IL-17A Differentially Regulates Corneal Vascular Endothelial Growth Factor (VEGF)-A and Soluble VEGF Receptor 1 Expression and Promotes Corneal Angiogenesis after Herpes Simplex Virus Infection

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Ocular infection with HSV causes corneal neovascularization (CV), an essential step in the pathogenesis of the blinding immunoinflammatory lesion stromal keratitis. The infection results in IL-17A production, which contributes to CV in ways that together serve to shift the balance between corneal concentrations of vascular endothelial growth factor A (VEGF-A) and the soluble vascular endothelial growth factor receptor 1 molecule, which binds to VEGF-A and blocks its function (a so-called VEGF trap). Accordingly, animals lacking responses to IL-17A signaling, either because of IL-17 receptor A knockout or wild-type animals that received neutralizing mAb to IL-17A, had diminished CV, compared with controls. The procedures reduced VEGF-A protein levels but had no effect on the levels of soluble vascular endothelial growth factor receptor 1. Hence the VEGF trap was strengthened. IL-17A also caused increased CXCL1/KC synthesis, which attracts neutrophils to the inflammatory site. Neutrophils further influenced the extent of CV by acting as an additional source of VEGF-A, as did metalloproteinase enzymes that degrade the soluble receptor, inhibiting its VEGF-blocking activity. Our results indicate that suppressing the expression of IL-17A, or increasing the activity of the VEGF trap, represents a useful approach to inhibiting CV and the control of an ocular lesion that is an important cause of human blindness. The Journal of Immunology, 2012, 188: 3434–3446.
of IL-17A in WT HSV-infected mice, showed reduced CV. IL-17RAKO mice had reduced production of VEGF-A, but sVEGFR-1 levels remained unchanged. In addition, IL-17A induced IL-6 production by corneal stromal fibroblasts, and IL-6, in combination with IL-17A, acted in concert to further upregulate VEGF-A production. IL-17A also directly enhanced MMP-9 production, which can break down sVEGFR-1 into inactive fragments but had no effect on VEGF-A bioavailability. IL-17A also induced the neutrophil chemoattractant CXCL1/KC in the cornea, with the recruited neutrophils further affecting the balance between VEGF-A and sVEGFR-1 by providing an additional source of preformed VEGF-A as well as sVEGFR-1-degrading MMPs. Taken together, our data show that IL-17A plays a central role in regulating ocular angiogenesis and does so, at least in part, by limiting the efficacy of the VEGF-A trap.

Materials and Methods

Mice, virus, and cell lines

IL-17RAKO mice on a C57BL/6 background were obtained from Amgen (Thousand Oaks, CA). C57BL/6 mice were purchased from Harlan Sprague Dawley, Indianapolis, IN. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. All animals were housed in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care. HSV-1 eye infection was performed under anesthesia (tribromomethanol [Avertin]), and all efforts were made to minimize animal suffering. HSV-1 RE Tumpey virus was grown in Vero cell monolayers (ATCC no. CCL-81). The virus was concentrated, titrated, and stored in aliquots at −80°C until use. The MK/T-1 cell line (immortalized keratocytes from C57/BL6 mouse corneal stroma) was kindly provided by Dr. Reza Dana, Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston MA. The MK/T-1 cell line was derived from cultures of mouse stromal cells transfected with a human telomerase transcriptase to attain immortalization (22). These cells were fibroblastic in appearance with no cellular transformation in cell culture over several passages. Although these cells shared similar characteristics with parental cells, they show some structural and morphological alterations, such as expression of smooth muscle α-actin when stimulated with cytokines such as TGF-β (22).

Corneal HSV infection and clinical scoring

Corneal infections of mice were performed with the mice under deep anesthesia induced by i.p. injection of Avertin. The corneas of mice were scarified with a 27-gauge needle, and a 3-μl drop containing 1 × 10⁴ PFU of virus was applied to the eye. The eyes were examined on different days, postinfection (p.i.), for the development and progression of clinical lesions, by slit-lamp biomicroscope (Kowa, Nagoya, Japan). The progression of angiogenesis of individually scored mice was noted. The severity of angiogenesis was recorded as described previously (23). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The scores of the four quadrants of the eye were then summed to derive the neovessel index (range, 0–16) for each eye at a given time point.

Subconjunctival injection

Subconjunctival injections of anti–IL-17 were performed as described previously (24). Briefly, these injections were done using a 2-cm, 32-gauge needle and syringe (Hamilton) to penetrate the perivascular region of the conjunctiva, and the required dose of anti–IL-17 mAb (5 μg in 10-μl volume) was delivered into the subconjunctival space. Control mice received isotype mAb.

Flow cytometry

Corneas were excised, pooled according to group, and digested with 60 U/ml Liberase for 35 min at 37°C in a humidified atmosphere of 5% CO₂.

FIGURE 1. IL-17RAKO mice display diminished HSV-induced CV. To compare the development and progression of CV, WT and IL-17RAKO mice were infected with 1 × 10⁴ PFU of HSV by corneal scarification. (A) Development of new blood vessels in normally avascular cornea was assessed on days 8, 11, 15, and 21 p.i. (B) CV score of individual mice on day 21 p.i. n = 8–12 mice per group from two independent experiments; *p < 0.03, ***p = 0.0003. (C and D) Mice were sacrificed on day 15 p.i., and corneas were pooled according to group for flow cytometry analysis. (C) Representative FACS plots for corneal CD31⁺ endothelial cells between WT and IL-17RAKO mice. (D) Bar graphs show reduced total CD31⁺ endothelial cell number per cornea from the IL-17RAKO mice group, compared with the WT group. n = 5 for WT and 6 for IL-17RAKO mice. Each sample is representative of two corneas. Data represent means ± SEM.
**FIGURE 2.** Systemic and local IL-17A neutralization reduces severity of HSV-induced CV. WT mice were infected with $1 \times 10^3$ PFU of HSV by corneal scarification. (A) For early IL-17A neutralization, animals were injected subconjunctivally with 5 μg in 10 μl per eye of anti–IL-17A or control IgG1 mAb, as indicated. (B) The development of new blood vessels in normally avascular cornea was assessed on days 5 and 7 p.i. (C) CV score of individual mice on day 15 p.i. *n* = 16 corneas per group from two independent experiments; *p* ≤ 0.037. (D) For late IL-17A neutralization, mice were injected subconjunctivally with 5 μg of anti–IL-17A or control IgG1 mAb, as indicated. (E) The development of response in normally avascular cornea was assessed on days 8, 11, and 15 p.i. (F) CV score of individual mice on day 15 p.i. *n* = 8 corneas per group from two independent experiments; ***p* = 0.0003. (G) Representative eye photograph of naive, uninfected, local isotype mAb- or anti–IL-17A mAb–treated mice on day 15 p.i. In uninfected mice, cornea is clear and devoid of any blood vessels (left panel). By day 15 p.i., new blood vessel development reaches to the center of the cornea in isotype mAb-treated mice (middle panel), but only halfway in anti–IL-17A–treated mice (right panel), as indicated by arrows. (H and I) Mice from late IL-17A neutralization groups were sacrificed on day 15 p.i., and corneas were pooled according to group for flow cytometry analysis. (H) Representative FACS plots for corneal CD31+ endothelial cells between isotype mAb- and anti–IL-17A mAb–treated mice. (I) Bar graphs show reduced total CD31+ endothelial cell numbers per cornea from anti–IL-17A–treated group as compared with isotype-treated group. *n* = 4, and each sample is representative of two corneas. Data show mean values ± SEM from at least two different independent experiments.
FIGURE 3. IL-17A differentially regulates corneal VEGF-A and sVEGFR-1 expression. WT and IL-17RAKO mice were infected with 1 × 10^4 PFU of HSV in PBS or were mock infected with only PBS by corneal scarification. VEGF-A (A) and sVEGFR-1 (B) mRNA expression was examined and compared between WT and IL-17RAKO mice by qRT-PCR. VEGF-A and sVEGFR-1 mRNA levels in mock-infected mice were set to 1 and used for relative fold upregulation at various days p.i. n = 4. (C) Local neutralization of IL-17A was carried out using anti–IL-17A mAb by subconjunctival injection during early and late stages of HSV infection, as indicated. (D) VEGF-A mRNA expression was examined and compared between isotype mAb- and anti–IL-17A mAb–treated mice by qRT-PCR. n = 4. (E) Corneal VEGF-A protein levels were analyzed from WT and IL-17RAKO mice at indicated time points. n = 3. (F) Corneal VEGF-A protein levels were analyzed and compared between isotype mAb- and anti–IL-17A mAb–treated mice at day 7 p.i. n = 3. (G) Corneal sVEGFR-1 protein levels were analyzed from WT and IL-17RAKO mice at day 7 p.i. n = 3. MK/T-1 cells (corneal stromal keratocytes) were stimulated under different concentrations of IL-17A in the presence or absence of anti–IL-17A mAb for 24 h. Supernatants were collected for VEGF-A protein estimation by ELISA, and cells were collected and pooled for analysis of VEGF-A and sVEGFR-1 mRNA expression by qRT-PCR. The mRNA levels in control media were set to 1 and used for relative fold upregulation at various days p.i. Relative fold change in VEGF-A (H) and sVEGFR-1 (I) mRNA expression, compared with control media. n = 4. (J) IL-17A stimulated VEGF-A production by MK/T-1 cells in a dose-dependent manner. n = 15.

Data show mean values ± SEM from at least two different independent experiments.
FIGURE 4. IL-17A stimulates IL-6 production by corneal stromal fibroblast cells, and both cytokines further stimulate VEGF-A production. WT and IL-17RAKO mice were infected with $1 \times 10^3$ PFU of HSV in PBS or were mock infected with only PBS by corneal scarification. (A) IL-6 mRNA expression was examined and compared between WT and IL-17RAKO mice by qRT-PCR. $n = 4$. (B) Local neutralization of IL-17A was carried out using anti–IL-17A mAb by subconjunctival injection during early and late stages of HSV infection, as indicated. (C) IL-6 mRNA expression was examined and compared between isotype mAb- and anti–IL-17A mAb–treated mice by qRT-PCR. IL-6 mRNA levels in mock-infected mice were set to 1 and used for relative fold upregulation at various days p.i. $n = 4$. (D and E) MK/T-1 cells were stimulated under different concentrations of IL-17A in the presence or absence of anti–IL-17A mAb for 24 h. Supernatants were collected for IL-6 protein estimation by ELISA, and cells were collected and pooled for analysis of IL-6 mRNA expression by qRT-PCR. (D) Relative fold change in IL-6 mRNA expression, compared with control media. $n = 4$. The mRNA levels in control media were set to 1 and used for relative fold change in mRNA expression. (E) IL-17A stimulated IL-6 production by MK/T-1 cells in a dose-dependent manner. $n = 9$. (F) MK/T-1 cells were stimulated with IL-17A (10 ng/ml) in the presence or absence of anti–IL-6R mAb for 24 h. (Figure legend continues)
MK/T-1 cell assay

Immortalized corneal stromal fibroblast (MK/T-1) cells were stimulated in vitro with different concentrations of rIL-17A, IL-6, and IL-1β (R&D Systems) either in the presence or the absence of neutralizing anti–IL-17A mAb or anti–IL-6 receptor mAb (BD Biosciences) in DMEM supplemented with 5% FBS for 24 h at 37°C in 5% CO2. After 24 h of stimulation, supernatants were collected and stored at −80°C until further use. Supernatants were analyzed for VEGF-A, IL-6, and CXCL1/KC production, using sandwich ELISA kit. Cells were collected for gene expression analysis.

Quantitative real-time PCR

Cells (from eight pooled corneas per sample or cultured MK/T-1 cells) were lysed, and total mRNA was extracted using TRIzol LS reagent (Invitrogen). Total cDNA was made with 500 ng RNA, using oligo (dT) primer. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) with iQ5 real-time PCR detection system (Bio-Rad). The expression levels of different molecules were normalized to β-actin, using ΔCt calculation. Relative expression between control and experimental groups was calculated using the 2−ΔΔCt formula. The PCR primers used were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>VEGF-A</td>
<td>F5'-GTTCATGATTGTCAGCAAGGAC-3'</td>
<td>R5'-GAA-GATGATCTCTCATCGCGG-3'</td>
</tr>
<tr>
<td>MMP-2</td>
<td>F5'-CCGGCTCCTACACCTACACCAAGAAC-3'</td>
<td>R5'-CCAG-TACCGCTGATACATACG-3'</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>F5'-CCTTACAGAGACTTTACTCCTGG-3'</td>
<td>R5'-GACAA-GTTTCTCAGATTAGGAG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>F5'-ATGCTTGGACATAAGCGACTGATGTG-3'</td>
<td>R5'-CTGGAAGATGAGAAGTGTGGCC-3'</td>
</tr>
<tr>
<td>MMP-7</td>
<td>F5'-CCGATTCCTACACCATCACAAGAAGAC-3'</td>
<td>R5'-CCAG-TACCGTGTACATATCAG-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>F5'-CCTTACAGAGACTTTACTCCTCGG-3'</td>
<td>R5'-CCAG-TACCGTGTACATATCAG-3'</td>
</tr>
<tr>
<td>sVEGFR-1</td>
<td>F5'-GCTTCGCGACCTCCGAATTC-3'</td>
<td>R5'-GCCTCGGGCA-3'</td>
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Western blot analysis

The supernatants from lysed corneal cells were quantified using the BCA Protein Assay Kit (Thermo Scientific). Samples with equal protein concentrations were denatured by boiling in Laemmli buffer. Polypeptides were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA in Tris-buffered saline containing 0.05% Tween 20 (20 mM Tris [pH 7.4], 137 mM NaCl, and 0.1% Tween 20) overnight at 4°C and probed with specific primary and secondary Abs. Proteins were detected using chemiluminescent HRP substrate (Millipore). The membrane was kept in stripping buffer for 10 min and reprobed using anti-β-actin Ab. The Abs used were rat anti–sVEGFR-1 (141515; R&D Systems), mouse anti-β-actin (AC74; Sigma-Aldrich), goat anti-rat IgG-HRP (R&D Systems), and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology).

Immunofluorescence staining

For immunofluorescence staining, the eyes from naïve uninfected mice were enucleated and snap frozen in OCT compound. Sections 6 μm thick were cut, air dried, and fixed in acetone-methanol (1:1) at −20°C for 10 min. Sections were blocked with 10% goat serum containing 0.05% Tween 20 and 1:200 dilution of Fc block (Clone 2.4G2; BD Biosciences). Rat anti-Ly6G (Clone RB6-8C5; eBioscience) was diluted in 1% BSA containing 0.1% Triton X and incubated at room temperature for 1 h. After incubation, sections were washed several times using PBS with Tween 20 and then stained with rabbit anti-rat Alexa 488 for 45 min. The corneal sections were repeatedly washed using PBS with Tween 20 and mounted with media containing propidium iodide as a nuclear stain (Vector Laboratories) and visualized under an immunofluorescence microscope.

ELISA

The pooled corneal samples (four to eight corneas per sample) were homogenized using a tissue homogenizer, and supernatant was used for analysis. The concentrations of VEGF-A, sVEGFR-1, CXCL1/1-KC (R&D Systems), and IL-6 (eBioscience) were measured by sandwich ELISA kits per the manufacturer’s instructions.

Statistics

A Student t test was performed to determine statistical significance, and data are expressed as mean ± SEM. For some experiments, as mentioned in the figure legends, a one-way ANOVA test was applied.

Results

Lack of IL-17AR signaling diminishes severity of HSV-induced CV

To study the role of IL-17AR signaling effects on HSV-induced CV, the eyes of IL-17RAKO mice were infected with HSV, and the severity of CV was compared with age- and sex-matched WT infected control mice. At all time points, IL-17RAKO mice showed significantly less CV than did WT animals (Fig. 1A, 1B). The onset of visible angiogenic sprouting was delayed, angiogenesis scores were lower, and more IL-17RAKO mice had diminished angiogenesis scores compared with their WT counterparts (Fig. 1A). In addition, not only the incidence but also the severity of angiogenesis (scores of ≥8) was reduced in the IL-17RAKO group (two of eight eyes at day 21) compared with WT counterparts (9 of 11 eyes at day 21) (Fig. 1B). Analysis by FACS of pooled corneal samples from IL-17RAKO and WT mice in each group at day 15 p.i. revealed significantly reduced numbers of CD31+ cells per cornea (a marker for blood vessel endothelium) in IL-17RAKO, compared with WT animals (Fig. 1C, 1D). Although IL-17RAKO mice showed diminished CV scores, compared with WT animals, the inhibition was not complete because additional angiogenic factors and inducers of VEGF-A, such as Cpg, IL-6, and IL-1, were still present (3, 15, 25). Collectively, these findings indicate that IL-17A plays a role in HSV-induced CV.

To further demonstrate the participation of IL-17A, WT HSV-infected mice were given neutralizing anti–IL-17A or isotype mAb (5 μg in 10 μl) on days 1, 3, and 5 p.i. by the subconjunctival route (Fig. 2A). As shown in Fig. 2B and 2C, the neutralizing mAb treatment significantly reduced angiogenesis scores, compared with those of control mAb-treated mice. Consistently, local subconjunctival administration of anti–IL-17A mAb starting on day 7, followed by days 9, 11, and 13, showed inhibitory effect on the onset as well as severity of CV (Fig. 2D–G). Moreover, the frequency of severe angiogenesis (scores of ≥8) was significantly decreased in animals from the anti–IL-17A mAb recipients (1 of 8 eyes) as compared with isotype mAb recipients (7 of 8 eyes) (Fig. 2F). Furthermore, corneal samples collected at the end of the experiment revealed reduced total numbers of CD31+ cells per cornea in the IL-17A–neutralized group, compared with isotype mAb recipients (Fig. 2H, 2I). Taken together, Supernatants were collected for VEGF-A protein estimation by ELISA (n = 6). (G) MK/T-1 cells were stimulated under different concentrations of IL-17A, IL-6, and IL-1β for 24 h. Supernatants were collected for VEGF-A protein estimation by ELISA. VEGF-A production by MK/T-1 cells under different concentrations of IL-17A, IL-6, and IL-1β, as indicated. n = 6–15. Data represent means ± SEM from at least two independent experiments. One-way ANOVA with Bonferroni’s multiple comparison test was used to calculate the level of significance. *p < 0.05, **p < 0.001, ***p < 0.0001.
FIGURE 5. IL-17A promotes MMP-2, -7, and -9 expression and affects VEGF-A bioavailability through sVEGFR-1 degradation. WT and IL-17RAKO mice were infected with 1 × 10^4 PFU of HSV in PBS or were mock infected with only PBS by corneal scarification. MMP-2 (A), MMP-7 (B), and MMP-9 (C) mRNA expression was examined and compared between WT and IL-17RAKO mice by qRT-PCR. n = 4. (D and E) Local neutralization of IL-17A was carried out using anti–IL-17A mAb by subconjunctival injection during early (D) and late (E) stages of HSV infection, as indicated. MMP-2 (left), MMP-7 (middle), and MMP-9 (right) mRNA expression was examined and compared between isotype mAb- and anti–IL-17A mAb–treated mice by qRT-PCR. MMP-2, -7, and -9 mRNA levels in mock-infected mice were set to 1 and used for relative fold upregulation at various days p.i. n = 4. (F) sVEGFR-1 expression was analyzed by Western blot in corneal samples from isotype mAb- and anti–IL-17A mAb–treated mice at day 7 p.i. β-actin was used as loading control. (G) MKT-1 cells were stimulated under different concentrations of IL-17 in the presence or absence of anti–IL-17A mAb for 24 h. Cells were collected and pooled for analysis of MMP-9 mRNA expression by qRT-PCR. Relative fold change in MMP-9 mRNA expression was compared with that in control media. n = 4. The mRNA levels in control media were set to 1 and used to measure relative fold change in mRNA expression. Data show mean values ± SEM from at least two different independent experiments.
FIGURE 6. IL-17A promotes neutrophil infiltration in the cornea after HSV infection through CXCL1/KC induction. WT and IL-17RAKO mice were infected with \(1 \times 10^4\) PFU of HSV in PBS or were mock infected with only PBS by corneal scarification. (A) CXCL1/KC mRNA expression was examined and compared between WT and IL-17RAKO mice by qRT-PCR. CXCL1/KC mRNA levels in mock-infected mice were set to 1 and used for relative fold upregulation at various days p.i. (B) Corneal CXCL1/KC protein levels were analyzed from WT and IL-17RAKO mice at indicated time points. \(n=3\). (C) Local neutralization of IL-17A was carried out using anti–IL-17A mAb by subconjunctival injection during early (left panel) and late (right panel) stages of HSV infection, as indicated. (D) CXCL1/KC mRNA expression was examined and compared between isotype mAb- and anti–IL-17A mAb–treated mice by qRT-PCR. CXCL1/KC mRNA levels in mock-infected mice were set to 1 and used for relative fold upregulation at various days p.i. \(n=4\). (E) Corneal CXCL1/KC protein levels were analyzed and compared between isotype mAb- and anti–IL-17A mAb–treated mice at day 7 p.i. \(n=4\). (F) HSV-infected WT and IL-17RAKO mice were sacrificed on days 1, 2, and 15 p.i., and corneas were pooled according to group for flow (Figure legend continues).
these data indicate that IL-17A signaling affects the extent of CV that follows ocular infection with HSV.

**Mechanisms for IL-17A–induced CV after HSV infection**

IL-17A differentially regulates VEGF-A and sVEGFR-1 expression after HSV infection. Although IL-17A could be directly involved in causing CV, a more likely explanation is that the cytokine affects the VEGF-A response and, conceivably, its regulation by sVEGFR-1. In support of this notion, we could show that WT infected animals, compared with uninfected controls, markedly upregulated VEGF-A, but not sVEGFR-1 mRNAs over the day 1 to 15 p.i. observation period (Fig. 3A). However, compared with controls, IL-17RAKO animals failed to upregulate VEGF-A mRNA and their sVEGFR-1 mRNA levels were modestly increased (Fig. 3A, 3B). Similarly, WT infected animals given neutralizing anti–IL-17A mAb failed to upregulate VEGF-A mRNA (Fig. 3C, 3D), with little or no effect on sVEGFR-1 mRNA expression (data not shown). VEGF-A protein levels were also recorded over the 15-d observation period in WT and IL-17RAKO animals. As is evident in Fig. 3E, WT animals expressed readily detectable VEGF-A protein levels, but both IL-17RAKO and WT animals receiving neutralizing anti–IL-17A mAb had reduced VEGF-A protein levels (Fig. 3E, 3F). In addition, when sVEGFR-1 protein levels in naive uninfected and WT infected day 7 corneal samples were compared, WT mice showed significantly reduced corneal sVEGFR-1 levels. However, when sVEGFR-1 protein levels in infected and in day 7 infected IL-17RAKO mice were compared, no significant reduction was observed between these two groups (Fig. 3G).

Furthermore, in vitro experiments using a murine corneal stromal fibroblast cell line (MK/T-1 cells) were performed to measure the effects of IL-17A stimulation on the production of VEGF-A and sVEGFR-1. Real-time PCR analysis showed that IL-17A induced upregulation of VEGF-A mRNA up to 150-fold (Fig. 3H) but had no major effect on sVEGFR-1 mRNA expression (Fig. 3I). Stimulation of MK/T-1 cells with IL-17A also significantly increased VEGF-A protein levels in a dose-dependent manner (Fig. 3J). The IL-17A response could be blocked by anti–IL-17A mAb, indicating that the response was IL-17A specific. Taken together, these in vivo and in vitro experiments demonstrate that IL-17A causes CV by stimulating VEGF-A production but had little or no effect on sVEGFR-1. The outcome would be increased bioavailability of VEGF-A to drive CV after HSV infection.

**IL-17A induces IL-6 expression and in combination expands production of VEGF-A by corneal stromal fibroblasts.**

Although IL-17A promoted VEGF-A expression by the cornea, it was not clear if the effect was direct or indirect, with additional molecules such as IL-6 involved as intermediaries (15, 26–28). To assess the role of IL-17A in IL-6 expression, real-time PCR was used to compare levels of corneal IL-6 mRNA in HSV-infected IL-17RAKO versus WT counterparts at various days p.i. Corneal samples from IL-17RAKO mice, compared with those from WT mice, showed markedly diminished expression of IL-6 at all tested days p.i. (Fig. 4A). Moreover, local neutralization of IL-17A during the early as well as the late phase of HSV infection resulted in reduced IL-6 mRNA expression on day 7 and day 15 p.i. (Fig. 4B, 4C). In vitro experiments also showed that stimulation of corneal fibroblasts with IL-17A caused the upregulation of IL-6 mRNA and protein (Fig. 4D, 4E). Furthermore, the addition of anti–IL-17A mAb completely blocked IL-6 mRNA and protein production, indicating a direct effect of IL-17A on the IL-6 response (Fig. 4D, 4E). These experiments suggest that IL-17A plays a direct role in inducing IL-6, which itself is known to induce VEGF-A (15). Although IL-17A could induce IL-6, the latter would not seem to be responsible for VEGF-A production by IL-17A–stimulated fibroblasts. Accordingly, VEGF-A expression levels were unaffected by the presence of anti–IL-6 mAb in cultures (Fig. 4F). Nevertheless, to further determine whether IL-17A stimulation was sufficient to induce maximal amounts of VEGF-A expression, in vitro experiments were performed to compare the stimulatory effects of IL-17A with those of the cytokines IL-6 and IL-1β. As shown in Fig. 4G, the addition of IL-17A, IL-β, or IL-6 given individually could all promote VEGF-A production by MK/T-1 cells. However, when MK/T-1 cells were stimulated with a combination of IL-17A with IL-6 and/or IL-1β, VEGF-A production by MK/T-1 cells was significantly increased, compared with the response to a single cytokine. In sum, our experiments indicate that IL-17A may have a direct role in inducing VEGF-A, but the magnitude of VEGF-A induction can be increased by coexposure to other cytokines such as IL-6 and IL-1β, a likely scenario in corneal stroma after HSV infection.

**IL-17A drives increased expression of various MMPs responsible for sVEGFR-1 degradation.**

It is known that MMPs facilitate the growth of new blood vessels through the extracellular matrix (29) and that some MMPs may differentially break down VEGF-A and sVEGFR-1 into nonfunctional fragments (13, 30). To determine if IL-17A could influence the production of MMP enzymes, corneal samples from HSV-infected IL-17RAKO and WT mice were compared for MMP mRNA levels by real-time-PCR analysis. From day 1 through 15 p.i., MMP-2, -7, and -9 mRNA levels were lower in samples from IL-17RAKO than in those from WT mice. The differential expression levels ranged from 5- to 10-fold and were evident at all time points tested (Fig. 5A–C). Maximal differences in expression levels were noted with MMP-9 on day 11 and 15 p.i. (Fig. 5C).

In additional experiments, WT HSV-infected mice received neutralizing anti–IL-17A mAb starting from day 1, followed by days 3 and 5 p.i. (Fig. 5D). This resulted in diminished expression of MMP-2 (12-fold), MMP-7 (3-fold), and MMP-9 (25-fold) on day 7 p.i., when compared with isotype mAb-treated animals (Fig. 5D). Even effects of IL-17A neutralization were observed when the treatment was begun on day 7, followed by treatment on days 9, 11, and 13 p.i. Levels were reduced 4-fold for MMP-2, 10-fold for MMP-7, and 15-fold for MMP-9 in the anti–IL-17A mAb recipient group compared with the isotype control mice. However, the magnitude of downregulation of MMPs in the IL-17A neutralized groups did not correlate with the reduction of VEGF-A expression levels seen in the IL-17A neutralized group.
FIGURE 7. Scheme depicting various critical events orchestrated by IL-17A after HSV infection causing CV. (A) Model proposing the critical role of IL-17A in the deviation of physiological balance between VEGF-A and sVEGFR-1 after HSV infection. Normally, naive, uninfected cornea constitutively secretes large amounts of sVEGFR-1 and small amounts of VEGF-A. This secretion leads to a perfect physiological balance between these two molecules, sVEGFR-1 counteracting angiogenic properties of VEGF-A. The net result is the absence of any blood vessels in normally uninfected corneas (left panel). However, early after HSV infection, expression of IL-17A in the cornea is increased, which is mainly contributed by early innate cells such as γδ T cells. The increased levels of IL-17A then further contribute to increased VEGF-A expression by direct stimulation of corneal stromal fibroblast cells as well as indirectly through increased production of IL-6, (Figure legend continues)
group, compared with isotype mAb-treated mice (Fig. 5E). Furthermore, Western blot analysis of corneal samples from mice treated with anti–IL-17A, compared with isotype mAb-treated mice, revealed reduced degradation of sVEGFR-1 (Fig. 5F).

In other experiments, MK/T-1 cells were stimulated with IL-17A for 24 h. This stimulation resulted in increased MMP-9 mRNA expression in a dose-dependent manner, an effect inhibited by neutralizing anti–IL-17A mAb, indicating that the increased MMP-9 expression was IL-17A specific (Fig. 5G). Collectively, these results indicate that IL-17A could influence the efficacy of the VEGF-A trap by increasing the synthesis of some MMPs that can cause sVEGFR-1 degradation, making more VEGF-A available to induce angiogenesis.

**IL-17A promotes corneal infiltration of neutrophils through increased CXCL1/KC expression.** Neutrophils form a prominent part of the inflammatory response in corneas after HSV infection (3, 31). These cells contribute to the extent of CV by acting as an additional source of VEGF-A, as do MMP enzymes that can degrade the sVEGFR-1 into fragments that fail to block VEGF-A (13, 32). To measure the extent of IL-17A participation in neutrophil recruitment, experiments were done to compare levels of the neutrophil-recruiting chemokine CXCL1/KC in WT and animals with blunted signals from IL-17A. Our results show that levels of the CXCL1/KC mRNA and protein in corneal extracts from IL-17RAKO mice were significantly lower than in extracts from WT (Fig. 6A, 6B), or from WT mice that received neutralizing mAb to IL-17A (Fig. 6C–E). The differences were apparent at all time points tested (Fig. 6A–E). Further evidence for the role of IL-17A in corneal infiltration of neutrophils after HSV infection came from comparing the corneal-infiltrating neutrophils of HSV-infected WT mice with those of IL-17RAKO mice. Accordingly, IL-17RAKO mice had significantly reduced total cell numbers of neutrophils when compared with WT counterparts (Fig. 6F).

Moreover, in vivo neutralization of IL-17A in WT HSV-infected mice, compared with isotype Ab-treated animals, showed diminished infiltration of neutrophils, further demonstrating the critical role of IL-17A in the migration of these cells to the site of inflammation (Fig. 6G–I).

Finally, in vitro experiments were performed to determine whether a corneal cell line stimulated with different concentrations of IL-17A would produce increased amounts of CXCL1/KC chemokine. These experiments revealed an increase in CXCL1/KC expression when stimulated by both IL-17A and IL-6 (Fig. 6F, 6K). The effect of IL-17A was inhibited by neutralizing mAb to IL-17A, but not anti–IL-6R mAb, indicating that IL-17A induction occurred independently of involvement with IL-6 (Fig. 6L).

Collectively, these experiments indicated that IL-17A could influence the function of the VEGF-A trap by elevating the production of a neutrophil-recruiting chemokine, with the neutrophils providing an additional source of VEGF-A, as well as enzymes that degrade sVEGFR-1 (13). Our overall results are summarized in Fig. 7.

**Discussion**

The normal eye uses numerous strategies to maintain the corneal transparency necessary for optimal vision (1). For example, the VEGF-A present in normal corneas does not cause vision-imparing CV because it is bound by its soluble receptor, forming a so-called VEGF-A trap (2). However, infection by HSV can overcome this homeostatic mechanism, and CV occurs, an essential step in the pathogenesis of the blinding immunoinflammatory lesion, SK (6). This article shows that the infection results in IL-17A production, which participates in causing CV in several ways. Accordingly, animals lacking responses to IL-17A signaling, either because of IL-17 receptor A knockout or WT animals that received neutralizing mAb to IL-17A, had diminished CV compared with controls. The procedures reduced VEGF-A protein levels but had no effect on the levels of sVEGFR-1. Consequently, the VEGF-A trap could act even more effectively. In addition, the sVEGFR-1 present was less subject to degradation by MMPs in animals without IL-17A signaling, because in normal circumstances IL-17A caused the elevated production of these enzymes. Furthermore, we showed that IL-17A also caused increased CXCL1/KC synthesis, which attracts neutrophils to the inflammatory site. Neutrophils further influence the extent of CV by acting as an additional source of VEGF-A, as do MMP enzymes that degrade the soluble receptor, inhibiting its VEGF-A–blocking activity. The MMPs also enhance angiogenesis by facilitating the movement of new blood vessels through the stromal matrix (29, 32). These results demonstrate that IL-17A plays a central role in causing CV and indicate that suppressing the expression of IL-17A could represent a logical approach to achieving antiangiogenesis and the control of SK.

The cytokine IL-17A has a range of biological activities and is well known to participate in several inflammatory reactions that include SK, as we and others recently reported (18, 33–35). A role for IL-17A in angiogenesis has also been documented, especially in tumor systems (19, 21), although whether IL-17A itself drives angiogenesis or acts via intermediary molecules is unclear. Thus, the cytokine can induce HUVEC cells, fibroblasts, and some cancer cells to produce VEGF-A, as well as some other angiogenic molecules (19, 21, 33), and these could explain the angiogenic effect. In this study, we also were able to show that corneal fibroblasts exposed to IL-17A upregulated VEGF mRNA and secreted VEGF protein. One report does demonstrate that IL-17A can induce angiogenesis in a rat corneal micropocket assay, consistent, perhaps, with a direct role in angiogenesis (21), although potential intermediaries were not excluded. Accordingly, we feel the balance of evidence favors the notion that IL-17A’s participation in angiogenesis is indirect and proceeds by stimulating cells with IL-17Rs to produce VEGF-A and perhaps other angiogenic factors (36–38). This participation was shown by in vitro studies, but these studies also demonstrated that the levels of VEGF-A produced were enhanced when cells were costimulated with IL-17A, along with other cytokines. Such additive effects were shown when cells were exposed to IL-17A, as well as to IL-6 or IL-18. A similar pattern of results was reported by others using fibroblasts isolated from tumors and from lesions of arthritis (20, 21, 37, 39).

Although our results demonstrate that IL-17A participates in the CV that follows HSVocular infection, the connection between virus infection and IL-17A production requires explanation. Thus, virus
replication is a brief event usually confined to the corneal epithe- lium, but the CV response occurs in the underlying stroma and progresses in magnitude beyond the time when the infection has been eliminated (3, 6). Moreover, corneal epithelial cells are an unlikely source of IL-17A, and we were unable to demonstrate that such cells produce IL-17A upon infection (A. Suryawanshi and B.T. Rouse, unpublished observations). More than likely, the initial source of IL-17A was innate cells, which began invading the stroma 24–48 h postinfection (18). Most of these innate cells were neutrophils, which in some systems were reported to produce IL-17A (40). We failed to confirm this observation, however, in the case of corneal neutrophils. Instead, we could show that γδ T cells in the cornea produced IL-17A, based on the observation that depleting such cells reduced IL-17A protein levels in corneal extracts (18). Others have also shown that γδ T cells are spontane- ous producers of IL-17A (41–43).

What needs to be explained is the link between the virus in- fection in the epithelium and the recruitment, and perhaps stimula- tion, of innate cells to produce IL-17A. We, as well as others, have shown that several cytokines and chemokines are produced in the cornea soon after infection (44, 45). Moreover, this induction is the consequence of TLR ligand activity of HSV, rather than being the product of infected cells themselves (46, 47). A few cytokines are produced briefly by infected cells, most notably, IL-6 (45). Furthermore, IL-6 is known to be a regulator of IL-17A production, particularly in T cells (48, 49), which in the ocular system become the major source of IL-17A production after the initial phase of SK pathogenesis (18). It is also possible that the γδ T cells recruited to the cornea were promoted to rapidly produce IL-17A, as was shown to occur in vitro when these cells were exposed to cytokines such as IL-1β and IL-23 (41). Both of these activating cytokines have increased expression after HSV ocular infection (50, 51), and their action on γδ T cells could help explain how the infection relates to IL-17A production in the initial stages. In later stages, the virus is no longer present and the γδ T cells are almost absent (31, 52), yet commencing IL-17A neutralization at day 7 p.i. still resulted in significantly less CV. Conceivably, an alternative cellular source of the IL-17A cells could be Th17 cells, but such cells are barely detectable in corneal preparations until at least 15 d p.i. (18). Further studies are under way to define the cellular origin of IL-17A.

The novel aspect of the current study was to demonstrate, in an infectious model of inflammatory disease in which pathologi- cal angiogenesis is an essential component of pathogenesis, that IL-17A signaling played a major part in causing CV and appeared to act by shifting the balance between VEGF-A and its soluble receptor. This served to make VEGF-A more available with this molecule, rather than IL-17A itself, likely acting as the angiogenic factor. This conclusion was reached because in vivo and in vitro experiments both showed that when signaling responses to IL-17A were interrupted, the outcome was a significant reduction in the levels of VEGF-A, but no effect on sVEGFR-1 protein levels. Moreover, in the absence of IL-17A signaling, levels of three MMP enzymes that break down sVEGFR-1 were lower, indicating that IL-17A stimulates MMP production, as was shown in vitro for MMP9 with IL-17A–stimulated corneal fibroblasts. Others, too, have shown that tumor fibroblasts upregulate some MMPs upon in vitro stimulation with IL-17A (53). Thus, when the IL-17A response is normal, conditions favor both the production of VEGF-A and the breakdown of its soluble receptor, both of which will set the stage for CV. Subsequent events also impacted on the balance of VEGF and sVEGFR1 favoring angiogenesis because when neutrophils become a prominent component of the inflam- matory response, as occurs in the clinical phase of SK, these cells are an additional source of VEGF, as are MMPs that degrade any sVEGFR-1 (18). We also showed with in vitro experiments that IL-17A was stimulatory for VEGF-A gene expression in vitro, but it had the opposite effect on sVEGFR-1 expression. If a similar event also operates in vivo, this could help diminish the function of the VEGF trap. In consideration of all this information, the influence of IL-17A on CV is likely to be the consequence of effects on the VEGF-A/sVEGFR-1 balance, rather than only the action of IL-17A as an inducer of VEGF-A.

Finally, it is of interest to comment about the therapeutic implications of our observations. Basically, we interpret our studies to show that IL-17A participates in angiogenesis by shifting the balance between the concentrations of VEGF-A and the soluble form of its receptor, which acts to inhibit the angiogenic function of VEGF-A. The idea of using a synthetic VEGF-A trap to control angiogenesis has been advocated for use in a number of situations that include choroidal neovascularization and some tumors (54, 55). In these circumstances, the VEGF–A-binding molecules used are synthetic derivatives of VEGF-A receptors and have a very high affinity for VEGF-A. In our situation, we are dealing with the IL-17A–induced breakdown of a natural VEGF-A trap. Past studies using a mouse model of SK, as well as human corneal samples from HSV-infected SK patients, have shown that IL-17A is expressed after HSV infection (18, 34, 56). These studies, as well as our present article, show that IL-17A promotes CV and is also involved in the pathogenesis of SK. Therefore, we advocate, on the basis of results from this investigation, that control of angiogenesis induced by HSV infection could be achieved either by blunting the participation or by blocking events that limit the ef- ficiency of the VEGF trap. Blunting IL-17A might be achieved most effectively using nanoparticles that encode small interfering RNA targeting IL-17A gene expression or anti–IL-17A mAb. Facili- tating the activity of the VEGF trap could be accomplished by approaches that inhibit MMP enzymes or that boost the concen- tration of sVEGFR-1. Both approaches are currently being investi- gated.

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Disclosures
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