pavilack et al.\textsuperscript{27} who reported that IL-1β, tumor necrosis factor-α, and interferon-γ can upregulate the pericellular expression of ICAM-1 in the human cornea. It is known that ICAM-1, by binding to its counter ligands, lymphocyte function-associated antigen (LFA-1) and Mac-1, on neutrophils and monocytes, plays an important role in mediating leukocyte-interactions with activated vascular ECs and in mediating extravasation of leukocytic populations from the vascular compartment. The relevance of ICAM-1 in mediating leukocyte extravasation and stromal infiltration in this model was underscored by our data in ICAM-1-deficient mice displaying significantly diminished corneal infiltration compared with wild-type controls. This is particularly striking, given that ICAM-1+/− animals display significantly higher leukocyte counts, in particular neutrophils, in their blood.\textsuperscript{28}

The correlation of the decreased leukocytic infiltration with suppression of ICAM-1 expression by EC as a result of IL-1ra treatment in our experiments indicates that the anti-inflammatory effect of IL-1ra in the cornea is, at least in part, through downmodulating ICAM-1 expression. These data are compatible with a study showing profound suppression of neutrophilic inflammation after intratracheal administration of IL-1ra in experimental endotoxin-mediated pulmonary inflammation in which the authors suggest IL-1ra suppresses the inflammatory response in an indirect fashion\textsuperscript{29} but do not delineate the mechanism by which this occurs. Moreover, our data are consistent with a study in experimental glomerulonephritis in which administration of IL-1ra led to significant suppression of disease activity by inhibiting expression of ICAM-1.\textsuperscript{30}

It is critical to appreciate the limitations of our data. First, although we have focused on the expression of ICAM-1 protein immunohistochemically, further studies examining the regulation of ICAM-1 gene transcription by IL-1ra (e.g., by in situ hybridization or quantitative PCR) are warranted. Second, it is important to appreciate that our data do not suggest a direct regulation of ICAM-1 expression by IL-1ra per se but rather show that effective suppression of IL-1RI can downmodulate ICAM-1 expression by the ECs. Third, although our data show that expression of ICAM-1 by corneal limbal ECs is significantly correlated with corneal inflammation, it must be recognized that non-EC populations including resident corneal cells are also capable of expressing ICAM-1 (and other adhesion molecules). The nonvascular expression of adhesion factors, although not directly related to extravasation of leukocytes, may play an important role in recruitment of inflammatory cells to the cornea, as has been suggested by others.\textsuperscript{10,11} Fourth, although our data make a strong case for the functional relevance of ICAM-1 in corneal inflammation, other factors that may relate to the decrease of leukocyte infiltration in this model should be examined. The codependence of angiogenesis and inflammation, in which one response potentiates the other,\textsuperscript{11,32} underlies the concept that regulation of mediators of inflammation (such as cell adhesion molecules) should not be analyzed without regard for the neovascular response. For example, the limited antiangiogenic effect of IL-1ra in the cornea\textsuperscript{29} could mean decreased delivery to the cornea of inflammatory cells capable of expressing cytokines (e.g., tumor necrosis factor-α) that may themselves affect ICAM-1 expression. Furthermore, in vitro studies have shown that the corneal tissues may synthesize chemoattractants on stimulation by IL-1,\textsuperscript{33,34} providing yet one additional route by which leukocyte traffic to the cornea can be suppressed in conjunction with, but not directly caused by, suppressed ICAM-1 expression. In fact, our data showing that IL-1ra can suppress corneal leukocyte infiltration in ICAM-1 knockout mice (Fig. 3) strongly suggests that there are IL-1-independent, but ICAM-1-independent mediators that are active in corneal inflammation. One final point deserves comment. As stated earlier, to the extent that ICAM-1 mediates inflammatory cell recruitment to the corneal stroma, it may also be involved in the angiogenic response, because virtually any endothelial growth factor identified can be expressed by neutrophils and macrophages.\textsuperscript{32} However, it is important to recognize that although ICAM-1 expression by EC can be suppressed by IL-1ra as our data suggest, the antiangiogenic effect of IL-1ra is limited,\textsuperscript{29} and ICAM-1 knockout mice also showed corneal NV (data not shown), suggesting that, at least in this particular experimental model, ICAM-1 activity is not necessary for corneal angiogenesis. To our knowledge, this is the first report documenting the effect of topical application of a cytokine in relation to in vivo expression of adhesion molecules on corneolimbal and neo-vascular endothelium and the associated stromal leukocytic infiltration. Further work delineating the complex interactions between cytokines, adhesion molecules, and leukocyte activation and recruitment will increase our capacity to target critical steps that are involved in the regulation of immune and inflammatory responses in the cornea.

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